CSIRO PUBLISHING

Reproduction, Fertility and Development, 2019, **31**, 1894–1903
https://doi.org/10.1071/RD19201

Mare and stallion effects on blastocyst production in a commercial equine ovum pick-up–intracytoplasmic sperm injection program

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Abstract. This study retrospectively examined the degree to which success within a commercial ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) program varied between individual mares and stallions. Over 2 years, 552 OPU sessions were performed on 323 privately owned warmblood mares. For mares that yielded at least one blastocyst during the first OPU-ICSI cycle, there was a 77% likelihood of success during subsequent attempts; conversely, when the first cycle yielded no blastocyst, the likelihood of failure (no embryo) in subsequent cycles was 62%. In mares subjected to four or more OPU sessions, the mean percentage of blastocysts per injected oocyte was 20.5% (range 1.4–46.7%), whereas the mean number of blastocysts per OPU-ICSI session was 1.67 (0.2–4.2). Age did not differ significantly between mares that yielded good or poor results. The number of recovered oocytes per OPU was positively associated with the likelihood of success ($P < 0.001$). Although there were considerable between-stallion differences, most stallions (14/16) clustered between 15.6% and 26.8% blastocysts per injected oocyte, and the number of blastocysts per OPU (mean 1.4; range 0.2–2.2) was less variable than among mares. In conclusion, although both mare and stallion affect the success of OPU-ICSI, mare identity and the number of oocytes recovered appear to be the most reliable predictors of success.

Additional keywords: ICSI, IVF, IVM, oocyte, OPU.

Received 10 June 2019, accepted 12 September 2019, published online 22 October 2019

Introduction

In the past decade equine *in vitro* embryo production (IVEP) by ovum pick-up (OPU) followed by intracytoplasmic sperm injection (ICSI) has become popular among sport horse breeders, and has led to the development of several laboratories offering commercial equine OPU-ICSI in various parts of the world (Herrera 2018). OPU-ICSI was initially considered primarily a technique for salvaging fertility in subfertile, often aged, mares unable to conceive or produce a viable embryo using more standard breeding methods (natural mating or AI) due, for example, to oviduct occlusion, chronic uterine infection or fibrosis, irreparable damage to the reproductive tract or infertility of unknown origin. OPU-ICSI can also provide a solution for stallion subfertility (low sperm output, low numbers of morphologically normal spermatozoa, idiopathic infertility). However, there is also an increasing demand from horse breeders to use OPU-ICSI in reproductively normal, fertile mares to enable more efficient use of limited and costly frozen–thawed semen from valuable stallions (Claes *et al.* 2016).

Factors affecting the success or efficiency of breeding techniques based on *in vivo* fertilisation (i.e. natural mating and AI) have been examined in numerous surveys. Among these, it is accepted that the status (maiden, foaling or barren; Squires *et al.* 2006; Allen *et al.* 2007) and age (Vanderwall and Woods 1990; Carnevale and Ginther 1995; Allen *et al.* 2007; Marinone *et al.* 2015) of the mare are major contributors to reproductive success and efficiency. Conversely, the intrinsic fertility of individual mares has not been considered to have a major effect on reproductive efficiency in intensively managed thoroughbred stud farms, not least because mares can only produce a single foal per year whereas over 90% of mares become pregnant by the end of the breeding season (Bosh *et al.* 2009). However, stallions typically cover many mares in a season and because the intrinsic fertility of individual stallions varies greatly stallion identity has a significant effect on the overall reproductive efficiency (Allen *et al.* 2007). Allen *et al.* (2007) included data from 36 different thoroughbred stallions that covered at least 30 mares during a single breeding season, and found per cycle pregnancy rates of individual stallions to range between 29% and 89%, although half the stallions ($n = 18$) achieved a per cycle pregnancy rate between 60% and 70% (Allen *et al.* 2007).

In contrast with *in vivo* fertility, the relative effects of individual mares and stallions on the overall efficiency of an OPU-ICSI program are not well documented, primarily because this breeding technique has only recently been used on a

commercial scale. In addition, several additional factors may significantly affect the success of equine IVEP (i.e. OPU procedure, stage of oocyte maturation at collection, ICSI laboratory expertise, sperm preparation etc.), which makes meaningful comparison between studies difficult. However, to best inform and manage clients' expectations, it is important to identify the primary factors that influence the outcome of an OPU-ICSI session (i.e. likelihood of producing a transferable embryo), so that a better prognosis or recommendation can be made on a case-by-case basis.

The objective of the present retrospective study was to identify the major factors influencing the success and efficiency of a commercial OPU-ICSI program, measured in terms of blastocysts per injected oocyte and the number of embryos produced per OPU-ICSI session.

Materials and methods

Animals

In all, 323 mares with a mean (±s.d.) age of 13.0 ± 6.3 years (range 1–29 years) were subjected to OPU-ICSI between February 2017 and the end of January 2019 on 1–10 occasions (a total of 552 OPU cycles). Most of the mares (99.1%) were warmbloods (show jumping or dressage type) with a small percentage of Arabian horses (0.9%); they were presented for clinical IVEP for one of three major reasons, or combination of the three: (1) the mare was actively participating in high-level sporting competition and it was therefore not desirable that she carry a foal to term; (2) the mare had a previous history of subfertility or infertility (e.g. due to advanced age, recalcitrant uterine infection or infertility of unexplained origin); and (3) for efficient use of spermatozoa of limited availability (e.g. from deceased stallions or from semen doses of high economic value).

Following each OPU procedure, ICSI was performed using frozen–thawed semen from one of 114 different stallions, matching the breed and type of donor mare and chosen by the mare's owner. ICSI performed with spermatozoa subjected to two cycles of freeze–thawing ('refrozen' semen) was not used in the analysis.

Ovum pick-up

Perioperative antibiosis, analgesia and sedation were initiated by administering gentamycin sulfate (6.6 mg kg^{-1}, i.v.) and benzyl penicillin sodium (20 000 IU kg^{-1}, i.v.), flunixin meglumine (1.1 mg kg^{-1}, i.v.; Dechra Veterinary Products), detomidine hydrochloride (0.01 mg kg^{-1}, i.v.; Dechra Veterinary Products) and butorphanol tartrate (0.01 mg kg^{-1}, i.v.; Intervet International) immediately before the OPU procedure. Caudal epidural anaesthesia was induced with 8 mL of 2% lidocaine hydrochloride (Bela-Pharma). After aseptic preparation of the perineum, a urinary catheter was inserted. OPU was performed by transvaginal ultrasound-guided follicle aspiration using a 12-G double-lumen needle attached via a collection bottle to a vacuum pump (Cook Medical Europe) set to aspirate 20 mL fluid in approximately 40 s. All visible antral follicles (i.e. >2–3 mm) were punctured and follicular fluid was aspirated; when the follicle was empty, the needle was vigorously rotated in both directions and each follicle was flushed eight times with 0.5–5 mL (depending on follicle size) oocyte flushing medium (Equipro OPU Recovery Medium; Mofa Group) prewarmed to 37°C. The needle was rotated again at the end of each flush. Follicular fluid and flushing medium were collected into sterile 500-mL glass bottles prewarmed to 37°C and then maintained in a polystyrene box containing prewarmed gel packs to buffer the drop in temperature towards 22°C. The collected fluid was poured through a sterile 70-μm embryo filter (Emcom; IMV Technologies Netherlands) immediately after the end of the OPU procedure. The filtered contents were emptied into a sterile Petri dish, and oocytes were identified under a stereomicroscope (Olympus SZ60; Olympus Nederland), washed three times with modified HEPES-buffered synthetic oviductal fluid (mH SOF), transferred into a 2.5-mL cryovial containing mH SOF (Ritchie 2006; Galli *et al.* 2014) and shipped overnight at 22°C in a polystyrene box designed for transporting organs for transplantation (ChillTherm; Sonoco Thermosafe) to a dedicated equine ICSI laboratory in Italy (Avantea) for IVM, ICSI, *in vitro* culture (IVC) of embryos and embryo cryopreservation.

IVM, ICSI, IVC and embryo cryopreservation

IVM of oocytes to the MII stage and Piezo-driven ICSI and IVC to produce blastocysts were performed as described previously (Colleoni *et al.* 2011). ICSI was performed using frozen–thawed spermatozoa following gradient selection and swim-up (Colleoni *et al.* 2011). Blastocysts were identified on Days 6, 7 or 8 after ICSI, cryopreserved by slow freezing in 10% glycerol and returned to Utrecht University in liquid nitrogen for storage and subsequent embryo transfer.

Experimental design

The following variables and factors were recorded during each OPU cycle for future analysis of the efficiency of OPU-ICSI: age of mare, consecutive number of OPU cycles, stallion used for ICSI, number and size of aspirated follicles (diameter <10 vs ≥10 mm) and date of the OPU (divided into 2-month periods: January–February, March–April, May–June, September–October and November–December) to examine the effect of time of year. No OPUs were performed during July and August. Finally, the 552 OPU cycles were divided into three consecutive chronological periods, with each period containing a similar number of mares ($n = 184$ cycles per period), so that potential overall improvements in OPU and ICSI laboratory performance could be accounted for.

To study the effect of individual mares and stallions on the success of blastocyst production, two different approaches were used. The first approach analyses outcome for mares subjected for two consecutive OPU-ICSI sessions ($n = 112$ mares; 224 cycles) in which the same stallion ($n = 37$) or two different stallions ($n = 75$) were used for ICSI in the two cycles. This enabled investigation of how changing the stallion used for ICSI affected the likelihood of success (the production of at least one blastocyst). The second approach compared mean blastocyst production for individual mares and stallions used for four or more OPU-ICSI sessions. For the analysis of individual stallions, only ICSI performed with oocytes from a mare on the first OPU cycle that the given combination was used was included.

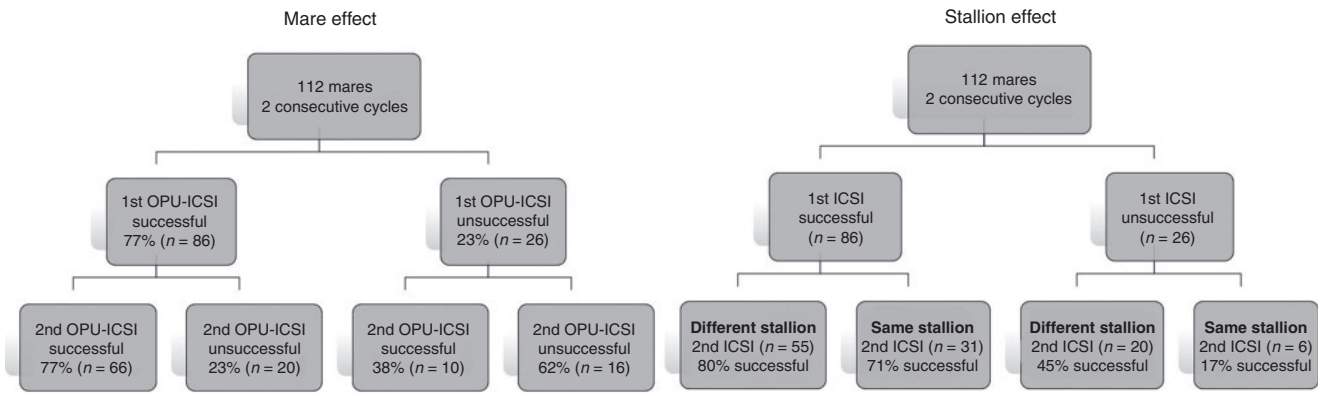

Fig. 1. Likelihood of success in a second ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) session for 112 mares divided on the basis of outcome of the first OPU-ICSI session, and regardless of the stallion used in the second session (mare effect; left panel) or taking into account the use of a different stallion in the second OPU-ICSI session (stallion effect). Successful OPU-ICSI was defined as the *in vitro* production of at least one Day 6–8 blastocyst. Unsuccessful OPU-ICSI sessions were defined as those that did not yield a blastocyst.

Outcomes

The following outcomes were evaluated:

- recovered oocytes (i.e. total number of oocytes recovered per OPU procedure)
- percentage oocyte recovery (number of oocytes recovered/number of follicles aspirated × 100)
- percentage of oocytes reaching the MII stage (number of oocytes (MII) injected/number of oocytes recovered; i.e. excluding oocytes that degenerated or failed to mature *in vitro*)
- percentage embryo cleavage (number of cleaved zygotes by Day 3 after ICSI/total number of injected (MII) oocytes × 100)
- percentage of blastocysts per injected oocyte (number of blastocysts produced 6–8 days after ICSI/total number of injected (MII) oocytes × 100)
- likelihood of success (calculated as the number of OPU-ICSI sessions resulting in the production of at least one blastocyst divided by the total number of OPU cycles (including those cycles in which ICSI was cancelled) and multiplied by 100).

Statistical analyses

Linear (continuous data) and logistic (binary data) regression models were used to test the effects of the various independent variables described above on the efficiency of the OPU-ICSI program in terms of blastocysts per injected oocyte, number of embryos per OPU-ICSI and the likelihood of success (one or more blastocysts). Paired Student's *t*-tests were used to determine the significance of differences in the number of oocytes between two consecutive OPU-ICSI cycles. Pearson's correlation for normally distributed data) and Spearman's ranked correlation (for data not normally distributed) were used to determine the repeatability of blastocysts per injected oocyte, and numbers of aspirated follicles and recovered oocytes between two consecutive OPU-ICSI cycles for individual mares. Significance was set at two-tailed $P \leq 0.05$, and a tendency was assumed for $P \leq 0.1$. Where appropriate, data are reported as the mean ± s.d.

Results

Over the 552 OPU cycles, the mean number of follicles aspirated was 22.2 ± 8.3 (range 7–67); this resulted in 12.8 ± 6.0 (range 0–49) recovered oocytes, with a mean oocyte recovery of 57.6% (range 0–100%). On five occasions (0.9%), ICSI was not performed after the OPU cycle either because of a failure to recover any oocytes ($n = 1$) or failure of any oocytes to reach the MII stage ($n = 4$) due to a low number of oocytes recovered (two mares with one oocyte each, and one mare with two oocytes) or degeneration of all oocytes recovered (one mare with 12 oocytes). The mean MII and cleavage rates were 58.6% (range 0–100%) and 68.7% (range 0–100%) respectively. The mean percentage of blastocysts per injected oocyte was 21.1% (range 0–100%), resulting in a mean of 1.6 ± 1.8 (range 0–10) embryos per OPU-ICSI session. The overall likelihood of success (i.e. ≥1 blastocyst) was 67.7%, with a mean of 2.4 embryos per successful OPU-ICSI session. The percentage of mares with none, one, two, three or four or more embryos per OPU-ICSI session was 32.3%, 27.0%, 16.2%, 11.6% and 12.9% respectively.

In the 112 mares in which two consecutive OPU-ICSI sessions were performed, the median interval between the first and second OPU sessions was 49 days (range 19–360 days). Mares with a successful first OPU-ICSI session (≥1 blastocyst) were more likely ($P < 0.001$) to have a positive outcome in the second OPU-ICSI cycle than mares with an unsuccessful first session (77% vs 38% chance of success respectively; Fig. 1). Changing the stallion for the second ICSI session did not affect ($P > 0.05$) the likelihood of success (Fig. 1). Conversely, the number of recovered oocytes did significantly effect the likelihood of success in the second cycle (Fig. 2). The strength of the association between the percentage of blastocysts per injected oocyte in the first and second OPU-ICSI sessions was weak ($r = 0.31$), and was not affected by the stallion used for ICSI (Fig. 3). The correlations for the number of follicles aspirated ($r = 0.63$; $P < 0.01$) and the number of oocytes recovered ($r = 0.53$; $P < 0.01$) between the first and second consecutive OPU-ICSI cycles were fairly high.

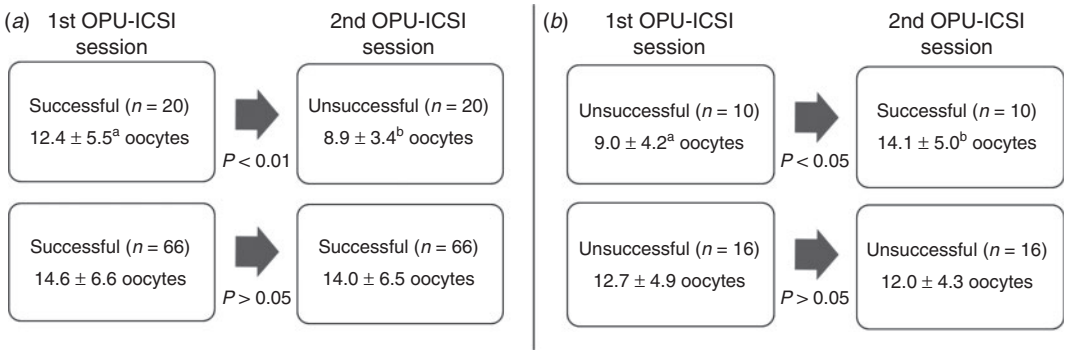

Fig. 2. Effect of the number of oocytes recovered on the likelihood of success in two consecutive ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) sessions in 112 mares with a (*a*) successful or (*b*) unsuccessful first OPU-ICSI session. Paired Student's *t*-test was used to examine whether there was a significant difference (*P*-values) in the mean number of oocytes recovered between the first and second OPU sessions. The number of oocytes is given as the mean ± s.d.

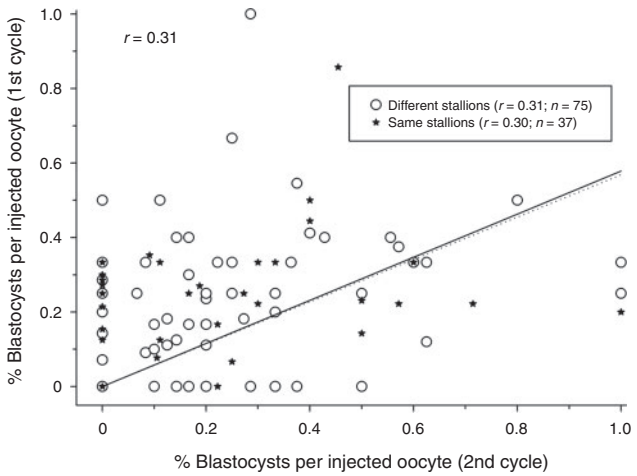

Fig. 3. Scatter plot of the percentage of blastocysts per injected oocyte in 112 mares during two consecutive ovum pick-up–intracytoplasmic sperm injection (ICSI) sessions, in which ICSI in the two cycles was performed with the same (stars) or different stallions (open circles). Spearman's ranked correlation coefficient (*r*) is shown. Regression lines of best fit for the percentage of blastocysts per injected oocyte for the two cycles with a similar (dotted) or different stallion (solid) are shown.

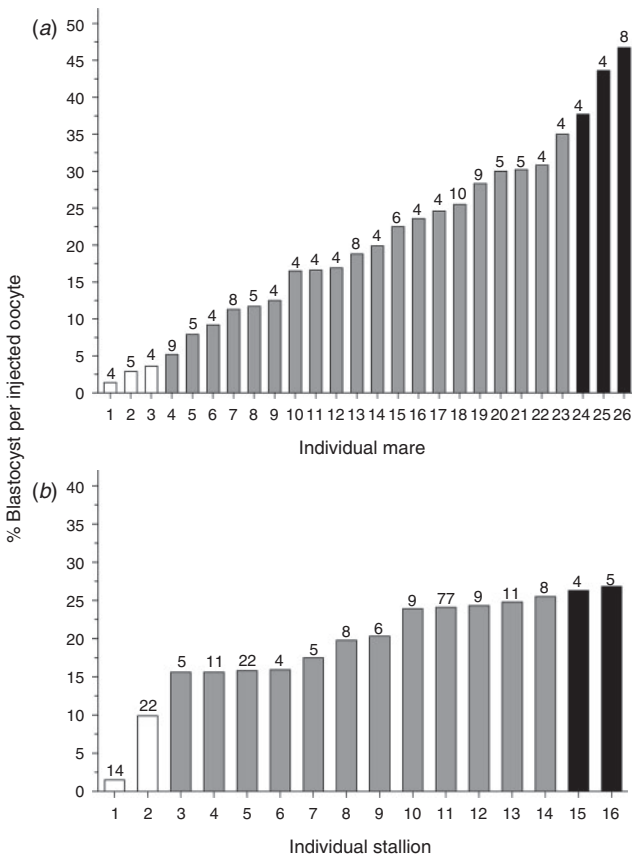

Fig. 4. Mean percentage of blastocysts per injected oocyte for individual (*a*) mares and (*b*) stallions used for four or more intracytoplasmic sperm injection (ICSI) sessions. In the stallion group, all ICSI sessions were performed with oocytes from different mares. The number of cycles for each individual mare and stallion is shown at the top of each column. Grey columns indicate the mean blastocyst rate for 75% of the population investigated.

The mean percentage of blastocysts per injected oocyte for individual mares ($n = 26$) and stallions ($n = 16$) subjected to four or more OPU-ICSI sessions ranged from $1.4 \pm 3.3\%$ to $46.7 \pm 12.8\%$ (75% of mares fell within the range 5–35%) for mares and from $1.5 \pm 4.3\%$ to $26.8 \pm 7.1\%$ (75% of stallions fell within the range 15–25%) for stallions (Fig. 4). The OPU-ICSI parameters for individual mares and stallions grouped or ranked according to mean blastocyst production per injected oocyte are given in Tables 1 and 2 respectively. The variation in percentage of blastocysts per injected oocyte over the consecutive OPU-ICSI sessions for four individual mares with varying blastocyst rates per injected oocyte is shown in Fig. 5. Of the 26 individual mares included with four or more OPU-ICSI sessions, eight (31%) yielded at least one embryo in every session, 10 mares (38%) yielded one embryo in all but one session, four (15%) yielded one embryo in approximately half the OPU-ICSI sessions (range 50–66%) and the remaining four mares (15%) had only one successful session. Furthermore, of the 26 individual

Table 1. Parameters for mares subjected to four or more ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) sessions classified into groups according to percentage of blastocysts per injected oocyte

Data show mean values, with the range given in parentheses. Within columns, different letters indicate significant differences ($P < 0.05$). Poor, <15% blastocysts per injected oocyte; medium, 15–30% blastocysts per injected oocyte; good, >30% blastocysts per injected oocyte

	No. mares	No. OPU sessions	Age (years)	No. follicles aspirated	No. oocytes recovered	MII (%)	Cleavage (%)	Blastocyst (%)	No. embryos per OPU session	Success rate (%)
Poor	9	5.8 (4–8)	13.3 (4–23)	22.1[a] (12–37)	12.9[a] (5–23)	51.3 (38–68)	60.3[a] (40–84)	7.1 (1.4–12.5)	0.52[a] (0–2.1)	41.0[a] (20–80)
Medium	10	5.2 (4–10)	16.9 (5–25)	21.2[a] (17–26)	12.8[a] (9–18)	62.1 (56–76)	63.5[a] (50–81)	21.3 (16.5–28.3)	1.6[b] (1–2.2)	80.8[b] (70–100)
Good	7	5.6 (4–9)	14.4 (11–21)	25.2[b] (17–39)	15.1[b] (8–20)	60.2 (50–68)	73.0[b] (66–88)	36.3 (30–46.7)	3.1[c] (1.8–4.2)	96.8[b] (78–100)

Table 2. Parameters for individual stallions used for intracytoplasmic sperm injection (ICSI) on oocytes from four or more mares for the first time they were subjected to ovum pick-up–ICSI

Stallion ID	No. mares	Mean no. injected oocytes	Cleavage (%)	Blastocyst (%)	No. embryos per ICSI	Success rate (%)
1	14	7.6	42.9	1.5	0.2	7.1
2	22	7.1	65.0	9.9	0.7	50.0
3	11	5.4	47.1	15.6	0.9	54.5
4	5	9.0	57.0	15.6	1.4	60.0
5	22	7.2	77.1	15.8	1.2	50.0
6	4	12.0	34.0	15.9	1.8	100
7	5	7.6	53.6	17.5	1.4	80.0
8	8	5.1	75.2	19.8	0.9	62.5
9	6	9.0	95.9	20.3	2.2	83.3
10	9	6.7	58.2	23.9	1.8	66.7
11	77	7.7	73.4	24.1	2.0	75.3
12	9	7.3	71.7	24.3	1.8	77.8
13	11	6.7	66.7	24.8	1.5	72.7
14	8	6.2	83.7	25.5	1.5	87.5
15	4	5.8	79.9	26.3	1.5	75.0
16	5	6.8	75.2	26.8	1.3	80.0

mares with four or more OPU-ICSI sessions, three (11.5%) yielded no embryo despite producing nine or more oocytes in at least 50% of their OPU sessions. Finally, of those 26 mares, two (7.7%) yielded eight or fewer oocytes in every OPU session; these two mares were 4 and 23 years old at the beginning of the study, with four and five OPU sessions each and success (i.e. embryos produced) in one in four (25%) and two in five (40%) sessions respectively.

The number of the OPU cycle (first vs second vs third or higher) did not affect ($P > 0.1$) the percentage of blastocysts per injected oocyte (20.1%, 22.4% and 22.4% respectively), the number of embryos per OPU-ICSI session (1.6, 1.6 and 1.8 respectively) or the likelihood of success (65.6%, 68.4% and 73.0% respectively).

The number of follicles aspirated ($P < 0.001$; odds ratio (OR) 1.051, 95% confidence interval (CI) 1.026–1.077; Fig. 6) and the number of oocytes recovered ($P < 0.001$; OR 1.132, 95% CI 1.089–1.178; Fig. 7) affected the likelihood of success. There was a stepwise increase in the likelihood of success with the number of aspirated follicles up until approximately 20 follicles per mare. However, increasing the number of follicles aspirated above 21 was not accompanied by a further increase in the likelihood of success (Fig. 6). Similarly, the likelihood of success increased in a stepwise manner with the number of oocytes recovered: success ranged from 10% to 35% for mares with less than five oocytes, from 45% to 55% for mares with five to eight oocytes collected and >60% when nine or more oocytes were recovered (Fig. 7). The diameter of the aspirated follicles (<10 vs ≥10 mm) affected oocyte recovery efficiency ($P = 0.001$; higher recovery efficiency when the percentage of <10-mm follicles was higher) and the total number of oocytes recovered ($P = 0.003$), but not the percentage of blastocysts per injected oocyte or the number of embryos per OPU-ICSI session ($P > 0.1$ for both; Table 3).

The time of year tended to affect ($P = 0.09$) the follicle count at the time of OPU (Table 4), with the highest number of aspirated follicles per OPU observed during May and June, and the lowest number in January–February. In addition, the time of the year affected ($P = 0.01$) both oocyte recovery efficiency and the percentage of aspirated follicles that had a diameter of <10 mm (Table 4). Conversely, the percentage of blastocysts per injected oocyte, embryos per OPU-ICSI session and the likelihood of success were not affected by time of year ($P > 0.1$; Table 4).

Increasing mare age was associated with a decrease ($P < 0.001$) in the number of follicles aspirated, reduced oocyte

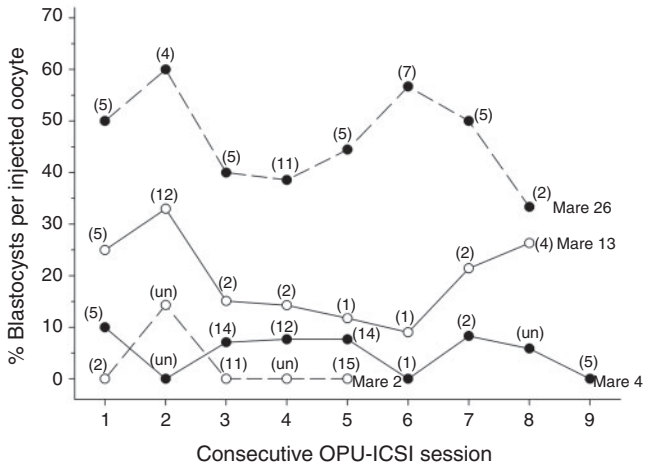

Fig. 5. Variation in the percentage of blastocysts per injected oocyte in four individual mares during all their ovum pick-up–intracytoplasmic sperm injection (ICSI) cycles during the study period. Numbers in parentheses are the ID for the stallion used in each ICSI session (see Table 2). Some stallions were unclassified (un), because they were used for ICSI on fewer than four mares. The age and mean number of oocytes recovered per session for the four individual mares were as follows: Mare 2, 6 years and 10.6 oocytes; Mare 4, 16 years and 22.9 oocytes; Mare 13, 18 years and 15.7 oocytes; Mare 26, 17 years and 13.1 oocytes.

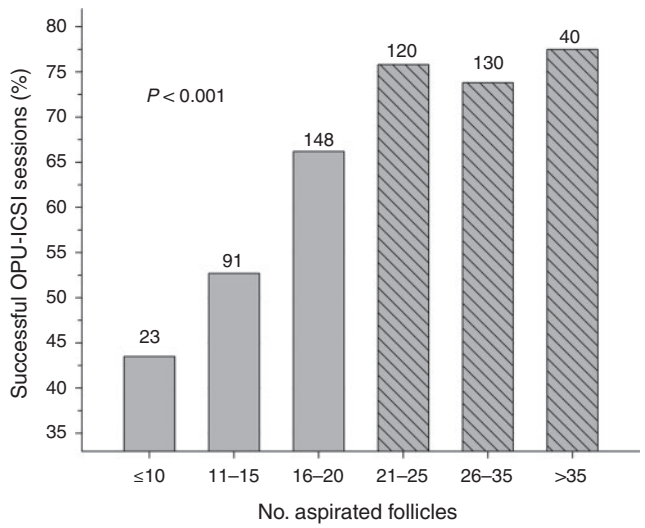

Fig. 6. Effect of the number of aspirated follicles on the likelihood of success of an equine ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) session (i.e. the production of at least one blastocyst). Numbers at the top of the columns indicate the number of OPU-ICSI cycles for each group of aspirated follicles. The logistic regression model showed a significant ($P < 0.001$) association between the number of follicles aspirated and the likelihood of success, although the likelihood of success in OPU-ICSI sessions with ≥21 aspirated follicles did not differ ($P > 0.1$).

recovery efficiency and a lower number of oocytes recovered (Fig. 8). In contrast, mare age did not affect the number of embryos per OPU-ICSI session, the blastocysts per injected oocyte or the likelihood of success when the regression model

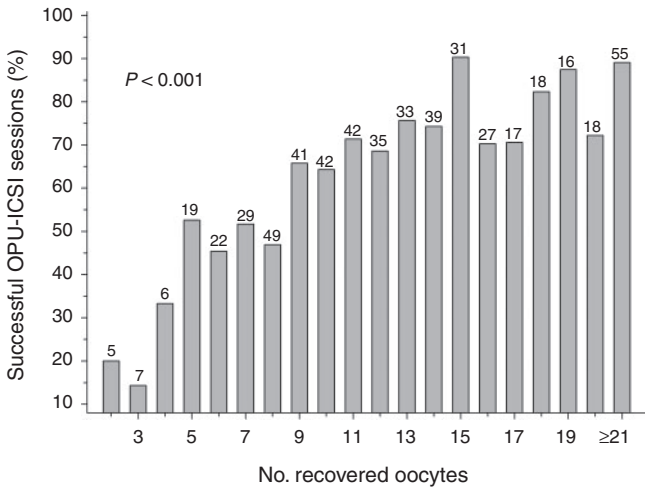

Fig. 7. Effect of the number of recovered oocytes on the likelihood of success of an equine ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) session (i.e. the production of at least one blastocyst). Numbers at the top of the columns indicate the number of OPU-ICSI cycles for each group of recovered oocytes. The logistic regression model showed a significant ($P < 0.001$) association between the number of oocytes recovered and the likelihood of success.

was adjusted for the number of oocytes injected ($P > 0.1$ for all; Table 5).

Finally, the period within the study during which the OPU was performed affected the success of the OPU-ICSI program (Table 6), with an overall improvement in success during the last third of the study (most recent period between November 2018 and February 2019).

Discussion

It appears that once an ICSI laboratory is established and is able to achieve consistent and commercially viable results (i.e. ≥1.5 blastocysts per OPU-ICSI session appears to meet warmblood mare owner expectations), the main two factors affecting the likelihood of success in a commercial OPU-ICSI program are mare identity and the number of oocytes recovered, where the latter is primarily a function of the mare's antral follicle count. Given a subjective threshold number of recovered oocytes (i.e. at least nine oocytes for a warmblood mare to achieve the mean percentage of success of the whole population, ~65% of sessions with at least one blastocyst), most mares (~65%) will yield at least one embryo, and will do so repeatedly as long as they have enough follicles to reach the minimum oocyte threshold. On occasions, some of these mares may fail to yield enough oocytes (i.e. less than eight oocytes) for various reasons (poor oocyte recovery rate, too few follicles at the time of OPU etc.), leading to failure of the OPU-ICSI procedure to yield an embryo.

Conversely, a small percentage of mares (~10%; 3/26 (12%) in the present study) fail to produce an embryo despite yielding sufficient oocytes (i.e. nine or more). Most breeders may give up after one or two failed attempts. However, in some cases, due to the high financial or genetic value of the mare or the impossibility of obtaining offspring via alternative methods, an owner may

Table 3. Effect of the percentage of small follicles aspirated on oocyte developmental competence

Data show mean number per OPU session performed in a group of mares. % Follicles <10 mm (of all follicles aspirated) at the time of OPU. NS, not significant ($P > 0.1$). OPU, ovum pick-up.

Variable	% Follicles <10 mm (of all follicles aspirated)						*P*-value
	100	80–99	60–79	40–59	20–39	0–19	
No. OPU sessions	17	59	159	182	120	15	–
No. follicles aspirated	17.8	23.0	23.5	22.8	21.4	21.3	NS
No. oocytes recovered	12.8	14.4	13.9	12.7	11.9	10.8	0.003
Oocyte recovery (%)	67.2	60.7	58.4	56.5	55.9	49.2	0.001
MII (%)	49.9	56.8	57.6	59.2	62.7	60.5	0.001
Cleavage (%)	66.1	72.1	70.6	67.3	66.1	77.5	NS
No. embryos per OPU session	1.6	1.8	1.8	1.5	1.5	1.7	NS
Blastocyst (%)	22.5	20.9	23.1	19.7	20.2	23.7	NS

Table 4. Effect of time of year on various ovum pick-up (OPU)–intracytoplasmic sperm injection parameters

Data show mean number per OPU session performed in mares, within the given time period. NS, not significant ($P > 0.1$)

	Jan.–Feb.	Mar.–Apr.	May–June	Sep.–Oct.	Nov.–Dec.	*P*-value
No. OPU sessions	184	91	30	73	168	
No. follicles aspirated	21.2	22.6	24.9	23.1	22.5	0.09
No. oocytes recovered	12.7	12.3	13.6	12.5	13.2	NS
% Follicles <10 mm	59.2	50.7	49.7	50.3	56.4	0.01
Oocyte recovery (%)	59.6	54.4	54.5	56.2	58.5	0.01
Blastocyst (%)	22.2	16.7	20.3	19.8	22.9	NS
No. embryos per OPU session	1.7	1.3	1.6	1.5	1.8	NS
Success rate (%)	69.0	56.5	73.3	67.1	71.7	NS

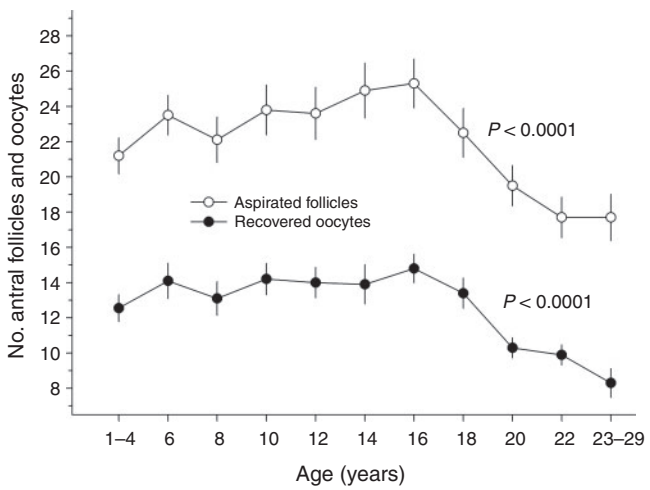

Fig. 8. Mean (± s.d.) number of follicles aspirated and oocytes recovered in ovum pick-up cycles from mares of different ages. Mares aged from 5 to 22 years are combined in 2-year groups (5–6, 7–8, 9–10 years etc.). Mare age significantly affected the number of follicles aspirated and oocytes recovered ($P < 0.0001$).

continue to try OPU-ICSI; in these cases, the ultimate success rate (production of at least one blastocyst) was approximately 20–25% (i.e. four to five OPU-ICSI sessions were needed to obtain at least one embryo). Finally, some mares (usually very young or of advanced age) have a poor success rate (<40% of sessions yield one or more blastocyst), but in these cases the primary contributing factor appears to be a low number of antral follicles and, in turn, a low number of oocytes recovered.

The observation that the identity of the mare is one of the main predictors of OPU-ICSI success contrasts with the results of *in vivo* fertility surveys. In large thoroughbred farms, mares that fail to conceive at the first cover have the same chance of conceiving during the second oestrous cycle as the whole population had during the first cycle (Allen *et al.* 2007; Mateu-Sánchez *et al.* 2016). That is, the first cycle pregnancy rate (338/498; 67.9%) was no different to the second cycle pregnancy rate (98/160; 61.3%; Mateu-Sánchez *et al.* 2016). In contrast, for OPU-ICSI the likelihood of success for the first session was 77%, whereas in mares that yielded no embryo during the first OPU-ICSI session the likelihood of success at the second session was only 38%. This implies the existence of some factor(s) intrinsic to individual mares that affects the developmental competence of the oocytes recovered.

The main effect of increasing mare age on reproductive efficiency in the OPU-ICSI program was a lower number of follicles that could be aspirated. The effect of mare age on the antral follicle number has been documented previously (Claes *et al.* 2015). The reduction in antral follicle count in aged mares results in fewer oocytes being recovered per OPU, and therefore fewer oocytes injected and fewer embryos produced per ICSI session. Conversely, increasing mare age did not appear to have

Table 5. Effect of mare age on the efficiency of a commercial ovum pick-up (OPU)–intracytoplasmic sperm injection program

	Age of mare (years)											*P*-value
	1–4	5–6	7–8	9–10	11–12	13–14	15–16	17–18	19–20	21–22	23–29	
No. OPU sessions (n)	49	47	44	41	43	55	73	68	50	45	37	
Mean no. aspirated follicles	21.2	23.5	22.1	23.8	23.6	24.9	25.3	22.5	19.5	17.7	17.7	0.0001
Mean no. recovered oocytes	12.5	14.1	13.1	14.2	14.0	13.9	14.8	13.4	10.3	9.9	8.3	0.0001
Mean oocyte recovery (%)	58.8	60.3	59.2	61.7	59.2	57.6	58.9	59.6	54.4	54.6	45.7	0.001
Mean no. day 6–8 blastocysts per OPU-ICSI session	1.9	1.6	1.6	1.7	2.4	1.8	2.0	1.6	1.0	1.3	0.9	0.389
Blastocysts per injected oocyte (%)	24.9	19.4	21.2	18.0	27.8	22.9	24.1	19.3	15.1	19.7	17.4	0.131
Likelihood of succesful OPU-ICSI session*	71.0	70.2	68.2	73.2	81.4	71.0	75.3	67.6	50.0	66.7	43.2	0.127

*% of sessions with 1 or more blastocysts

Table 6. Effect of time after onset of the program on ovum pick-up (OPU)–intracytoplasmic sperm injection (ICSI) efficiency

A, February 2017–January 2018; B, February 2018–October 2018; C, November 2018–January 2019; NS, not significant ($P > 0.1$)

Variable	Period of study			*P*-value
	A	B	C	
No. OPU sessions	184	184	184	–
No. oocytes per OPU	13.1	12.7	12.6	NS
MII (%)	58.6	60.1	57.2	NS
Cleavage (%)	64.7	67.4	74.3	<0.0001
Blastocyst (%)	17.9	18.1	27.1	<0.0001
No. embryos per OPU-ICSI	1.43	1.42	2.04	0.001
Success rate (%)	64.3	63.0	75.7	0.02

a significant effect on the developmental competence of the oocytes (blastocysts per injected oocyte). These results broadly agree with previous studies in which ICSI was performed following aspiration of immature (Cuervo-Arango *et al.* 2019*a*) or preovulatory (Altermatt *et al.* 2009) follicles. It appears that OPU-ICSI somehow bypasses the negative effects of advanced mare age on embryo quality (Claes *et al.* 2019) that are observed following *in vivo* fertilisation (Cuervo-Arango *et al.* 2019*a*) and, because they cannot be remedied by transfer to the oviduct of a young mare, are assumed to originate from mare age-related oocyte defects (Carnevale and Ginther 1995).

There is also a stallion effect on the efficiency of OPU-ICSI, as evidenced by the considerable differences in the mean percentage of blastocysts per injected oocyte between individual stallions when analysing the first ICSI session performed on oocytes from at least four different mares. However, stallion identity appeared to have a less marked effect on the overall efficiency than either mare identity or the number of oocytes recovered. This conclusion is supported by two observations: (1) most individual stallions used for ICSI on oocytes from four or more different mares yielded a blastocyst rate somewhere between 15% and 27% (14/16 stallions; 88%), and a success rate (one or more embryos) of 50–80% (12/16; 75%), which is very close to the mean for the entire dataset in terms of blastocyst production and likelihood of success (21.1% and 67.7% respectively); and (2) changing stallion for a subsequent ICSI session did not significantly affect the outcome for 112 mares with records of two OPU-ICSI sessions. Furthermore, eight of 26 mares on which OPU-ICSI was performed four to eight times yielded at least one blastocyst in all cycles, despite using different stallions. Conversely, there was one stallion that performed extremely poorly (1.5% blastocysts per injected oocyte and 7.1% successful cycles following ICSI on oocytes from 14 different mares), and another stallion with fairly poor performance (9.9% blastocysts per injected oocyte and 50% success rate over 22 different mares). These two stallions were clearly below the mean for the rest, and can therefore be considered outliers. Nevertheless, it is clear that certain stallions (~10–15% of the population in the present study) may severely negatively affect the efficiency of ICSI. For mares that fail to produce embryos following ICSI with such stallions, despite an adequate number of oocytes recovered, it would be advisable to try a different stallion of known acceptable ICSI performance. One limitation of the present study is that different straws from individual stallions, which could have been frozen at different locations and times, were used, but this was not accounted for during analysis. This could have been a confounding factor for the performance of individual stallions, as has been reported previously (Galli *et al.* 2016).

The time of year when the OPU-ICSI sessions were performed did not have an apparent effect on oocyte developmental competence. Moreover, there appeared to be only a minor effect of month on the antral follicle count, with the number of follicles aspirated tending to be higher in the months of May and June compared with the rest of the year. The present study showed an effect of month on oocyte recovery, with the highest percentage of oocyte recovery per aspirated follicle in the months of November–February (non-breeding season), when it is more likely that mares are in anoestrus (Ginther 1992). This higher oocyte recovery in the winter months may reflect a higher proportion of small follicles among the total population of aspirated follicles in non-cyclic mares (Purcell *et al.* 2007; Iacono *et al.* 2014). In fact, the results of the present study suggest that oocyte recovery in mares in which all follicles aspirated were <10 mm in diameter was higher than in mares with larger follicles (i.e. >80% of aspirated follicles >10 mm in diameter; 67.2% vs 49.2% respectively). It is possible that scraping of the follicle wall is more thorough (and therefore more effective in detaching the oocyte) in small follicles because the needle's bevel can reach and scrape a greater

proportion of the surface of the wall than in larger follicles as it is rotated during the collapse and refilling of the follicle, resulting in more efficient oocyte recovery. An abattoir study (Hinrichs and Schmidt 2000) similarly showed no difference in the number of follicles between the breeding and non-breeding seasons, whereas follicle size has been reported to be smaller in mares subjected to OPU during anoestrus compared with cyclic mares (Iacono *et al.* 2014).

Unfortunately, the reproductive phase of mares (anoestrus, oestrus or dioestrus) at the time of OPU was not recorded during the present study. However, it is likely that a higher proportion of mares in January and February would have been in transitional anoestrus (Ginther 1992), and yet still showed similar blastocyst per injected oocyte percentages and yielded a similar number of embryos per OPU-ICSI as mares treated during the breeding season (i.e. May–June and September–October). These results corroborate a previous report in which stage of the breeding season (transitional vs cyclic mares) did not affect the developmental competence of oocytes, measured as blastocyst production (Suh *et al.* 2006). Conversely, Colleoni *et al.* (2004) suggested that although oocytes collected during the non-breeding season were just as capable of completing meiosis during IVM, they were less capable of communicating with the surrounding cumulus cells than oocytes collected during the breeding season. However, Colleoni *et al.* (2004) did not use ICSI to determine whether the observed differences affected blastocyst production efficiency between oocytes collected during the breeding and non-breeding seasons.

In our clinical program, oocytes were not collected separately according to follicle size and our ability to establish an association between the diameter of the aspirated follicle and the developmental competence of the recovered oocytes was limited to an association with the percentage of small follicles (<10 mm) among the total population of aspirated follicles. Nevertheless, during 17 OPU session (17 different mares) only small follicles were aspirated (<10 mm in diameter); these yielded a similar blastocyst per injected oocyte rate (22.5%) to the rest of the population. Previous studies using abattoir material, in which the oocytes could be collected separately and classified according to the size of the follicle, reported a lower IVM rate for oocytes recovered from follicles <10 mm in diameter (Del Campo *et al.* 1995; Brück *et al.* 1996; Hinrichs and Schmidt 2000). Moreover, Hinrichs (1991) showed a positive correlation between follicle size and the percentage of viable oocytes, with 21%, 42% and 83% of oocytes classified as viable for follicles with a diameter <10, 10–19 and ≥20 mm respectively. In contrast, Suh *et al.* (2006) reported no difference in the percentage of oocytes reaching the MII stage or in the developmental competence (blastocyst production) of oocytes recovered from follicles 10–20 or >20 mm in diameter. The present study indicated a lower MII percentage for OPU sessions with a higher proportion of small (<10 mm) follicles, but no effect on blastocyst production per injected oocyte. The discrepancies between studies may indicate that the size of the follicle *per se* is not a good indicator of the developmental competence of the recovered oocyte; instead, whether the follicle is growing or regressing (becoming atretic) may be more predictive. Further research should be conducted to map and follow-up follicles over time before aspiration to elucidate the relationship between size, phase within a follicular wave (growing or decreasing in size) and developmental competence of the recovered oocytes.

The clear increase in overall efficiency of the ICSI program observed in the last period of the study (November 2018 to January 2019) indicates continuing improvement in the skills of the laboratory staff where all oocytes were sent. In fact, since the start of the combined Utrecht–Avantea OPU-ICSI program at the end of 2014, with over 1000 OPU-ICSI sessions performed, the overall efficiency of the commercial program has increased gradually but markedly. The earlier years (2014–16) of our program yielded 0.95 embryos per OPU with 12.2% blastocysts per injected oocyte and a likelihood of success of 52.1% over the first 400 OPU-ICSI sessions. This has improved markedly over the past 2 years (1.63 embryos per OPU, 21.1% blastocyst per injected oocyte rate and 67.7% success), which may reflect changes in the OPU procedure (Cuervo-Arango *et al.* 2019*b*) and probably indicates further potential room for improvement, not least because the success of the program improved further in the last 3 months of the study period. One possible confounding factor is the expectation that more fertile or successful mares are more likely to be subjected to repeated OPU-ICSI.

The main limitation of the present study is its retrospective nature and the fact that the ICSI laboratory was external, such that subtle changes (not known to the authors) in procedures for the processing of the oocytes, spermatozoa and embryos (IVM, ICSI and IVC) may have affected the results.

In conclusion, mare identity and the number of oocytes recovered per OPU appear to have the greatest effect on the likelihood of success within a commercial OPU-ICSI program. The individual stallion, although relevant, had a less marked effect on the overall efficiency of the program, with only two stallions (12.5%) identified as poor blastocyst producers. The number of follicles aspirated (affected by mare age) was highly correlated with the number of oocytes recovered, and therefore with the likelihood of success. However, mare age, time of year during which OPU was performed or the size of follicles (5–45 mm) did not appear to affect the developmental competence of the oocytes recovered.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This research did not receive any specific funding.

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