Factors affecting post-breeding endometritis, pregnancy rate and embryonic/fetal death in sport mares in two French commercial stud farms: special focus on age, parity and lactating status effects

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Abstract

Fertility in mares depends on management and reproduction mode, but overall analyses have shown that there is an important and progressive decline in fertility in older mares. Age is often related to maternal parity, but the role of parity in the fertility decline in old mares is not known. Moreover, the positive or negative impacts of breeding mares yearly are a subject of controversy. The aim of this study was to identify whether these factors, as well as reproductive management, played a role on post-insemination endometritis and fertility in sports mares in commercial studs. Altogether, the breeding records of 277 mares (506 cycles) over one breeding season in two French commercial stud farms were analyzed. Age, parity, whether the mare was barren or suckling, follicular size before ovulation, the use of estrus and/or ovulation induction, artificial insemination (AI) protocol, post-breeding inflammation and treatment, Day 14 pregnancies and number of embryos, as well as subsequent foaling the next year were recorded. Data were analyzed using multivariate logistic regression models. The pregnancy rate by cycle was 41.9% and 76.5% of mares were pregnant at the end of the season. Mares older than 10 years, barren mares or mares inseminated in July or August were more likely to have inflammatory reaction after insemination ($p<0.0001$, OR=3.29, 5.389 and 3.329, respectively). Mares >10 years and mares inseminated with frozen semen were less likely to be pregnant on Day 14 vs younger mares and mares inseminated with fresh and refrigerated semen ($p<0.05$, OR=0.622 and 0.582, respectively). As expected, mares with multiple ovulations were more likely to be pregnant on Day 14 compared to mares that had only one ovulation ($p<0.05$, OR=1.791). Altogether, parity only tended to improve the likelihood of being pregnant on Day 14 ($p=0.07$, OR=1.434 in parous vs nulliparous) but in older mares (age >10), parity increased pregnancy rates (44.09% vs 30.89% in parous vs nulliparous, $p<0.05$). Post-breeding inflammation was not related to pregnancy rate on Day 14 (37.8% *vs 44.2%*, $p=0.16$) but all mares with post-breeding inflammation received a treatment. No effect of suckling at insemination was observed. None of the studied factor

could be related to embryo/fetal mortality. In conclusion, maternal age seems to be more important than parity and lactating status for reproductive success in mare. Cumulative effects on parity and aging were observed on pregnancy rates.

Keywords

Horse, equine, reproduction, insemination, lactation, fertility

1. Introduction

Effectiveness of mare reproduction is an important economical factor in the horse industry. Breeding a mare is an expensive procedure with no guarantee: stallion fees, gynecological monitoring and mare maintenance are costly. Therefore, once mares begin their reproductive career, breeders generally aim to produce one foal per year per mare to remain profitable. This goal can only be achieved if mares remain fertile throughout their life span. Several factors may affect mare fertility such as their age, parity and lactation status during the breeding period.

The decline in mare fertility associated to age has been well documented in various breeds [1–18]. Moreover, Langlois et al. demonstrated that if fertility was the only factor for culling broodmares and if a 10% of fertility decline was chosen as cutoff value, then mares older than 8 should not be bred [2]. A survey sent to breeders participating in competing events, however, showed that mare's age was the least important consideration for the selection of mares for reproduction [19]. Moreover, since a mare's reproductive career often begins after their sporting career, it is common in sport horses to breed older mares. Indeed, in Canada, 37% of the standardbred mares were shown to be older than 11 years old at insemination [20]. These figures are even higher in Finland where 53% of Standardbred and 63% of Finnhorse mares exceed this age [3].

Mares can be bred shortly after foaling and breeders often aim to produce foals yearly, so that mares are often nursing at the time of breeding. Lactation affects mares' metabolism [21] but there is no consensus on whether it may induce effects on fertility and embryo/fetal loss [22–25].

As for lactation, the putative effects of mare's parity on fertility and embryo loss are controversial [2–5,9,10,12–16,18]. Horse production is unique in that mares may be both nulliparous and old at the same time, which is not the case in other animal production. As a matter of fact, in Finland, 20.5% of Finnhorse and 15.5% of Standardbred broodmares are both older than 10 and nulliparous [3].

77 Transient uterine inflammation is a normal physiological reaction in mares after breeding. It is
78 thought to be required for the elimination of bacteria and the excess of spermatozoa introduced in
79 the uterine lumen. For some mares, however, a persistent infection can develop and interfere with
80 fertility outcomes (for review [26]). Ultrasound images of uterine fluid accumulation are the most
81 commonly used diagnostic criterion for post-breeding endometritis. They are observed more
82 frequently in mares >8 years old compared to younger mares. In a study on 57 mares, Woodwards et
83 al. observed that 45% (N=22) of the 8-16 years old mares and 88% of the older mares (≥17 years old,
84 N=26) had uterine fluid retention 48h after insemination with frozen semen, whereas none of the 9
85 younger mares (<8 years old) were affected [27]. To the authors' knowledge, the specific effects of
86 maternal parity and/or lactation on the post-breeding inflammation has not been explored.

87 The Thoroughbred studbook only allows hand breeding whereas the French Trotter studbook allows
88 insemination but only with fresh semen. There is no such limitation in sport horse breeds but studies
89 on fertility in sports mares are scarce and relatively ancient. There is no recent study considering at
90 the same time the method of reproduction and the effects of age, parity and lactation on mare
91 fertility.

92 The aim of this study was thus to identify effects of mare age, parity and nursing on post-breeding
93 endometritis, fertility and embryonic/fetal death in commercial stud conditions for sport mares.

94 2. Material and methods

95 *2.1. Ethics*

96 This is an observational study performed in commercial farms. All procedures were carried out
97 according to the European Union Directive 98/58/CE concerning farm animals protection and the
98 French legislation about animal care in rural codification.

99 *2.2. Animals*

100 Data were recorded in 2 French commercial stud farms, one located about 60km south west of Paris,
101 the other 150 km south of Paris, both managed by one artificial insemination cooperative during one
102 breeding season (2019).

103 From beginning of February to late August 2019, the study enrolled 277 sport mares over 506 estrus
104 periods. For each mare, date of birth, breed, number of previous breedings and foalings were
105 extracted from the national database (Infochevaux: https://infochevaux.ifce.fr/fr/info-chevaux).
106 Mares were clustered according to age in two (≤ 10 years old *vs* > 10 years old) or four classes (≤ 5
107 years old,]5, 10],]10, 15], > to 15 years old). They were also clustered in 2 classes according to
108 previous fertility (number of previous foaling/number of previous breedings <0.7 or >0.7) and in 2
109 classes according to parity: nulliparous (never foaled before) vs parous (foaled at least once before)
110 at the beginning of the breeding season.

111

112 *2.3. Breeding management*

113 *2.3.1. Estrus monitoring*

114 First, luteolysis was induced in mares that had a persistent corpus luteum or that were sent for
115 breeding late in the season, using prostaglandin F2α analogs: Alfaprostol (Alfabedyl©) or Luprostol,
116 (Prosolvin®, 1 to 1.5 mL IM according to mare size).

117 Estrus detection was performed using uterine horn firmness as estimated by rectal palpation
118 together with ultrasound examination where the uterine oedema score (0-5) [28] and the largest
119 follicle diameter were determined. Mares were monitored once or twice daily (according to breeding
120 method, see below) from the time when the largest follicle diameter reached 35 mm and until
121 ovulation was detected.

122 As ovulation approached (follicle diameter ≥35 mm and reduction of the uterine oedema score),
123 ovulation was induced with hCG (Chorulon®, 1500 to 5000 IU IM related to mare's weight) or with a
124 GnRH analog (Decapeptyl®, 0.1 mg triptorelin in 1 mL SC). The GnRH analog option was chosen when
125 mares had a known resistance to hCG or when hCG had already been used before during the
126 breeding season and was not effective to induce ovulation.

127 ### *2.3.2. Insemination management*

128 All mares were bred using artificial insemination (AI). Mares were inseminated with fresh (FAI),
129 cooled transported (CTAI) or frozen semen (FZAI). Mare management differed according to the type
130 of semen used.

131 Mares entitled to be inseminated with fresh semen were examined once daily during estrus. A first
132 insemination was performed before ovulation (follicle diameter ≥ 35 mm and decrease in the uterine
133 oedema score) and mares that had not ovulated 48h later were inseminated a second time. This was
134 repeated until ovulation was observed.

135 Mares entitled to be inseminated with cooled transported semen were also examined once daily and
136 inseminated before ovulation but subsequent inseminations were performed every 24h until
137 ovulation.

138 Mares bred with frozen semen were examined every 12h if the number of available straws was >4
139 while examinations were performed every 6 h when the number of available straws was ≤4. In this
140 latter case, deep horn insemination was performed. The aim was to inseminate just before ovulation,
141 as determined by ultrasound observation of preovulatory follicular wall thickening and follicle
142 deformation, or just after ovulation.

143

144 ### *2.3.3. Post-breeding endometritis and pregnancy diagnosis*

145 All mares were examined by ultrasound the day after insemination. If uterine oedema and/or fluid
146 accumulation were observed, mares were treated either with a single dose of oxytocin (Ocytocine
147 S©, 10-20 UI in 1 to 1.5 mL IM or IV), or oxytocin in association with uterine lavage (one or two litters
148 of warm, sterile saline solution), or in association with antibiotics or by uterine lavage alone.
149 Treatment was decided by the veterinary surgeon and depended on the volume of fluid
150 accumulated.

151 Pregnancy was assessed 14 days (D14) after AI by ultrasonography. In the case of twin pregnancy,
152 squeezing was recommended to the breeder but not always performed. Pregnancy was confirmed on
153 Day 30 if the mare was brought back to stud. In addition, some mares came back again in autumn for
154 late pregnancy diagnosis.

155

156 ***2.3.4. Data recording***

157 All data were recorded in the same dedicated software (Gynebase©, Equidéclic, France) by the
158 veterinarians and technicians of the 2 studs.

159 Age, parity, lactational status at breeding (nursing *vs* non nursing), number of monitored cycles for
160 each mare, estrus induction (yes/no), heat duration (number of days of observation by
161 ultrasonography), size of the preovulatory follicle, induction of ovulation (yes/no), hormone used to
162 induce ovulation (hCG or GnRH analog), ovulation observed (yes/no), number of follicles ovulated, AI
163 mode (fresh semen, cooled transported or frozen semen), number of AI during estrus, date of
164 insemination (last AI if more than one AI were performed during estrus), stallion identity, number of
165 straws used (for frozen semen), uterine fluid accumulation after AI (yes/no), treatment used
166 (oxytocin alone vs other), presence of single or twin embryos, squeezing when twins were diagnosed
167 (yes/no), pregnancy diagnosis on D14 as well as on D30 or later, when possible, were recorded. The
168 number of the monitored cycles needed to obtain a gestation was classified as 1 *vs* >1 when

169 pregnancy was achieved after more than one cycle. The number of straws used for frozen
170 insemination was classified as either <8 *vs* ≥ 8 or ≤ 4 vs > 4. Cutoff values of 4 and 8 were chosen
171 according to previous (8 straws for 400 million mobile spermatozoa) and the current (4 straws of 50 x
172 10^6 mobile spermatozoa, 4 mL each) recommendation of the French Institute for Horses and Horse-
173 riding (IFCE) for frozen semen.

174 Pregnancy rate was calculated as the ratio between the number of pregnant mares at the end of the
175 breeding season and total number of mares bred (overall pregnancy rate at the end of the season)
176 and as the ratio of number of positive diagnoses on D14 / total number of used cycles (pregnancy
177 rate per cycle). Embryonic loss was estimated by the number of mares not pregnant on D30 after a
178 positive pregnancy diagnosis on D14. Late embryonic loss was calculated by the number of mares not
179 pregnant during the autumn after a positive pregnancy diagnosis on D14. Foal birth was checked the
180 next year using the national database. Total embryonic/fetal loss was defined as the number of
181 mares that did not foal after a positive pregnancy diagnosis on D14. Mares were only considered as
182 non-foaling when abortion/stillbirth or new breeding without foaling were recorded in the national
183 database in 2020.

184

185 ## *2.4. Statistical analysis*

186 Data were analyzed using SAS® Studio 3.8 (SAS® University Edition).

187

188 ### *2.4.1. Univariate & multivariate analysis*

189 Univariate analysis was used to evaluate the effects of qualitative variables on (1) the incidence of
190 post-breeding endometritis, on (2) pregnancy rate and (3) embryonic/fetal loss. For (1) and (2), all

recorded cycles were used while for (3), only pregnant mares on D14 were considered. The statistical unit was, therefore, the cycle for (1) and (2) while it was the mare for (3).

For each criterion analyzed, results were compared between the different classes using a Chi-Squared test. Variables associated at $p < 0.20$ were included in the second step of the analysis.

As a second step, multivariate analysis was conducted using logistic regression (GLIMMIX procedure of SAS® Studio). Individual effects were considered by including mare identity in each model as a random effect. A backward stepwise elimination of non-associated ($p > 0.10$) variables was performed to develop multivariate models. Models presenting the lowest Akaike's Information Criterion were retained.

For these analyses, breeds and stallions could not be used as their numbers were too high without enough repeats to be considered in the statistical analysis.

For quantitative data, results are presented as mean ± standard error.

2.4.2. Analysis of age, parity and nursing status

The analyses were performed on data, using the estrous cycle as the reference unit. Young (≤ 10 years) *vs* old (>10 years), nulliparous *vs* parous, nursing *vs* non nursing mares were compared using T test for quantitative variables and Xi Square for qualitative ones. Using Xi Square analysis, effects of mares' suckling status on post-breeding inflammation and effects of mares' parity on Day 14 pregnancy rates were assessed in each population of young or old mares.

2.4.3. Data availability statement

Data supporting the conclusions of this article are available in the Data INRAE repository, available at: https://doi.org/10.15454/4OIUKH

3. Results

3.1. Descriptive analysis

3.1.1. Population of study

The 277 mares belonged to 32 breeds approved by the French Institute of the Horse (IFCE), with French saddlebred (Selle français) being the most represented breed (56% of mares included in this study). No other breed reached more than 15 individuals. Eight mares did not belong to any French approved breed. In addition, 13 mares belonged to racehorse breeds (3 English Thoroughbreds, 10 French Trotters). There were also 22 ponies and one draft horse. All mares were used for sport and leisure.

Characteristics of the 277 mares are detailed in Table 1. The average age was 12.7 years old (min 2, max 23, σ = 4.8). Altogether, 196 mares had been bred at least in one previous breeding season and the overall average of previous breeding seasons was 2.5 (min 0, max 17, σ = 3.0). For previously bred mares, mean fertility (number of foalings/number of breeding) was 0.71 (min 0, max 1, σ = 0.31). The interval between previous breeding and the 2019 reproductive season ranged from 1 to 13 years. Altogether, 66 (21.9%) mares were nulliparous and aged more than 10 years and 44 (15.8%) were suckling and older than 10 years.

Table 1: Characteristics of the 277 mares bred in 2019.

Variable	n	Percent
Age in 2 classes (Years)		
≤ 10	96	34.7

> 10	181	65.3
Age in 4 classes (Years)		
≤5	24	8.7
5-9	72	26.0
10-14	94	33.9
> 15	87	31.4
Parity		
Nulliparous	98	35.4
Parous	179	64.6
Lactation status in 2019		
Suckling	76	27.4
Non suckling	201	72.6
Fertility at previous breeding (n=196)		
< 0.7	82	41.8
≥ 0.7	114	58.2
Month of first AI		
March	36	13.0
April	70	25.3
May	86	31.0
June	67	24.2
July - August	18	6.5

3.1.2. Pregnancy rate per mare

234 Mares were bred with 154 different stallions. Altogether, 212 mares were diagnosed pregnant on
235 D14 at the end of the season (76.5% overall fertility rate). These performances were obtained within
236 an average of 1.8 cycle per mare (min 1, max 6). From the 154 mares that came back for pregnancy
237 confirmation, 130 were still pregnant at Day 30, so that embryonic loss was 9.1%. Only forty-six
238 mares came back later in the autumn, of which 44 mares were confirmed still pregnant (late
239 embryonic loss: 4.3%).

240 Foaling information in 2020 were obtained for 206 pregnant mares on Day 14. Among them, 168
241 foalings were recorded indicating a total embryonic/fetal loss of 18.4%.

242

243 ### 3.1.3. Breeding management

244 Data recorded per estrus period are presented on Table 2.

245 Among the 506 estrus recorded periods, 92 were induced by luteolysis of a previous corpus luteum
246 using prostaglandins (18.2%). Data were recorded in average during 5.1 days per estrus period (min
247 1, max 16, σ = 2.1).

248 Of the 506 estrous periods, fresh semen was used for 90 cycles (FAI, 17.8%), cooled transported
249 semen for 142 cycles (CTAI, 28.1%) and frozen semen for 274 (FZAI, 54.1%). Ovulation was induced in
250 360 estrous periods (71.2%). The induction of ovulation was performed in 40.0% of the FAI, 74.0% of
251 the CTAI and 80.0% of the FZAI. Mostly hCG was used (n=322, 89.4%) with GnRH analog used in the
252 other cases (n=38, 10.6%). Preovulatory dominant follicle mean diameter was 42.4 mm (min 28, max
253 60, σ = 4.8). Mares were inseminated in average 1.2 times per estrus (min 1, max 5, σ = 0.6). For FZAI,
254 the mean number of straws used was 5.1 (min 1, max 12, σ = 2.4, recorded in 251 AI by 274).

255 Ovulation was observed by ultrasonography in 501 of the 506 estrous periods (99%). In most cases,
256 only one ovulation was observed (n=425, 84.8%). Double or triple ovulations were observed in 75
257 (15.0%) and 1 estrus, respectively.

258 After insemination, uterine fluid accumulation was observed in 180 estrous periods (35.6%). Oxytocin
259 was used for 173/180 treatments, mostly alone (139/180), sometimes associated with uterine lavage
260 (27 cases). Antibiotics were used for local treatment (10/180) associated to oxytocin (n=3), to uterine
261 lavage (n=4) or alone (n=3).

262 After AI, 212 cycles among the 506 led to a pregnancy on D14 (pregnancy rate per cycle: 41.9%).
263 Twins were detected in 27 cases (5% of breeding and 12.7% of pregnancies). Squeezing was
264 performed for 25/27 pregnancies. After squeezing, pregnancy was checked on Day 30 and 21/24
265 mares were still pregnant (87.5%).

266

267 **Table 2: Characteristics of the 506 monitored estrus**

Variable	n	Percent
Cycle number		
1	277	54.7
2	144	28.5
3	65	12.8
4	18	3.7
5	1	0.2
6	1	0.2
Luteolysis before AI		
No	414	81.8
Yes	92	18.2
Induction of ovulation		
No	146	28.8
Yes	360	71.2

Number of observed ovulations (n=501)		
1	425	84.8
2	75	15.0
3	1	0.2
Method of semen preservation		
Fresh semen (FAI)	90	17.8
Cooled transported (CTAI)	142	28.1
Frozen (FZAI)	274	54.1
Number of straws (n= 251)		
< 8	177	70.5
≥ 8	74	29.5
Number of straws (n= 251)		
≤ 4	126	50.2
> 4	125	49.8
Month of AI		
March	36	7.1
April	104	20.6
May	150	29.6
June	155	30.6
July-August	61	12.1
Number of AI by estrus period		
1	431	85.2
2	57	11.2
3	11	2.2
4	5	1.0
5	2	0.4

Observed ovulation		
Yes	501	99.0
No	5	1.0
Uterine fluid accumulation/endometritis after AI		
Yes	180	35.6
No	327	64.4
Uterine treatment (n=180)		
Ocytocin alone	139	77.2
Other	41	22.8

3.2. Univariate & multivariate analysis

3.2.1. Factors associated with post-breeding inflammation

After univariate analysis, variables associated with post-breeding inflammation/endometritis at the threshold of 20% were: mare's age (using 10 years old as cutoff value), parity, lactation (suckling *vs* non suckling), induction of estrus by luteolysis, cycle number and month of AI (Figure 1). Post-breeding inflammation was not associated with semen conditioning (33.6, 40.2 and 37.9% respectively for FAI, CTAI and FZAI, p=0.45), number of AI per estrus (35.2% for 1 AI *vs* 40.3% for more than one AI per estrus, p=0.40), nor number of straws used (33.9 vs 33.8% for number of straws < 8 vs ≥ 8, p=0.98 and 37.3 vs 30.4% for number of straws ≤ 4 vs > 4, p=0.25).

After multivariate analysis, only 3 variables were significantly associated ($p<0.05$) with post-breeding inflammation: mare age, lactation and month of AI (Supplementary table 1). Inflammation was more frequent in mares older than 10 years than in younger mares (Figure 1). Non suckling mares at the time of insemination were also more affected in comparison with suckling ones (Figure 1). There was no interaction between age and nursing status ($p = 0.38$) but increased age amplified the number of

283 post-breeding inflammations in both non-suckling and suckling mares. Respectively, 22.9% and 8.2%
284 of the non-suckling and suckling young mares were affected by post-breeding inflammation while
285 50.7% and 18.3% of mares older than 10 years old were affected.

286 The risk of inflammation was also increased in July and August compared to previous months (Figure
287 1).

288

289 **3.2.2. Factors associated with pregnancy rate per cycle**

290 After univariate analysis, the following factors were associated with pregnancy rate at the 20%
291 threshold: age in 2 classes, parity, cycle number, AI modality, month of insemination, number of
292 observed ovulations and post-breeding inflammation. All the associated variables were introduced in
293 multivariate models. As significant effect of number of ovulations was observed after univariate
294 analysis, only estrous cycle with ovulations that were observed by ultrasonography were considered
295 (*i.e.*, 501 estruses among the 506 recorded).

296 Stepwise regression and backward elimination led to a model containing only 3 significant variables
297 ($p<0.05$): mare age, number of ovulations and semen conditioning (Supplementary Table 2). Data
298 that significantly influenced pregnancy rates are summarized in Figure 2. Pregnancy rate was higher
299 in mares younger *vs* older than 10 years mares. Pregnancy rate was increased when a multiple *vs*
300 single ovulation was observed. FAI and CTAI resulted in more pregnancies on Day 14 than FZAI.
301 Trends were observed ($p < 0.10$) for the effects of month of AI and parity. Pregnancy rate tended to
302 be higher in April, May, July and August vs March and June. Parous mares tended also to have better
303 pregnancy rates than nulliparous mares ($p=0.07$, OR=1.434 in parous mares).

304 The study of the interaction between maternal age and parity showed that in mares aged of 10 years
305 or less, being nulliparous or parous did not alter pregnancy rates (49.25 and 46.15% of pregnancy
306 rate per cycle for young nulliparous and multiparous respectively, $n = 158$, $p = 0.70$). In mares older

307 than 10 years old, however, nulliparity accentuated the decrease in pregnancy rates. Indeed, the
308 pregnancy rates per cycle was only 30.89% for old nulliparous mares *vs* 44.09% for old multiparous
309 mares (OR = 1.96 in parous mares, p = 0.016).

310 After multivariate analysis, pregnancy rate was not significantly affected by post-breeding
311 inflammation (37.8% of pregnancy rate after inflammation vs 44.2% in healthy mares, p = 0.16). In
312 treated mares, treatment after post-breeding treatment did not affect pregnancy rate (37.4% of
313 pregnancy for treatment with oxytocin alone, n = 139 *vs* 39.0% for other treatment, n = 41, p = 0.85).

314

315 #### 3.2.3. Factors associated with embryonic/fetal loss between Day 14 and foaling

316 After the univariate analysis, age in four classes, induction of ovulation and month of insemination
317 were associated with embryonic/fetal loss at the threshold of 20%. However, after multivariate
318 analysis, none of these factors affected embryonic/fetal loss.

319

320 ### 3.3. Focus on maternal age, parity and suckling status

321 #### 3.3.1. Effect of maternal age

322 Mares 10 years old or younger were 7.2 ± 0.2 years old while mares older than 10 years were 15.6 ±
323 0.2 years old.

324 Neither parity, induction of the estrus cycle or of ovulation, month of AI, number of cycles required
325 for a gestation at Day 14, number of straws in case of FZAI or the number of twin embryos at Day 14
326 were significantly related to maternal age (Supplementary Table 3).

327 Nevertheless, more young mares were pregnant at Day 14 and less had a post-breeding
328 inflammation compared to old mares (Table 3). In addition, 31% of young were nursing while it
329 represented only 20.7% of old mares ($p < 0.05$). Multiple ovulations occurred more frequently in old

330 mares compared to young mares. Young mares tended to be more inseminated using fresh and
331 frozen and less with refrigerated semen compared to older mares.

332 Estrus tended to be longer in old *vs* young mares (respectively, in average, 5.2 ± 0.1 *vs* 4.8 ± 0.2 days,
333 p = 0.086). The preovulatory follicle diameter tended to be smaller in old compared to young mares
334 (respectively, in average, 42.2 ± 0.2 *vs* 43.0 ± 0.4 mm, p = 0.086).

335 **Table 3: Characteristics in young (≤10 years old) vs old mares (>10 years old)**

Variable	**n**	**Young**	**Old**	**p**
		≤ 10 years old	**> 10 years old**	
		n=158	**n=343**	
Pregnancy rates on Day 14	501	47.5%	39.4%	0.021**
Post-breeding inflammation	501	18.3%	44.0%	< 0.0001**
Lactation				
Non-nursing	381	69.0%	79.3%	0.012
Nursing	120	31.0%	20.7%	
Method of the semen preparation				
*				
FAI	87	19.0%	16.6%	0.093
CTAI	140	21.5%	30.9%	
FZAI	274	59.5%	52.5%	
Number of ovulations per cycle				
1				
> 1	425	90.5%	82.2%	0.016
	76	9.5%	17.8%	

336 * AI: Artificial insemination, FAI: insemination with fresh semen, CTAI: insemination with cooled
337 transported semen, FZAI: insemination with frozen semen

338 ** Results from multivariate analysis

339

340 ### 3.3.2. Effect of maternal parity

341 Neither age, induction of the estrus cycle, semen preparation, month of AI, number of cycles or AI
342 required for a gestation at Day 14, number of straws in case of FZAI nor the number of ovulation or
343 twin embryos at Day 14 were significantly affected by maternal age (Supplementary Table 4).

344 Nulliparous mares tended to be less pregnant on Day 14 (Figure 2). Parity tended to be related to
345 post-breeding inflammation. Maternal parity was different according to maternal age. More
346 nulliparous mares were present in 5 or less and 10-15 years old group while less nulliparous were
347 observed in 5-10- and 16 or more-years old groups. Nulliparous mares were mostly older than 10
348 years (64.7%, Supplementary Table 4) but nulliparous were in average younger than parous mares
349 (Table 4) with a mean 2-year difference between nulliparous and parous mares (in average,
350 respectively, 11.6 ± 0.3 *vs* 13.7 ± 0.3 years old, $p < 0.0001$). Moreover, suckling status was obviously
351 related to parity and 38.6% of parous mares were suckling at insemination. Ovulation was induced
352 more frequently in parous than in nulliparous mares. Parity did not modify estrus duration (5.1 ± 0.2
353 days for nulliparous *vs* 5.1 ± 0.1 days for parous mares, $p = 0.11$) but the size of the preovulatory
354 follicle at ovulation was reduced in nulliparous compared to parous mares (respectively, 41.9 ± 0.3
355 and 42.8 ± 0.2mm in diameter, $p=0.03$).

356 **Table 4: Characteristics in nulliparous vs parous mares**

Variable	n	Nulliparous	Parous	p

		n=190	n=311	
Pregnancy rates on D14	501	37.4%	44.7%	0.066**
Age				
≤ 5	40	17.4%	2.3%	< 0.0001
5 < Age ≤ 10	118	17.9%	27.0%	
10 < Age ≤ 15	185	43.2%	33.1%	
Age > 15	158	21.6%	37.6%	
Lactation				
Non nursing	381	100.0%	61.4%	< 0.0001
Nursing	120		38.6%	
Induction of ovulation				
No	145	35.8%	24.8%	0.008
Yes	356	64.2%	75.2%	

AI: Artificial insemination, FAI: insemination with fresh semen, CTAI: insemination with cooled transported semen, FZAI: insemination with frozen semen

** Results from multivariate analysis

3.3.3. Effect of nursing at insemination

Neither induction of the estrus cycle nor ovulation induction, semen preparation, number of AI per estrus required for a gestation at Day 14, number of straws in case of FZAI nor the number of ovulations or twin embryos at Day 14 were significantly affected by maternal suckling status (Supplementary Table 5).

366 Significantly less suckling mares were affected by post-breeding infection than non-suckling ones
367 (Table 5) but pregnancy rates were not affecting by lactational status (45.8% and 40.7% of pregnancy
368 rate per cycle for, respectively suckling and non-sucking mares, p = 0.32).
369 There were more suckling mares younger than 10 years of age and more non-suckling mares aged
370 more than 10 years. Mares younger than 5 and mares aged between 10 to 15 years were less
371 frequently nursing than mares aged 5-10. In the group of 5-10 years old, more suckling mares were
372 present in comparison to non-suckling ones while the lactational status was similarly represented in
373 16 years old and older mares. In average, however, the age of suckling *vs* non-suckling mares was not
374 different (respectively 13.0 ± 0.2 *vs* 12.6 ± 0.2 years old, p = 0.39). Less nursing mares were bred in
375 March and April but more were bred in June than non-nursing ones (Table 5). Moreover, more
376 nursing mares were pregnant at Day 14 within the first exploited cycle than non-nursing ones.
377 Estrus was shorter (respectively, 4.7 ± 0.16 *vs* 5.2 ± 0.1 days, p = 0.03) and preovulatory follicle
378 diameter was larger (respectively 43.8 ± 0.4 *vs* 42.0 ± 0.2, p < 0.0001) in nursing vs non-nursing
379 mares.
380 **Table 5: Characteristics in non-suckling *vs* suckling mares**

Variable	**n**	**Non suckling**	**Suckling**	**p**
		n=381	**n=120**	
Post-breeding inflammation	501	42.8%	14.2%	< 0.0001
Age				
≤ 10 years old	158	28.6%	40.8%	0.012
> 10 years old	343	71.4%	59.2%	
≤ 5	40	9.7%	2.5%	< 0.0001
5 < Age ≤ 10	118	18.9%	38.3%	

10 < Age ≤ 15	185	39.6%	28.3%	
Age > 15	158	31.8%	30.8%	
Month of AI				
March	35	8.1%	3.3%	0.005
April	103	22.8%	13.3%	
May	149	29.7%	30.0%	
June	153	26.7%	42.5%	
July - August	61	12.6%	10.8%	
Number of cycles for pregnancy				
1	276	52.5%	63.3%	0.037
> 1	225	47.5%	36.7%	

* AI: Artificial insemination, FAI: insemination with fresh semen, CTAI: insemination with cooled transported semen, FZAI: insemination with frozen semen

4. Discussion

Data presented here are summarized in Figure 3.

4.1. Post-breeding inflammation

4.1.1. Prevalence

In this study, more than a third of the monitored cycles were followed by post-breeding uterine fluid accumulation. In a normal population of Thoroughbred mares, around 15% of mares are susceptible to post-breeding inflammation that persists for several days [29]. In a more recent study in Thoroughbreds in UK, however, post-breading fluid accumulation occurred in 47.7% of analyzed

392 pregnancies [17]. The observed rate in the present study is therefore in the range of the literature. In
393 comparison to other studies, however, the post-breeding inflammation rate was maybe
394 overestimated. Indeed, as mares could be kept on the breeding stud for several cycles, the same
395 mares could have had several inflammations over the entire breeding period, as this condition is
396 generally persistent [30].

397

398 #### 4.1.2. Effect of maternal age, parity and lactation status

399 In the studied population, there was twice more post-breeding endometritis in mares older than 10
400 years than in younger mares. These results agreed with literature. Indeed, one recent study in
401 Thoroughbred stud farms showed that the percentage of mares with post-breeding intrauterine fluid
402 accumulation [17], as well as the frequency of endometritis in other breeds, increase with mare age
403 [27,31–35].

404 In addition, suckling at insemination appeared to have a protective effect against post-breeding
405 endometritis. To the authors' knowledge, there is no study on the effect of nursing at insemination
406 on post breeding endometritis. Since lactating mares are producing endogenous oxytocin, the well-
407 known and widespread treatment against post-breeding inflammation [36] could explain the
408 observed improvement in nursing mares. Indeed, nursing induces oxytocin release in the blood
409 circulation, reaching a peak of around 10mIU/L in nursing pony and broodmares [37,38]. Plasma
410 oxytocin concentrations of 10-7 mIU/L were reported to be present 20min after the beginning of the
411 suckling period [37]. Since sucking bouts last around 20min during the first month after birth, and
412 since foals nurse at hourly intervals, the release of oxytocin happens in a regular manner throughout
413 the day [38].

414

415 #### 4.1.3. Effect of the method of semen preservation and semen quantity

416 In this study, nor semen preservation method nor the volume determined by number of frozen
417 straws, nor the number of inseminations were related to post-breeding endometritis. One study
418 showed that a similar volume of uterine fluid accumulated in the uterus after insemination with
419 cooled or frozen semen which is consistent with results obtained here [39]. One recent study,
420 however, showed that the uterine inflammatory response was positively corelated to the number of
421 spermatozoid used for the insemination with frozen semen [40]. This high inflammatory response
422 was also faster to resume than the response for low doses of semen, which could explain the lack of
423 influence of the number of straws in the present study.

424

425 #### 4.1.4. Effect of month of insemination

426 Data showed that there were more inflammations/endometritis when insemination was performed
427 late in the season, i.e., during July and August. Several hypotheses could explain this result. The first
428 is that mares with good quality uteri needed less cycles to become pregnant and therefore that more
429 mares with reproductive troubles were inseminated late in the season. Another hypothesis could be
430 that there is more post-breeding inflammation from June/July onwards because of the increased
431 environmental temperature. So far, there is no study on the effects of outdoor temperature on the
432 incidence of endometritis in mares but in dairy cows, environmental heat, as evaluated by comparing
433 the possibility of sheltering or not from the sun, increased rectal temperature by almost one degree
434 [41] and reduced uterine blood flow [42]. The temperature increase, by acting directly on uterine
435 temperature and/or on uterine blood flow could therefore promote inflammation in the mares'
436 uterus.

437

438 #### 4.1.5. Treatment and pregnancy rates

439 In the present study, when a post-breeding inflammation was detected, treatment was
440 systematically applied. Most of the time, this treatment was limited to an injection of oxytocin. In a
441 recent study about therapeutic practices in intensively managed Thoroughbred mares, almost half of
442 the pregnant mares were treated with intrauterine antibiotics and the same proportion was treated
443 with oxytocin. Oxytocin combined with intrauterine antibiotics are used prophylactically in
444 Thoroughbreds in the UK to avoid uterine infections that breeders believe to be a cause of
445 conception failure and embryo loss [17]. In Thoroughbreds, however, less than 10% of early
446 pregnancy failures were associated with uterine inflammation [17,32]. Another study on 99
447 Thoroughbred mares showed that there was no association between post-breeding uterine fluid
448 accumulation and pregnancy rates nor embryonic death [43], as observed here. In all studies, the
449 systematic management to prevent/cure anormal inflammation could explain this lack of association.
450 Therefore, today, the post-breeding inflammation seems to be well handled by stud farms.

451 In different breeds, the combination of antibiotics with oxytocin was shown to be more efficient than
452 antibiotics or oxytocin alone to prevent decreased pregnancy rates after mating [44]. In another
453 study on warmblood mares artificially inseminated, however, oxytocin alone was sufficient to
454 increase pregnancy rates in comparison to no treatment [36]. In most of cases, here, oxytocin alone
455 was applied and was sufficient to avoid altered pregnancy rate. This, therefore suggested that this
456 treatment alone is efficient to avoid decreased pregnancy rates in artificially inseminated mares.

457

458 ## 4.2. Pregnancy rates

459 ### 4.2.1. Prevalence

460 In studies on different breeds in several countries using artificial insemination, pregnancy rates were
461 between 40-80%, according to the semen preservation method [15,45–47]. In a comparable recent
462 study on 328 sport mares artificially inseminated in the Netherlands, 46.6% of gestations were

463 obtained on Day 12 – 18 [48]. Here, pregnancy rates per cycles were similar with 41.9% of mares
464 being pregnant by cycle regardless of the method used for semen preservation.

465

466 #### 4.2.2. Effect of maternal age, parity and lactation status

467 Pregnancy rates were reduced when mares were older than 10 years old at the time of insemination.
468 Several studies already showed a reduction of pregnancy rates with increased maternal age
469 [1,7,10,31,49–51]. Most studies, however, observed differences in mares older than 14 years old and
470 not as early as 10 years old. Here, the clustering in 4 classes (≤ 5, 5-9, 10-14 and ≥ 15 years old) was
471 not associated with alteration of pregnancy rates. This could be because pregnancy rates were only
472 slightly reduced with age and due to the limited number of cycles studied, the present study was not
473 able to demonstrate these changes.

474 Maternal parity tended to not reduce pregnancy rates in the overall population. Effect of maternal
475 parity on pregnancy rates are controversial and related to mares' age. Indeed, when nulliparous
476 mares were mostly older than 7 years, a detrimental effect of nulliparity was observed [2,15] but
477 when nulliparous mares were younger, no deleterious or a favorable effect of parity was observed
478 [2,14]. Here, more than 60% of nulliparous were older than 10 years and this could explain the
479 observed tendency.

480 When considering only mares older than 10 years, D14 pregnancy rates were reduced in nulliparous
481 mares while in the youngest group, the parity did not affect the incidence of pregnancy. Thus, the
482 present data indicate a deleterious cumulative effect of nulliparity and aging on fertility in mares.

483 Lactation at the time of insemination did not influence pregnancy rates, 14 days post ovulation.
484 Foaling mares, however, needed less cycles to become pregnant. In the literature, the effect of
485 lactation on fertility is controversial as some studies observed that nursing mares are more fertile
486 than non-nursing ones [2,8,31,50,51] while others do not observe any difference [4,7,10,14].

487 Insemination at foal heat was previously shown to reduce pregnancy rates [24,52] and to be
488 associated with increased embryonic death [22,23,52]. Here, most foaling mares were bred on foal
489 heat but this did not affect pregnancy rates.

490

491 #### 4.2.3. Effect the number of ovulations

492 The more ovulations were observed, the higher the likelihood that the mare was to be pregnant at
493 14 days post ovulation. The result obtained here seem obvious as double ovulations increase the
494 number of oocytes that could be fertilized.

495

496 #### 4.2.4. Effect of the method of semen preservation and semen quantity

497 As previously observed [45,46,48,53], the modalities of insemination influenced the probability for a
498 mare to be pregnant on Day 14. Indeed, pregnancy rate was higher after FAI or CTAI than after FZAI.
499 It is well known that stallion semen quality decreases with cryopreservation and that a higher critical
500 number of mobile spermatozoa per dose is required to reach same pregnancy rates than with fresh
501 semen [53]. In this study, the number of straws was not related to pregnancy rate, as also shown by
502 others [45]. The protocol involving ultrasonography every 6h and deep intra-uterine AI after
503 observed ovulation seemed to be effective to reach acceptable pregnancy rates with 4 straws or less.

504

505 #### 4.2.5. Effect of month of insemination

506 Month of insemination tended to influence the pregnancy rate, with reduced incidence of
507 pregnancies in March and June. In another French study, March was one of the more prolific month
508 in terms of foal productivity per mare but all breed were considered (Thoroughbred, Standardbred

509 and sport bred) [2]. In racehorse breeding, it is common to use light to advance the breeding season
510 [54] since foals born early in the year have an advantage when they are sold as yearling. In sport
511 horse breeding under European latitudes, however, March is often the beginning of the breeding
512 season. At this time, mare enter the spring transitional period and less mares are bred, which could
513 explain the reduced pregnancy rates on this month. The other French study also reported a fertility
514 decrease when mares were bred in June and after [2]. The June effect, here, is more complicated to
515 explain as July and August did not affect pregnancy rates. During their stay, all mares were housed in
516 individual stable with no access to fresh grass. They were fed with hay. A change in food quality in
517 June could not explain the present results. Nevertheless, increasing ambient temperature could
518 explain the differences in pregnancy rates as temperatures reached a maximum of 35.8°C in one stud
519 farm in June 2019. In July and August, temperature were also high with more than 33°C as maximal
520 temperature in the 2 studied stud farms. The lack of difference in July and August could be explained
521 by the fact that only few mares were bred during this period and that technicians and veterinarians
522 were maybe more caring.

523

524 ## 4.3. Effects of age on other reproductive outcomes

525 ### 4.3.1. Choice of semen preservation method

526 Maternal age did not influence the breeders' choice concerning ovulation management but
527 influenced the modality of AI. Indeed, less frozen and fresh semen were chosen for the insemination
528 of older mares. On one side, frozen semen is less efficient for producing a foal, as confirmed by
529 literature [53] and this study. Moreover, reduced fertility has been observed in old mares, both in
530 literature [for review see 47] and in the present study. The combination of both factors could explain
531 that older mares tended to be inseminated less with frozen semen as the financial risk might have
532 been too costly for breeders. Moreover, currently, equine breeders do not use more straws for old
533 than for young mares, even if it has been shown that increasing straw number could improve fertility

534 [53]. On the other side, as more than half of the mares older than 10 years were parous in the
535 present study, breeders could have been looking for stallions that are not available on the
536 reproductive centers for fresh insemination to look for genetic diversity, thus explaining why cooled
537 transported semen was preferred.

538

539 #### 4.3.2. Length of estrus and ovulation outcomes

540 This retrospective study shows that the length of estrus tended to increase with mare age (only 0.4
541 day more in old mares), with a smaller preovulatory follicle. This was already observed as a
542 prolonged interovulatory interval was associated with a prolonged follicular growth [56–63] and to a
543 reduction in follicular growth and preovulatory follicular size in oldest mares [64–67].

544 In the studied population, more multiple ovulations were also observed in older mares. Several
545 studies reported that the incidence of multiple ovulation continuously increases until the mare
546 reaches 20 years of age [62,67–69]. More multiple ovulations in older mares, however, is not
547 associated with more twinning at 14 days post ovulation. Studies have shown that even if fertilization
548 appears to be equal between young and old mares, early embryo mortality is increased in older
549 mares [49,70] and this could explain that there are no more twin embryos observed at 14 days in
550 older mares than in younger ones.

551

552 ### 4.4. Effects of parity on other reproductive outcomes

553 Nulliparous mares had smaller preovulatory follicles. As explained above, the monitoring of mares
554 was not performed more than twice a day: since follicles continuously grow until breakdown, this
555 observed difference should be considered with caution.

556

557 ### 4.5. Effects of suckling at insemination on other reproductive outcomes

558 In sport horses, mares are often bred and subsequently foal during the spring. Foaling mares are
559 most of the time pregnant at the beginning of the spring. It is, therefore, not surprising to observe a
560 peak of breeding in May and June in foaling mares while barren mare breedings were more spread
561 throughout the reproductive season. Moreover, as it appears that less estrus cycles were required to
562 obtain a pregnancy in lactating mares, it is not surprising either to not observed many lactating
563 mares in August.

564 Finally, the heat period was shortened and the preovulatory follicle was larger when mares were in
565 lactation without changing the number of ovulations. To the author knowledge, there is no study on
566 the effect of lactation at insemination time on estrus and follicle parameters.

567

568 ## 5. Conclusion

569 In conclusion, maternal age appeared to be the most important factor affecting both post-breeding
570 inflammation and pregnancy rates. Both could be explained by the degenerative changes that are
571 observed in older mares. Breeders, should, therefore, be encouraged to pay more attention to the
572 age of their broodmares and either, breed earlier in their life or culling them earlier to avoid
573 excessive costs. Older mares are also of less interest concerning genetics point of view [71] that
574 should also be considered by breeders. Moreover, as frozen semen was associated with decreased
575 pregnancy rates, the use of fresh or cooled semen for the insemination of old mares should be
576 advised.

577 In this study, it has also been shown that nulliparity as the same time to aging affected pregnancy
578 rates. For their first gestation, mares should not be more than 10 years old to increase chance of

579 pregnancy. One advice to breeders could be to use the mares' time before competition to produce
580 their first foal as it will help for the later inseminations.

581 Suckling at insemination prevented post-breeding inflammation. The most probable hypothesis could
582 be that sucking provokes oxytocin release that is acting as a natural treatment of uterine fluid
583 accumulation.

584 Here, pregnancy rates were not affected by post-breeding inflammation, although uterine fluid
585 accumulation were more frequent than in other studies. This suggests that endometritis is well
586 handled and that oxytocin only as a treatment is efficient.

587

Declaration of competing interest

None.

Acknowledgements

The authors tanks V. Lehuraux, A Jugland, B. Brisset and P. Drevillon from the Stud of “Charmoy” (89) and Stud of “Les Bréviaires” (78) for the availability of the data and for their hospitality during the visits of their organizations. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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810 # Figure legends

811 Figure 1: Odds ratio for factors influencing the likelihood of post-breeding endometritis in sport
812 mares

813 Figure 2: Odds ratio for factors influencing the likelihood of pregnancy in sport mares

814 Figure 3: Factors affecting post-breeding endometritis and pregnancy rates in the studied population
815 of sport mares

816 Red arrows significates that the factor increased the likelihood while blue arrows indicated that the
817 factor decreased the likelihood.

818

Supplementary data legends

Supplementary table 1

Sup1_PostBreedingInfla_OR.xlsx

Odds ratio for factors that did not influence the likelihood of post-breeding endometritis in sport mares

Supplementary table 2

Sup2_DG14_OR.xlsx

Odds ratio for factors that did not influence the likelihood of pregnancy in sport mares

Supplementary table 3

Sup3_Age_NS.xlsx

Factors that were not associated with mares' age

Supplementary table 4

Sup4_parity_NS.xlsx

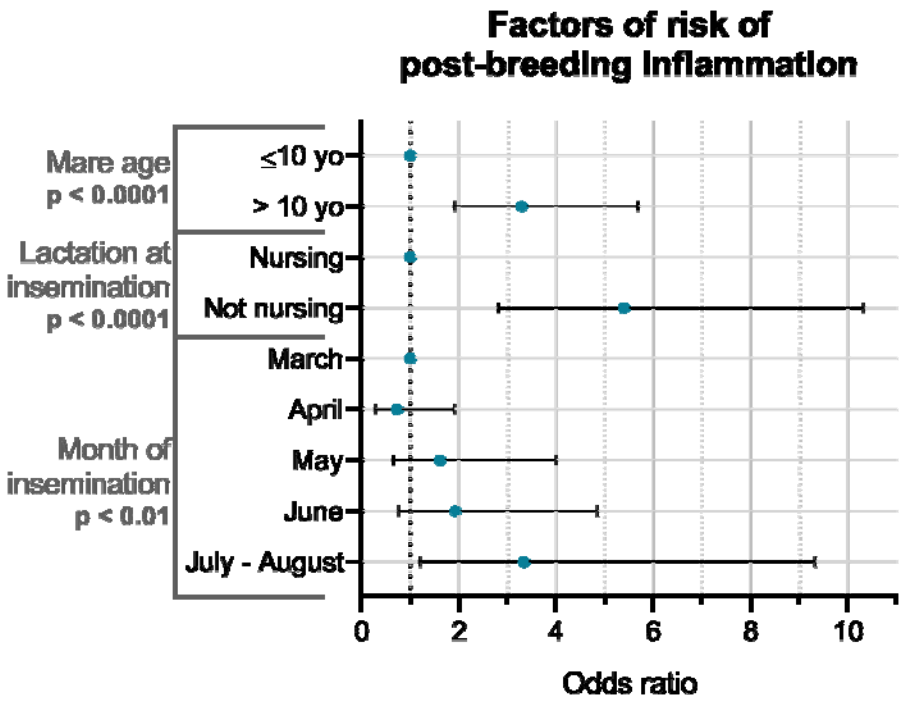
Factors that were not associated with mares' parity

Supplementary table 5

Sup4_nursing_NS.xlsx

Factors that were not associated with mares' nursing

841 **Figures**



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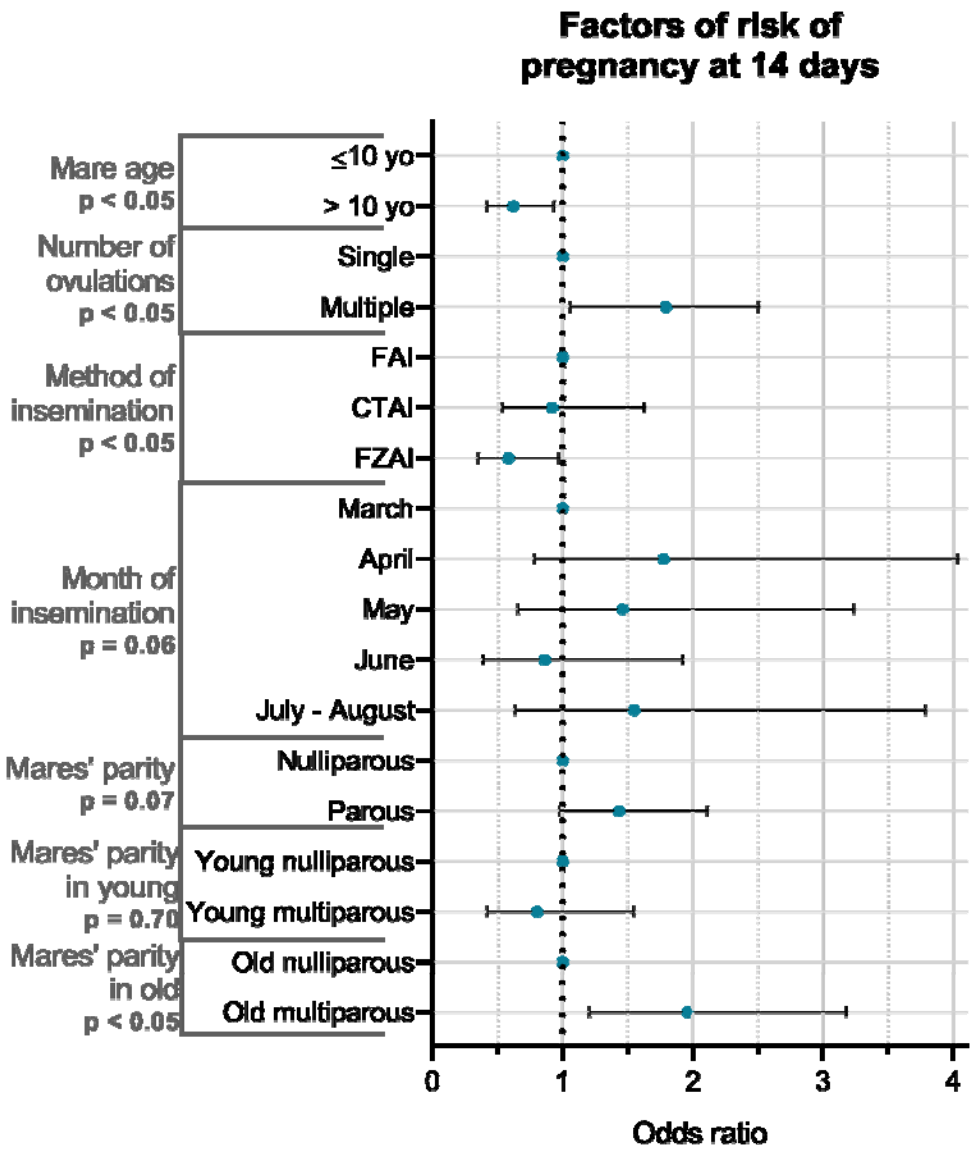

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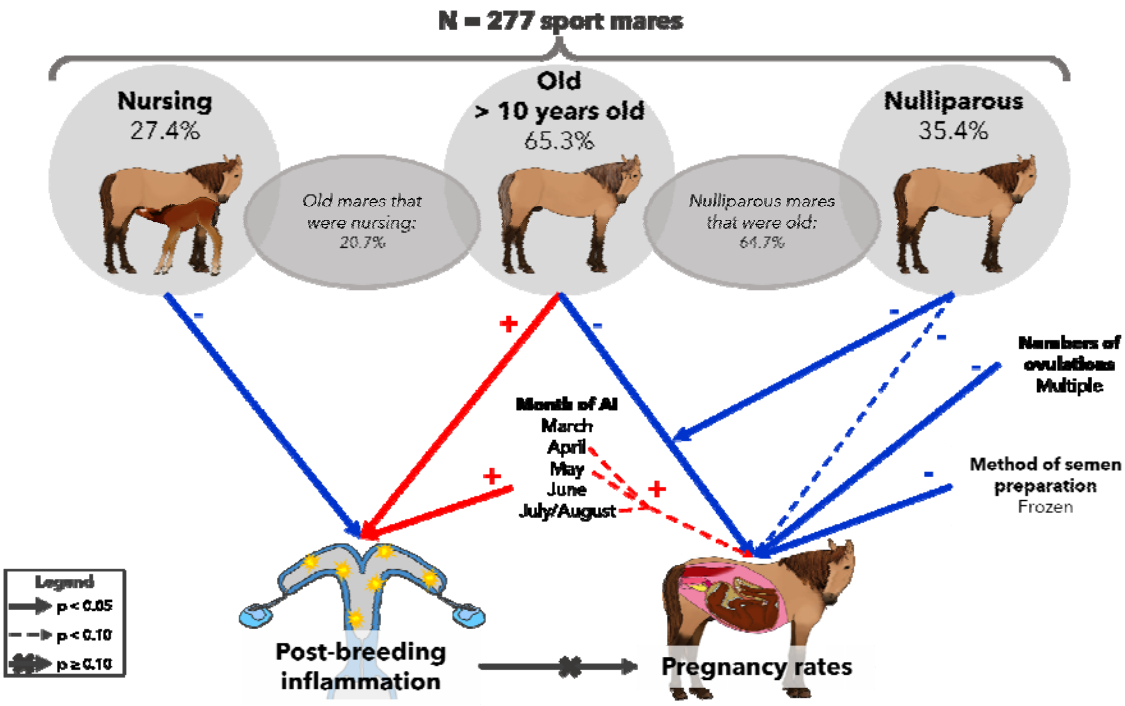
N = 277 sport mares
Nursing
27.4%
Old
> 10 years old
65.3%
Nulliparous
35.4%
Old mares that were nursing: 20.7%
Nulliparous mares that were old: 64.7%
Month of AI
March
April
May
June
July/August
Numbers of ovulations
Multiple
Method of semen preparation
Frozen
Legend
p < 0.05
p < 0.10
p ≥ 0.10
Post-breeding inflammation
Pregnancy rates

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