Retrospective study of factors affecting multiple ovulations, embryo recovery, quality and diameter in a commercial equine embryo transfer program

Panzani Duccio[1a,], *Rota Alessandra*[a], *Marmorini Paola*[b], *Iacopo Vannozzi, Camillo Francesco.*[a]

[a]Dipartimento di Scienze Veterinarie, Università degli Studi di Pisa, 56124 San Piero a Grado, Pisa (PI), Italy

[b]Private practitioner, Italy

[1]Corresponding author: Panzani Duccio, Dipartimento di Scienze Veterinarie, Università di Pisa, San Piero a Grado, Pisa, 56124, Italy; tel. +390502210164, fax +390502210182, d.panzani@vet.unipi.it

Abstract: In this study, 198 donor mares of different breeds, ages and reproductive category were inseminated with fresh, cooled, frozen or frozen and cooled semen at the embryo transfer station or in private AI centers during 10 breeding seasons. The results of this activity were retrospectively analyzed by Pearson Chi-Square test and logistic regression to evaluate factors affecting multiple ovulations, embryo recovery, embryo quality and embryo diameter. Out of the 661 cycles, 937 ovulations were recorded (mean ovulations/cycle: 1.42 ± 0.58). Ovulation rate and incidence of multiple ovulations were significantly affected by age, breed and reproductive category. Uterine flushings for embryo recovery were performed between 7 and 10 days after ovulation and resulted in the recovery of 338 embryos (51.1% embryos/cycle and 36.1% embryos/ovulation, respectively). At least one embryo was recovered in 298 flushings (45.1%). The factors affecting embryo recovery were age, breed, reproductive category, type of semen, number of ovulations and location of AI. Flushing protocol and day of flushing had no effect on embryo recovery. Age, type of semen, number of ovulations and day of flushing had a significant influence on embryo diameter (N= 215). None of the factors included in the model had an effect on embryo quality distribution.

Introduction

The first foal after embryo transfer (ET) was born in Italy in 1990 [1], two decades after the production by ET of the first hybrid and horse offspring in the world [2,3], and around 10 years after the first studbook (AQHA) admitted the registration of foals born with this procedure [4].

One of the major factors limiting the outcome of ET in mares is the lack of reliable protocols for induction of superovulation in clinical practice [5,6]. It was, on the other hand, well demonstrated that the occurrence of multiple ovulation enhance embryo recovery rate [7][8]: for this reason spontaneous multiple ovulations are highly desirable in embryo donors mares. Breed, age, reproductive status, season and the use of drugs to induce ovulation have been reported as affecting the number of double ovulations [9-15]

The effect on embryo recovery rate of age and intrinsic fertility of donor mares has been reported by many authors [16][17][5][18] who agreed that old age and a history of subfertility are related to a lower embryo recovery rate. On the other hand it was demonstrated that fillies as young as one year old are able to produce embryos, although at a lower rate, and that embryos can be recovered from two years old mares in a rate similar to the one of mature mares [19]. The effect reported in literature of sport activity on embryo recovery rate is controversial [20-22]while the effect of the donor's breed was only marginally investigated [23]

The use of fresh semen rather than chilled transported or frozen semen was associated with a higher embryo recovery rate in mares [5,17]. Finally, significant differences in embryo recovery were reported in some studies [24-26] but not in others [24-26] depending on the method used to flush the uterus.

The aim of this study was to retrospectively analyse some of the factors that affected ovulations (age, reproductive category and breed of the donor), embryo recovery (location of AI, flushing protocol, age, reproductive category and breed of the donor, type of semen, number of ovulations, day of flushing), embryo quality and diameter (age, reproductive category, breed of the donor, type of semen, number of ovulations, day of flushing) in a commercial ET program in mares.

Materials and Methods

During ten breeding seasons (2002-2011) 131 mares of different breeds (Arabian, Show Jumping, Haflinger, Quarter Horse and Standardbred) and aged 2-24 years, have been included as embryo donors into a commercial ET program at the former Dipartimento di Clinica Veterinaria of the Pisa University (Department). Thirty-eight mares were in the program for more than one season for a total of 198 donors entered in the program for the 10 seasons. To analyze the effect of the age, the donors were divided in the following classes: 2-10, 11-15, 16-20 and 21-24 years old. Nine mares

included for more than one year in the program changed age class in subsequent seasons.

Eighty-eight donors were housed, monitored and inseminated at Department (AI@department) and 110 were bred in different farms/AI centers and came to the Department only for the uterine flushing for embryo recovery (AI@home). Resident mares have been maintained in boxes during the nighttime and in paddocks during the daytime, and were fed with a balanced ratio of hay and commercial horse fodder.

General health and reproductive history were recorded and mares were submitted to a general and reproductive physical examination [27]. Reproductive examination included the perineum conformation and transrectal palpation and ultrasonographic examination of cervix, uterus and ovaries. Cytologic, bacteriologic and histologic uterine exams were performed when needed. Mares were included into 4 categories:

1. Healthy donors performing sport activity (n=39; SHD); all the donor of this category were non resident;
2. Healthy donors non performing sport activity (n=87; NSHD);
3. Donors with reproductive pathologies (mares with an history of endometritis and/or embryonic loss or abortion, n=45; or persistent mating induced endometritis, n=18; RPD);
4. Mares with non-reproductive pathologies (respiratory, n=3, neurologic or neuro-endocrine, n=5 and gastrointestinal, n=1, diseases; NRPD).

Twelve donors included for more than one year in the program changed reproductive category in subsequent seasons.

If a non-reproductive pathology was reported or discovered after the clinical examination, animals were submitted to a specific therapy. If a bacterial endometritis was diagnosed, specific intrauterine treatment was administrated. The chronic and/or non septic endometrites were treated during estrus with multiple uterine lavages with sterile saline and oxytocin [28,29]or infusions of a solution of saline and povidone iodine at 0.5-1% [28,30]. Mares showing post breeding induced endometrites, that recovered within 24 hours from AI spontaneously, or after uterine lavages and oxytocin, were considered as normal.

Donors' cycles were monitored by ultrasound and ovulations have been induced in estrus at the evidence of a follicle diameter ≥ 35 mm with 2000-3000 IU, iv of hCG (Vetecor 2000, Bio98, Bologna, Italy).

Artificial inseminations have been performed in the 48 hours or in the 24 hours before ovulation with fresh or cooled semen, respectively [31], or within 6 hours of ovulation when frozen semen was used [32]. In some cases, when mares didn't ovulate within 24 hours from a cooled semen AI, they were re-inseminated with a dose of frozen semen of the same stallion.

Embryo recovery: Donors were submitted to uterine flushing for embryo recovery between the 7th and the 10thday after ovulation. Flushes for embryo recovery were performed on the 9th and 10th day after ovulation on donors unable to provide embryos for at least 3 cycles or that produced very small embryos 8 or 9 days after ovulation, respectively. Donors were led to a stock, the rectum evacuated of feces, the tail wrapped and tied up, and the perineum washed three times with a povidone iodine soap (EsoformJod 75, Esoform S.p.a., Rovigo, Italy), rinsed and dried accurately.

For the first two years of the study donors' uteri were flushed three times for embryo recovery using a total of 3 L of DPBS flushing medium at 37°C added of 0.4% BSA (ZE067, IMV, L'Aigle, France) and a one-way tubing system (Equine Lavage Catether 32 Fr.®, Bivona, Gary, IN) without filter [33] (Flushing Protocol 1, FP1, n= 104 flushings). For the remaining part of the study uteri were washed up to 5 times with 1-2 liters (for maiden and foaled mare, respectively) of ringer lactate at 37°C (Galenica Senese, Siena, Italy) using a three ways 36Fr homemade silicon tubing system connected to a silicon cuffed 36 Fr catheter (Minitübe, Milan, Italy) and to an EZ-Way Filter (EZ-Way, A&E Int'l, Alta Vista, Kansas, USA) (Flushing Protocol 2, FP2, n=557 flushings). After the flushing, 3 mg/im alfaprostol (Gabbrostim, Vetem, Spa, Monza-Brianza, Italy) was administered to donors to induce luteolysis and embryos were searched by a stereomicroscope in a Petri dish (FP1) or directly into the filter (FP2) always under a laminar flow cap.

Recovered embryos were washed 10 times at 37°C with DPBS (IMV, L'Aigle, France), FP1, or EmCare Holding Solution (EHS; ICPbio, Ltd., Auckland, New Zealand), FP2, and submitted to morphological evaluation [34] by a stereomicroscope at 40 magnifications; in 215 occasions embryos were measured.

Statistical Analysis:

Data were analyzed using the software IBM® SPSS® Statistics (Version 22) and differences were considered statistically significant with $P<0.05$.

The ovulation rates were described as:

- Multiple ovulations = cycles in which > 1 ovulations occurred
- Ovulation rate: ovulations/cycles

The embryo recovery rates were described as:

- Positive flushings/cycles = flushings in which ≥ 1 embryo was recovered;
- Embryo recovery rate = embryos recovered/cycles;
- Embryo recovery per ovulation = embryos recovered/ovulations.

Pearson Chi-Square test has been used to evaluate differences between:

1. Donors age categories, breeds and reproductive catgories on ovulation rate and on the incidence of multiple ovulations ;
2. Location of AI, flushing protocols, donors age categories, breeds and reproductive categories, semen types, number of ovulations per cycle and days of flushing for embryo recovery;
3. Donors age categories, breeds, reproductive categories, semen types, number of ovulations per cycle and days of flushing for embryo quality.

To evaluate the influences of single factors in categories showing significantly different influencing factors after Pearson Chi-Square test

1. A forward stepwise univariate logistic regression model based on the Wald statistics criterion of P>0.10 was used to establish significant predictors for the incidence of multiple ovulations and positive flushes.
2. Logistic regression for ordinal responses, employing the negative Log-log function as the link function was employed on ovulation rate, embryos recovered per cycle and per ovulation.

Univariate ANOVA GLM and Fisher LSD post hoc test have been used to evaluate the influences of age categories, breeds, reproductive categories, semen types, number of ovulations per cycle and day of flushing on embryo diameter.

Results

Ovulation rates:

Out of 661 cycles, 937 ovulations were recorded (means 1.42 ± 0.58). The factors affecting the occurrence of multiple ovulations per cycle and ovulation rates are described in Tables 1 to 3.

177 *Table 1: Factors affecting the occurrence of multiple ovulations per cycle and ovulation rate in 661*
178 *cycles of 198 donors mares*

Age Class	**Multiple ovulations/cycle (%)**	**Ovulation rate (average ± sd)**
2-10	25/130 (19.23%)[a]	158/130 (1.22 ± 0.47)[a]
11-15	55/163 (33.74%)[b]	223/163 (1.37 ± 0.54)[a]
16-20	115/219 (52.51%)[c]	351/219(1.60 ± 0.64)[b]
21-24	52/149 (34.90%)[b]	205/149 (1.38 ± 0.54)[a]
Breed		
Show Jumping	206/453 (45.47%)a	685/453 (1.51 ± 0.61)a
Standardbred	21/85 (24.71%)b	107/85(1.26 ± 0.47)b
Quarter Horse	16/77 (20.78%)[b]	95/77 (1.23 ± 0.48)[b]
Haflinger	3/35 (8.57%)[b]	38/35 (1.09 ± 0.28)[a,b]
Arabian	1/11 (9.09%)[b]	12/11 (1.09 ± 0.30)[a,b]
Reproductive category		
SHD	17/76 (22.37%)[a]	94/76 (1.24 ± 0.46)[a]
NSHD	120/348 (34.48%)[b]	477/348 (1.37 ± 0.53)[a,b]
RPD	85/202 (42.08%)[b,c]	298/202 (1.47 ± 0.60)[b]
NRPD	25/35 (71.43%)[c]	68/35 (1.94 ± 0.80)[c]
Total	247/661 (37.37%)	937/661 (1.42 ± 0.58)

179 *[a,b,c]: Data designated by different superscripts differ significantly (p<0.05) Pearson Chi-Square test*
180
181 *Table 2: Factors significantly influencing the occurrence of multiple ovulations per cycle in 661*
182 *cycles of 198 donors mares*

		95% C.I.for Odds Ratio		
Category	B (Std. Error)	Lower	Odds Ratio	Upper
Age Class: 2-10	-1.10 (0.25)	0.20	0.33	0.54
Show Jumping	1.21 (0.20)	2.25	3.35	4.99
SHD	-0.85 (0.30)	0.24	0.43	0.77
NRPD	1.17 (0.398)	1.48	3.24	7.07

183 *R^2: 0.123 (Cox & Snell), 0.168 (Nagelkerke)*
184

Table 3: Factors significantly influencing the ovulation rate in 661 cycles of 198 donors mares

Category	Estimate	95% Confidence Interval	
		Lower Bound	Upper Bound
Age Class: 2-10	-0.74 (0.27)	-1,28	-0.21
Show Jumping	1.38 (0.59)	0.22	2.5
SHD	-0.65 (0.31)	-1.25	-0.06
NRPD	0.80 (0.25)	0.32	1.29

Link Function: Negative Log-log
R^2: 0.129 (Cox & Snell), 0.162 (Nagelkerke)

Embryo recovery:

Out of 661 flushings and 937 ovulations, 338 embryos were recovered (51.1% and 36.1% respectively). At least one embryo was recovered in 298/661 flushings (45.1%).

The factors affecting the occurrence of positive flushes and of embryos/cycles and embryo/ovulations rates are described in Tables 4 to 7

Table 4: Factors analyzed for embryo recovery in 661 cycles of 198 donors mares

	Positive flushes/cycle (%)	*Embryos/cycle(%)*	*Embryos/ovulations (%)*
Flushing protocol			
FP1	39/104 (37.5%)[a]	43/104 (41.3%)[a]	43/158 (27.2%)[a]
FP2	259/557 (46.5%)[a]	295/557 (53.0%)[a]	295/779 (37.9%)[a]
Age Class			
2-10	61/130 (46.9%)[a,b]	66/158 (41.8%)[a]	66/158 (41.8%)[a]
11-15	85/163 (52.1%)[a]	100/163 (61.3%)[b]	100/223 (44.8%)[a]
16-20	89/219 (40.6%)[b]	105/219 (47.9%)[a]	105/351 (29.9%)[b]
21-24	63/149 (42.3%) [a,b]	67/149 (45.0%)[a]	67/205 (32.7%)[a]
Breed			
Show Jumping	183/453 (40.4%)[a]	215/453 (47.5%)[a]	215/685 (31.4%)[a]
Standardbred	49/85 (57.6%)[b]	52/85 (61.2%)[b,c]	52/107 (48.6%)[b,c]
QuarterHorse	49/77 (63.6%)[b]	54/77 (70.1%)[b]	54/95 (56.8%)[b,c]
Haflinger	13/35 (37.1%)[a]	13/35 (37.1%)[a,c]	13/38 (34.2%)[a,c]

Arab	4/11 (36.4%)[a,b]	4/11(36.4%)[a,b,c]	4/12 (33.3%)[a,c]
Reproductive category			
SHD	41/76 (53.9%)[a]	42/76 (55.3%)[a]	42/94 (44.7%)[a]
NSHD	178/348 (52.6%)[a]	208/348 (59.8%)[a]	208/477 (43.6%)[a]
RPD	65/202 (32.2%)[b]	71/202 (35.1%)[b]	71/298 (23.8%)[b]
NRPD	14/35 (40.0%)[a,b]	17/35 (48.6%)[a,b]	17/68 (25.0%)[b]
Type of semen			
Fresh	81/148 (54.7%)[a]	90/148 (60.8%)[a]	90/197 (45.7%)[a]
Cooled	118/266 (44.4%)[a]	136/266 (51.1%)[a,c]	136/392 (34.7%)[b,c]
Frozen	87/226 (38.5%)[b]	99/226 (43.8%)[b,c]	99/318 (31.1%)[b,c]
Cooled+Frozen	12/21 (57.1%)[a]	13/21 (61.9%)[a,c]	13/30 (43.3%)[a,c]
Number of ovulations			
1	178/414 (43.0%) [a]	178/414 (43.0%)[a]	178/414 (43.0%)[a]
2	108/220 (49.1%) [a]	140/220 (63.6%)[b]	140/440 (31.8%)[b]
3	12/25 (48.0%) [a]	20/25 (80.0%)[b]	20/75 (26.7%)[b]
4	0/2 (0.0%) [a]	0/2 (0%)[a,b]	0/8 (0.0%)[a,b]
Day of flush			
7	16/35 (45.7%)[a]	16/35 (45.7%)[a]	16/47 (34.0%)[a]
8	253/546 (46.3%)[a]	287/546 (52.6%)[a]	287/770 (37.3%)[a]
9	21/63 (33.3%)[a]	27/63 (42.9%)[a]	27/99 (27.3%)[a]
10	8/17 (47.1%)[a]	8/17 (47.1%)[a]	8/17 (47.1%16)[a]
Location of AI			
AI@department	155/384 (40.4%)[a]	179/384 (46.6%)[a]	179/569 (31.5%)[a]
AI@home	143/277 (51.6%)[b]	159/277 (57.4%)[b]	159/368 (43.2%)[b]

Total	298/661 (45.1%)	338/661 (51.1%)	338/937 (36.1%)

196 [a,b,c]: Data designated by different superscripts differ significantly (P<0.05) *Pearson Chi-Square test*

197 *Table 5: Factors significantly influencing positive flushes in 661 cycles of 198 donors mares*

Category	B (Std. Error)	95% CI for Odds Ratio		
		Lower	Odds Ratio	Upper
AI@department	-0.456 (0.178)	0.449	0.634	0.895
Standardbred	0.569 (0.262)	1.057	1.767	2.953
RPD	-1.051 (0.191)	0.240	0.350	0.509
Frozen semen	-0.461 (0.178)	0.445	0.631	0.894
Number of ovulations	0.283 (0.143)	1.004	1.328	1.756

198 *R^2: 0.071 (Cox & Snell), 0.095 (Nagelkerke)*

199

200 *Table 6: Factors significantly influencing* embryo recovery per cycle *in 661 cycles of 198 donors*

201 *mares*

Category	Estimate (Std. Error)	95% Confidence Interval	
		Lower	Upper
Number of ovulations	0.41 (0.11)	0.20	0.62
Standardbred	0.90 (0.37)	0.18	1.62
NSHD	0.68 (0.17)	0.35	1.00
SHD	0.67 (0.26)	0.16	1.17
Frozen semen	0.73 (0.32)	0.09	1.36

202 *Link Function: Negative Log-log*

203 *R^2:0.094 (Cox & Snell), 0.114 (Nagelkerke)*

204

205

Table 7: Factors significantly influencing embryo recovery per ovulation in 661 cycles of 198 donors mares

		95% Confidence Interval	
Category	Estimate (Std. Error)	Lower	Upper
NSHD	0.55 (0.16)	0.222	0.869
SHD	0.58 (0.25)	0.078	1.076
Frozen semen	0.77 (0.31)	0.151	1.386

Link Function: Negative Log-log

R^2: 0.011 (Cox & Snell), 0.014 (Nagelkerke)

Embryo evaluation:

Three-hundred-and-twenty-six/338 (96.4%) recovered embryos were expanded blastocyst, 11/338 (3.2%) early blastocysts and 1/338 (0.02%) was a morula.

Embryo quality was grade I in 307/338 (90.8%) embryos, grade II in 18/338 (5.3%), grade 3 in 11/338 (3.2%) and grade IV in 2/338 (0.6%) embryos.

No differences has been found between categories in embryo quality (Pearson Chi-Square test, $P>0.05$).

Day of flushing ($P<0.0001$), age class ($P=0.0003$), type of semen ($P=0.0313$), number of ovulations ($P=0.0054$) had a significant influence on embryo diameter (Table 8), while donor's breed and reproductive category did not.

223 *Table 8: Factors affecting embryo diameter in 215 horse embryos*

Day of flushing	**Embryo diameter (µm, average± sd)**
7 (N= 12)	404.91 ± 306.50[a]
8 (N= 189)	660.29 ± 329.33[b]
9 (N= 10)	912.39 ± 665.11[c]
10 (N= 4)	1224.50 ± 821.04[d]
Age class	
2-10 (N= 47)	747.82 ± 332.62[a]
11-15 (N= 58)	740.81 ± 390.31[a]
16-20 (N= 70)	610.81± 363.25[b]
21-24 (N= 40)	570.13± 382.33[b]
Type of semen	
Fresh (N= 50)	752.5 ± 375.27[a]
Cooled (N= 96)	635.75 ± 423.79[a,b]
Frozen/thawed (N= 63)	638.61 ± 277.58[b]
Cooled + Frozen/thawed (N= 6)	797.77 ± 298.32[a,b]
Number of ovulations	
1 (N= 118)	681.46 ± 385.90[a]
2 (N= 87)	612.32 ± 362.54[a]
3 (N= 10)	1014.53 ± 711.89[b]
Total (N=215)	668.26 ± 373.15

224 [a,b,c]: Data designated by different superscripts differ significantly ($P<0.05$). Fisher LSD

225

Discussion:

In this study the effect of factors shown by the Pearson Chi-Square analysis to have a statistically significant influence on ovulation and embryo recovery rates were analyzed together by logistic regression in order to weight their influence on the outcome.

Ovulation rate:

Factors reported in literature to influence the number of double ovulations are breed, age, reproductive status, season and, potentially, treatments to induce ovulation [9-15]. This study was unable to test the last hypothesis as the donors' ovulations were always induced, however all the others tested parameters influenced significantly both ovulation rate and incidence of multiple ovulations, confirming the previous studies. Show Jumping mares had the highest frequency of cycles with multiple ovulations (45.47%) and the higher ovulation rate (1.51 ± 0.61), having a significant positive influence on them. Standardbred and Quarter Horses donors included in this study showed markedly higher ovulation rates than reported in literature [10] (24.71% vs 13-15% and 20.78% vs 8-10%, respectively); Haflingers and Arabians ovulations rates were similar to what previously reported for ponies [2,3,14] and Arabians [4,9].

In this study the incidence of multiple ovulations was significantly lower in the younger mares, ≤10 years old, compared to the older ones, aged 11-24 years, while the ovulation rate was significantly higher for the mares between 16 and 20^{t} years of age compared to the others. Younger mares showed also a significantly negative influence on multiple ovulations and ovulation rate.

Donors' reproductive category had a significant influence on multiple ovulations and ovulation rate. Mares with pathologies not affecting the reproductive system showed the highest incidence of multiple ovulations and a significant positive influence on multiple ovulations and on ovulation rates; this was probably due to the very small number of mares in this category, only 9, and to the large number of cycles (N= 23/35) of one donor with an high ovulation rate. The lower incidence of multiple ovulations and the negative influence on this parameter and on ovulation rates of healthy mares performing sport activity were consistent with their young age, being these donors in 67/76 cycles between 2 and 16 years old.

Embryo recovery:

The embryo recovery rate achieved in this study was similar to what described by Squires et al. [7,8,35] and by Stout [5,9-15] for a commercial equine ET program. The same authors reported day of recovery, number of ovulations, age of the donor, her reproductive history, and semen quality as the factors able to affect embryo recovery in mares. A significantly lower embryo recovery rate was

reported for day 6 compared to days 7 to 10 post ovulation: 42% versus 56-66%, using fresh semen [19,36].

In the present study positive flushing per cycle and embryo recovery per ovulation were not different between days 7, 8, 9 and 10. No flushings for embryo recovery were performed at day 6.

In this study, the occurrence of multiple ovulations enhanced embryo recovery rate, as previously described [5,7,8,17], however, the embryo recovery per ovulation was significantly lower after multiple ovulations compared to a single one. These data are similar to those of previous studies where multiple ovulations affected positively embryo recovery in non treated and superovulated mares [24-26,37]. In spontaneously multiple ovulating mares, ipsilateral double ovulations resulted in a lower embryo recovery rate and in more unsuccessful recovery attempts than bilateral double ovulations [24-26,37]. This is probably due to an alteration of the mechanism of oocyte transport to the oviduct, already described in superovulated mares, caused by an interference between two or more simultaneous ovulations in the limited space of the ovulation fossa [6,27,37,38].

The effect of age and intrinsic fertility of donors mares on embryo recovery rate have been reported by many authors [5,16-18,20,22,28,29,35,39]. All these studies agree that old age and a history of subfertility are related to a lower embryo recovery rate. Moreover, aged mares with fertility problems were reported to have a high rate of pregnancy loss between 2 and 14 days after ovulation [28,30,40]. In the present study healthy mares, performing or not sport activity, showed significantly higher positive flushings (flushing rates) than mares with a reproductive pathology; moreover healthy mares not performing sport activity showed an embryo recovery rate per ovulation almost double than mares with reproductive pathologies. Healthy mares, performing or not sport activity, had a positive influence on embryos per cycle and embryos per ovulations rate, while mares affected by reproductive pathologies had a negative influence on positive flushings rates.

Age classes influenced positive flushes, embryos/cycle and embryos/ovulation rates; in particular mares between 11 and 15 years old had a positive flushes rate and an embryo recovery per ovulation rate higher than mares between 16 and 20 years of age and the higher embryo recovery rate per cycle compared to all the others categories of age. This agree with the literature mentioned before in which young mares generally are intended as mares younger than 15 years old [5,8]; moreover the present study seems to suggest that the ideal age for a mare to be bred as embryo donors is between 11 and 15 years of age. This is probably due to the simultaneous presence in this category of age of a good quality of uterine environment and a multiple ovulation rate higher than younger mares. Partially in contrast with the literature already reported [5,16-18,20,22,31,35,39], in the present study the positive flushing and the embryo recovery per cycle rates were not different

between younger and older mares, 2-10 years old and 16-24 years old, respectively. This could be explained, once again, with the multiple ovulation rate higher in older than in younger mares and with the different management to which older mares were submitted: in particular any effort was made to use fresh or at least chilled semen instead of frozen semen in this age category.

In this study AI with fresh, cooled and the association of cooled plus frozen semen resulted in significantly more positive flushes per cycle than AI with frozen semen. Moreover, AI with fresh semen resulted in a significantly higher embryo recovery per cycle than frozen semen AI and in a significantly higher embryo recovery per ovulation than cooled or frozen semen AI. This result was expected as it is a common belief that fresh semen is more fertile than the frozen one, which in fact negatively influenced embryo recovery rate of this program [5,32]. Jasko et al. [41], inseminating the same mares in subsequent cycles with fresh, cooled and frozen/thawed semen of three stallions, reported a pregnancy rates of 76%, 65%, and 56% respectively. However, in this study, the addition of a frozen semen AI, when a mare inseminated with cooled semen would not ovulate within 24 hours (only 21 cycles), was able to improve embryo recovery, giving results similar to fresh semen AI.

Embryo recovery rate in different breeds was not often compared in literature, however a lower embryo recovery rate in Mangalarga versus Quarter Horse mares was reported in a paper [23,34]. In the present study Standardbred and Quarter horse mares had significantly higher positive flushes and embryo recovery per cycle and per ovulation rates than Show Jumping mares. Standardbred also showed a positive influence on flushes in which at least one embryo was recovered and on embryos per cycle recovery rates. The factor that may have had contributed to these differences, and that was not possible to analyze in this study, was the different management of the semen market in Italy. In Standardbred the most of the semen fee is paid after the birth of a living foal and for this reason poorly fertile semen is rapidly excluded from the market; in case of Show Jumping, more and more frequently, semen is sold dose per dose, independently from the semen quality and the outcome of AI. Obviously less controlled semen quality may have contributed to lower embryo recovery in Show Jumping mares.

The effect of exercise on embryo recovery rate reported in literature is controversial: it has been reported to have no effect [9-15,22,42], to lower embryo recovery, [20] or to have no effect or a negative effect (full and partial exercise, respectively) [39]. In the present paper, positive flushes, embryo recovery per cycle and per ovulation were very close for healthy donors performing or not sport activity, with a significant positive correlation effect.

Mares inseminated in private breeding stations or at home showed a significantly higher incidence of positive flushes and embryo recovery rate per cycle and per ovulation compared to the mares

inseminated at the Department. This was probably due to the different populations of mares involved and to the type of semen used. Show Jumping mares, characterized by the lower reproductive performance in the present study, represented 82.8% of the cycles at the Department and 48.7% externally; moreover 31.8% of the mares bred outside the Department were under 10 years of age, while only 2.9% of the mares bred at Department were of the same age class. Fresh semen, instead, was used for 12.0% and 36.8% of inseminations inside or outside the Department respectively, while frozen semen was used in 40.4% and 25.6% of the cases in the same two groups of animals, respectively. No difference has been found between the two flushing protocols, however the filter-method was less time consuming and more practical than the older protocol

Embryo quality and diameter

In this study embryo quality was subjectively judged observing the embryo morphology by a stereomicroscope at 40 magnifications: 96% of the recovered embryos were evaluated as quality 1 or 2, which is close to what commonly described in literature [34,35], without differences based on the factors included in this model.

In the present paper, the day of recovery, donor age class, type of semen, and number of ovulations per cycle significantly affected embryo diameter. Older mares, 16-24 years old, produced significantly smaller embryos than younger ones, 2-15 years old. A delayed embryo development in old mares was associated to poor oocyte quality and to a high risk of embryo loss [40,43].It would be interesting to confirm if this difference on embryo diameter may have an effect on recipients pregnancy rate following transfer as already reported in literature [27,42,44]. Embryos recovered after insemination with frozen semen were significantly smaller than the ones recovered after insemination with fresh semen. This was probably due to the different moment in which artificial insemination has been performed: before ovulation with fresh semen and after ovulation with frozen semen, with a probable delay of the time of oocytes fertilization in the last case. Similar findings were reported by others which showed that post-ovulation inseminations produced pregnancies with smaller embryonic vesicles compared to pre-ovulation AI, regardless of the type of semen employed [45].

In conclusion, the analysis of factors affecting ovulation rate, embryo recovery efficiency and embryo quality performed in this study confirmed most of the results already present in literature. Moreover, the specific condition of this study, working simultaneously on different breeds, with different types of semen and with donors managed in different breeding stations, allowed to perform some comparison often not possible in past studies. Standardbred and Quarter Horse showed to be significantly more efficient in this ET program. The use of frozen semen negatively

affected positive flushes, as mares with reproductive pathologies did, and no differences have been found between healthy mares performing sport activity or not. Finally, in the condition of this study, it was possible to achieve similar recovery rates from mares younger than 10 years and older than 20 years.

Acknowledgments: The authors thank Professor Gabriele Massimetti for the statistical analyses, and veterinary students of the Pisa University who collaborated to the management of mares.

References:

[1] Rizzi N, Minori D, Tamagna S. Esperimenti di embryo-transfer nel cavallo in Italia. Atti Della Società Italiana Delle Scienze Veterinarie, 1990, p. 135–8.
[2] Allen WR, Rowson L. Allen: Transfer of ova between horses and donkeys - Proc 7th Int Congr Anim Reprod A. I. Munich, 1972, p. 484-8
[3] Oguri N, Tsutsumi Y. Non-surgical egg transfer in mares. J Reprod Fertil 1974;41:313–20.
[4] Betteridge KJ. A history of farm animal embryo transfer and some associated techniques. Animal Reproduction Science 2003;79:203–44.
[5] Stout TAE. Equine embryo transfer: review of developing potential. Equine Veterinary Journal 2006; 38(5):467-478.
[6] Raz T, Card C. Efficiency of superovulation and in vivo embryo production in eFSH-treated donor mares after estrus synchronization with progesterone and estradiol-17β. Theriogenology 2009;72:169–78.
[7] Squires EL, McClain MG, Ginther OJ, McKinnon AO. Spontaneous multiple ovulation in the mare and its effect on the incidence of twin embryo collections. Theriogenology 1987;28:609–13.
[8] Losinno L, Aguilar JJ, Lisa H. Impact of multiple ovulations in a commercial equine embryo transfer programme. Havemeyer Foundation Monograph Series 2001;3:81–83
[9] Ginther OJ, Douglas RH, Lawrence JR. Twinning in Mares - a Survey of Veterinarians and Analyses of Theriogenology Records. Theriogenology 1982;18:333–47.
[10] Ginther OJ. Ultrasonic Imaging and Reproductive Events in the Mare Equiservices Publishing; 1986.
[11] Ginther OJ. Reproductive Biology of the Mare. Equiservices Publishing; 1992
[12] Morel M, O'Sullivan J. Ovulation rate and distribution in the thoroughbred mare, as determined by ultrasonic scanning: the effect of age. Animal Reproduction Science 2001;66:59–70.
[13] Morel M, Newcombe JR, Swindlehurst JC. The effect of age on multiple ovulation rates, multiple pregnancy rates and embryonic vesicle diameter in the mare. Theriogenology 2005;63:2482–93.
[14] Henry M, Coryn M, Vandeplassche M. Multiple Ovulation in the Mare. Zentralblatt Für Veterinärmedizin Reihe A 2010;29:170–84.
[15] McKinnon AO. Origin and Outcome of Twin Pregnancies. In: McKinnon AO, Squires EL, Vaala WE, Varner DD, editors. Equine Reproduction 2nd edition, vol. 2, Oxford: Wiley-Blackwell; 2011, pp. 2350–8.
[16] Vogelsang SG, Vogelsang MM. Influence of donor parity and age on the success of commercial equine embryo transfer. Equine Veterinary Journal 1989;21:71–2.
[17] Squires EL, McCue PM, Vanderwall D. The current status of equine embryo transfer. Theriogenology 1999;51:91–104.

[18] Losinno L, Alonso C, Rodriguez D, Douglas RH. Ovulation an embryo recovery rates in young and aged mares treated with hCG or Deslorelin. 7th International Symposium on Equine Embryo Transfer, Cambridge, 2008 pp.96–7.
[19] Panzani D, Rota A, Pacini M, Vannozzi I, Camillo F. One year old fillies can be successfully used as embryo donors. Theriogenology 2007;67:367-71.
[20] Mortensen CJ, Choi YH, Hinrichs K, Ing NH, Kraemer DC, Vogelsang SG, et al. Embryo recovery from exercised mares. Animal Reproduction Science 2009;110:237–44.
[21] Kelley DE, Smith RL, Gibbons JR, Vernon KL, Mortensen CJ. Effect of exercise on ovarian blood flow and embryo recovery rates in mares. Animal Reproduction Science 2010;121:284–5.
[22] Pessoa MA, Cannizza AP, Reghini MFS, Alvarenga MA. Embryo Transfer Efficiency of Quarter Horse Athletic Mares. Journal of Equine Veterinary Science 2011;31:703–5.
[23] Uliani RC, Ramires Neto C, Dell'Aqua JA, Pessoa MA, Camargo AL, Alvarenga R, et al. Effect of mare breed and age on embryo transfer efficiency. Animal Reproduction Science 2010;121:303–4.
[24] Hinrichs K. Work in progress: A simple technique that may improve the rate of embryo recovery on uterine flushing in mares. Theriogenology 1990;33:937–42.
[25] Alvarenga MA, Alvarenga FCL, Meira C. Modifications in the technique used to recover equine embryos. Equine Veterinary Journal 2010;25:111–2.
[26] Riera FL, McDonough J. Commercial embryo transfer in polo ponies in Argentina. Equine Veterinary Journal 2010;25:116–8.
[27] Carnevale EM, Ramirez R, Squires E. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology 2000;54:965–79.
[28] Brinsko SP. Procedings of the 47th Annual Convention of the American Association Of Equine Practitioners, San Antonio, Texas, 2001, pp. 407-411 2001.
[29] Hurtgen JP. Pathogenesis and treatment of endometritis in the mare: a review. Theriogenology 2006;66:560–6.
[30] LeBlanc MM, Causey RC. Clinical and Subclinical Endometritis in the Mare: Both Threats to Fertility. Reprod Domest Anim 2009;44(3):10–22
[31] Pickett BW, Squires EL, McKinnon AO. Procedures for collection, evaluation and utilization of stallion semen for artificial insemination. Bulletin / Colorado State University, Animal Reproduction Laboratory 1987;03:45-8.
[32] Miller CD. Optimizing the use of frozen–thawed equine semen. Theriogenology 2008;70:463–8.
[33] Lagneaux D, duchamp G, Palmer E. La transplantation embryonnaire chex la jument. 14ème journée d'étude. C.E.R.E.O.P.A, Paris: n.d., pp. 163–81.
[34] McKinnon AO, Squires EL. Equine embryo transfer. Vet Clin North Am Equine Pract 1988;4:305–33.
[35] Squires EL, Carnevale EM, McCue PM, Bruemmer JE. Embryo technologies in the horse. Theriogenology 2003;59:151–70.
[36] Jacob JCF, Santos GO, oliveira JP, Gastal MO, Gastal EL. Evaluation of reproductive parameters in a commercial equine embryo transfer program. Animal Reproduction Science 2010;121:305–6.
[37] Riera FL, Roldán JE, Hinrichs K. Ipsilateral double ovulation is associated with reduced embryo recovery in mares. Havemeyer Foundation Monograph Series 2005;14:108–9.
[38] Carmo MT, Alvarenga MA, Losinno L, Aquilar JJ. Oocyte transport to the oviduct of superovulated mares. Animal Reproduction Science 2006;94:337–9.
[39] Kelley DE, Smith RL, Gibbons JR, Vernon KL, Mortensen CJ. Effect of exercise on ovarian blood flow and embryo recovery rates in mares. Animal Reproduction Science 2010;121:284–5.

[40] Ball BA, Little TV, Hillman RB, Woods GL. Pregnancy rates at Days 2 and 14 and estimated embryonic loss rates prior to day 14 in normal and subfertile mares. Theriogenology 1986;26:611–9.
[41] Jasko DJ, Moran DM, Farlin ME, Squires EL, Amann RP, Pickett BW. Pregnancy rates utilizing fresh, cooled, and frozen-thawed stallion semen. Proceedings of the 38th Annual Convention of the American Association of Equine Practitioneers, Orlando, Florida, 1992;649–60..
[42] Foss R, Wirth N, Schiltz P, Jones N. Nonsurgical embryo transfer in a private practice (1998). American Association of Equine Practioners 1999;45:210–2.
[43] Carnevale EM, Griffin PG, Ginther OJ. Age-associated subfertility before entry of embryos into the uterus in mares. Equine Veterinary Journal 2010;25:31–5.
[44] Camargo CE, Weiss RR, Kozicki LE, Duarte MP, Garcia Duarte MC, Lunelli D, et al. Some Factors Affecting the Rate of Pregnancy after Embryo Transfer Derived from the Brazilian Jumper Horse Breed. Journal of Equine Veterinary Science 2013;33(11):924-9.
[45] Cuervo-Arango J, Aguilar J, Newcombe JR. Effect of type of semen, time of insemination relative to ovulation and embryo transfer on early equine embryonic vesicle growth as determined by ultrasound. Theriogenology 2009;71:1267–75.