Analysis of factors affecting the pregnancy rate of mares after inseminations with cooled transported stallion semen

Kareskoski, Maria

2019-03-15

Kareskoski , M , Venhoranta , H , Virtala , A-M & Katila , T 2019 , ' Analysis of factors affecting the pregnancy rate of mares after inseminations with cooled transported stallion semen ' , Theriogenology , vol. 127 , pp. 7-14 . https://doi.org/10.1016/j.theriogenology.2018.12.036

http://hdl.handle.net/10138/308875
https://doi.org/10.1016/j.theriogenology.2018.12.036

Analysis of factors affecting the pregnancy rate of mares after inseminations with cooled transported stallion semen

Kareskoski Maria[a], Venhoranta Heli[a], Virtala Anna-Maija[b], Katila Terttu[a]

[a]University of Helsinki, Faculty of Veterinary Medicine, Paroninkuja 20, 04920 Saarentaus, Finland

[b]University of Helsinki, Faculty of Veterinary Medicine, PO Box 66, 00014 Helsinki, Finland; anna-maija.virtala@helsinki.fi

Corresponding author: Katila Terttu, University of Helsinki, Paroninkuja 20, 04920 Saarentaus, Finland; terttu.katila@helsinki.fi

Abstract

Artificial insemination (AI) with cooled stallion semen has increased markedly during the last decades in all countries, but fertility is often lower than when fresh semen or natural mating is used. The objective of this study was to examine field data (1634 inseminations, 523 Standardbred (SB) mares, 575 Finnhorse (FH) mares, and 90 stallions) using multivariable logistic regression for factors influencing the pregnancy rate (PR) after AI with cooled transported semen from SB and FH stallions. The PR per cycle for the material was 47%: Finnhorses 42% and Standardbreds 53%. When assessed with multivariable logistic regression analyses with a generalized linear mixed model, variables that affected the PR were breed, the number of inseminated estrus cycles, the percentage of progressively motile sperm (PMOT) in the ejaculate/AI dose at the time of shipment, and the number of progressively motile sperm in the AI dose at the time of insemination. In Standardbreds, variables that increased the per cycle PR were the number of AI per estrus cycle (multiple inseminations increasing the probability of pregnancy compared to only one insemination), the number of inseminated cycles, and PMOT in the AI dose at the time of insemination. In Finnhorses, the number of AI per estrus cycle (two and three inseminations increasing the probability of pregnancy compared to only one), the number of spermatozoa in the ejaculate and in the AI dose, and PMOT in the ejaculate/AI dose at the time of shipment increased the per cycle PR. Non-significant factors for the whole material included the type of artificial vagina (open-ended or closed), transport time, place of AI (stud farm or home stable), insemination done by veterinarian or technician, weekday, month, age of the mare (all age classes combined), age of the stallion, ejaculate parameters (sperm concentration, total number of sperm), and insemination dose parameters (volume

proportion of seminal plasma, sperm concentration, PMOT, total number of sperm). In conclusion, breed, breeding opportunity in more than one cycle, more than one insemination/estrus, PMOT of the ejaculate/AI dose and the number of progressively motile sperm in the AI dose at the time of insemination are important for the outcome of inseminations with cooled semen.

Keywords: horse, fertility, insemination dose, seminal plasma, progressive sperm motility, sperm numbers

1. Introduction

The use of artificial insemination (AI) by transported cooled semen has markedly increased during the last decades in all countries, whereas on-site AI and natural mating have become less popular. From the year 1991 to 2005, the use of transported semen increased from 15 to 43% of all registered matings among Standardbreds in Finland [1].

Successful storage of fresh semen for transportation requires dilution by adding extender and cooling of semen [2]. The optimal temperature for semen storage is from +5ºC to +10ºC, which temperature the styrofoam boxes used in Finland keep for 33 h in room temperature [3]. In practice, the boxes are exposed to various ambient temperatures, but in an Austrian study, the average temperature was still 9.8ºC after an average transport time of 26.5 h [4]. The duration of transport varies but typically it is around 24 h, frequently from 12 to 48 h.

Dilution of semen is necessary to decrease the sperm concentration; 25 x 10^6/mL maintains motility best during storage [5] but concentrations between 50 and 100 x 10^6/mL are more practical because of smaller volumes in the transport containers [6]. Another important goal in the dilution of semen is to decrease the volume proportion of seminal plasma (SP) in the stored insemination dose. A high proportion (more than 20%) of SP is harmful for the maintenance of sperm motility during long-term storage [7]. According to Jasko et al. [8], 5 to 20% of SP should be present in the shipment dose. If the semen is to be stored for over 24 h, the doses should ideally contain no more than 10% of SP [7, 9]. It has also been shown that the complete removal of SP protects sperm DNA integrity during 24 and 48 h of cooled storage [10]. Centrifugation of semen to reduce the proportion of SP is particularly important for "poor cooling" stallions [11].

A reduction of SP proportion is easily accomplished by adding extender to the semen, but concentrating the semen may be necessary, if the initial sperm concentration is low. A quick and practical concentration method is fractionated semen collection with open-ended artificial vagina (AV). In this method, semen is collected directly from the urethra using a large funnel to direct the desired jets into the collection device. The Krakow model AV developed by Marian Tischner was easily available in Finland when AI started in the early 1980's and it is still commonly used. Semen concentration and volume decrease with each successive jet during the ejaculation [12]. Usually the first two or three jets are collected, and additional advantages are avoidance of gel fractions and bacteria [13, 14]. Centrifugation of semen is not practiced in the Finnish stud farms.

It is to be expected that extra handling of semen, cooling and long storage decrease the quality and fertilizing capacity of sperm. When compared to on-site AI, pregnancy rates (PR) after AI with transported semen are significantly lower [1,15]. To compensate for the decrease in semen quality during storage, larger AI doses (0.5 to 1 x 10^9 sperm) are recommended for shipped semen [16], in Finland the recommendation is 1 x 10^9 progressively motile spermatozoa.

There are large differences between stallions in initial semen quality [17, 18], but particularly in the tolerance for cooled storage [15]. The center responsible for semen collection and handling has a profound effect on semen quality parameters at the time of AI [4]. There are also many other important reasons for the reduction in fertility that are not related to the stallion, such as inadequate mare management and veterinary care [16].

The objective of this study was to examine field data using multivariable logistic regression for factors influencing pregnancy rates after inseminations with cooled transported semen.

2. Material and methods

The data, 3557 inseminations, originate from all reported inseminations with cooled transported semen of Finnhorses (FH) and Standardbred (SB) trotters in Finland during the year 2007. Practically all matings are reported each year to the Finnish trotting and breeding association Suomen Hippos, as only foals born from registered matings are registered and allowed to race.

In Finland, a form is enclosed in every semen transport container to give information to the inseminator about the ejaculate of the stallion, preparation of the AI dose and the type of AV used as well as the time of semen collection. The form is returned to the semen collection centre with information regarding inseminated mares, inseminator, place and time of AI and progressive sperm motility (PMOT), if possible. One copy is sent to Suomen Hippos at the end of the season. We requested and received all copies from Suomen Hippos from one breeding season for analysis. The mating records are available for everyone and are published yearly by Suomen Hippos.

In Finland, semen is available on Mondays, Wednesdays and Fridays meaning that mares are usually inseminated every other day until ovulation - apart from the weekend. For each insemination, the following data were calculated and recorded for both the original ejaculate and the insemination dose prepared from the ejaculate: volume (mL), sperm concentration (10^6 sperm/mL), total number of spermatozoa (10^9), PMOT (%, subjective assessment with light microscopy) and number of progressively motile spermatozoa (10^9). In addition, PMOT at the time of AI was reported for 781 inseminations allowing the calculation of the number of progressively motile sperm at insemination. The AI doses were classified according to SP content: not more than 25%, 25 to 33%, or at least 34% SP. Per cycle pregnancy rates were determined using the forms: new semen orders and inseminations indicated a previous non-pregnant cycle. The result of the last estrus was based on foaling results from Suomen Hippos: all mares reported having foaled or aborted were considered pregnant.

The following factors were included in the analysis: breed (FH or SB), type of AV (open-ended or closed), transport time (h), place of AI (stud farm/clinic or home stable), insemination done by veterinarian or technician, number of AI cycles, number of inseminations per cycle, weekday, month, age of the mare, age of the stallion, ejaculate parameters (sperm concentration, total number of sperm, PMOT), and insemination dose parameters at the time of shipment (volume proportion of SP, sperm concentration, PMOT, total number of sperm, and number of progressively motile sperm) and at the time of insemination (PMOT and the number of progressively motile sperm), and the outcome of the insemination (pregnant or non-pregnant cycle). Progressive sperm motility of the ejaculate and of the AI dose at the time of sending is the same.

All warmblood riding horses, horses of unknown breed, and insemination forms with incorrect calculations or deficient or incomprehensive information were excluded. Insemination forms with the following extreme values were also excluded: sperm concentration more than 500 x 10^6 sperm/mL in ejaculates collected with a closed AV, sperm concentration more than 1000 x 10^6 sperm/mL in ejaculates collected with an open-ended AV, total number of spermatozoa/ejaculate more than 20 x 10^9, or progressive motility before storage more than 80%. Six stud farms had had less than ten shipments, wherefore their data of 20 shipments were also excluded. All records of one studfarm were excluded because the examination of semen was obviously not correctly done and subsequent dilution calculations were also wrong. Some of the report forms had been filled in incompletely, and therefore the number of observations vary between parameters. If the mare had been inseminated more

than once during the same estrus, only the last insemination dose was included in the calculation of the PR per cycle resulting in 1634 last inseminations, 523 Standarbred mares, 575 Finnhorse mares and 90 stallions. All inseminations, a total of 2928 inseminations, were assessed for the number of inseminations per season and per estrus.

The 95% confidence intervals (CI) for pregnancy rates were calculated with Wilson's method [19] using an internet-based epidemiological calculator [20]. After description of the data, the association of each factor separately with the per cycle PR (mare pregnant or not) was calculated using crude logistic regression analyses with generalized linear mixed model in the SAS software (GLIMMIX procedure, SAS Institute Inc., Cary, NC, USA); mare, stallion and stud farm were included as random effects. Factors with a Wald's p-value <0.2 were included in multivariable multilevel logistic regression analyses with generalized linear mixed models. Random intercepts for mare, stallion and stud farm were included in logistic models to account for the hierarchical structure of the data. In multivariable models, factors with Wald's $p \leq 0.05$ were considered significant. Several multivariable models were created since all factors describing the ejaculate could not be included in same model if they biologically represent the same phenomenon.

3. Results

The pregnancy rate per cycle of the whole material was 47.0%: FH 42.1% and SB 52.8%; the difference was significant when assessed with 95% CIs (Table 1). Foaling rates were 59.4% for FH and 73.5% for SB, 65.9 % for the whole material as

reported by Suomen Hippos. The pregnancy rates for all other categorical variables are shown in the same table. Tables 2a and 2b show descriptive statistics for semen quality and inseminations resulting in pregnancy or non-pregnancy separately for the two breeds. It seems that in the Standardbreds semen volume, sperm concentration and PMOT of the ejaculate, as well as PMOT at the time of insemination, were higher than in the Finnhorses both for pregnant and non-pregnant mares.

3.1. Crude logistic regression analyses

Variables that differed significantly between pregnant and non-pregnant mare groups in the crude logistic regression analyses were breed, the number of inseminated estrus cycles, age of the mare (2-9 vs. 17-22 years), PMOT in the ejaculate/AI dose at the time of shipment, and PMOT and the number of progressively motile sperm in the AI dose at the time of insemination (Table 3).

3.2. Multivariable logistic regression analyses

In the multivariable logistic regression models, variables that increased significantly the PR were breed, the number of inseminated cycles, PMOT in the ejaculate/AI dose, and the number of progressively motile sperm in the AI dose at the time of insemination (Table 4).

In the FH population, the number of inseminations per estrus cycle (two and three inseminations increasing the probability of pregnancy compared to only one insemination), higher numbers of spermatozoa in the ejaculate and in the AI dose, and higher PMOT in the ejaculate/AI dose increased the per cycle PR (Table 5). In the SB population, variables that increased the per cycle PR were the number of

inseminations per estrus cycle (multiple inseminations increasing the probability of pregnancy compared to only one insemination), the number of cycles (≥2 inseminated cycles better than one cycle), and higher PMOT in the AI dose at the time of insemination (Table 5).

4. Discussion

Breed had a significant effect on the per cycle PR, with the FH having a lower per cycle PR (42%) than the SB (53%). In addition to genetic traits within a breed, this is likely to be related to differences in mare management and to selection of mares to the breeding population. In the study of Langlois and Blouin [21], French draught breeds had lower foaling rates than saddle breeds and trotters, possibly related to amateurism, dystocia, and differences in the reporting of mating and born foals. Similarly, FH breeders are often less professional than SB breeders, which may affect mare management and the use of veterinary expertise.

Mare age

Katila et al. [1] reported lower foaling rates for the FH than for the SB in Finland (all breeding methods included). This was explained by a different structure of the mare populations: FH mares were older, and there were more barren mares and fewer foaling mares in the FH mare population compared to the SB. The FH seemed to enter the barren mare category at a younger age and the maiden mare category at an older age than the SB. Also in the present study, the FH mares were about one year older than the SB mares. The negative effect of ageing on mare fertility has been reported in all studies where mare age has been included as a factor [1,21-27]. Age

affects many aspects of reproduction, such as uterine function (uterine contractility, intrauterine fluid accumulation, endometrial inflammation and fibrosis), PR and pregnancy loss [22].

In our models, age was only almost significant (the youngest mares from 2 to 9 years having a higher PR than the oldest mares from 17 to 22 years). Its p-value increased to being clearly not significant, if the variable called "Number of progressively motile spermatozoa in the insemination dose (10^9) at the time of insemination" was included in the model. This variable had to be included since it had a confounding effect on the "Progressive sperm motility in the ejaculate/AI dose (%)" almost doubling its odds ratio from 6 to 12-14; therefore, these both had to be included in the model. Because mare age is so important for fertility, we included it into our multivariable models, regardless of its low significance.

Stallion age

Finnhorse stallions were approximately one year older than SB stallions, but stallion age did not come out as a significant factor in our analysis. However, Dowsett and Knott [18] reported that both age and breed of the stallion exerted significant effects on semen parameters. In our model, the total number of spermatozoa both in the ejaculate and in the AI-dose were significant factors for FH but not for SB suggesting that sperm production is lower and therefore more critical for fertility in FH stallions than in SB stallions.

Breeding opportunity

Mares inseminated in one estrus only had significantly lower foaling rates than mares inseminated in two or more cycles. The number of breeding cycles is the decision of the breeder, depending on choices on financial investments in breeding and when breeding is started and when it is finished for each mare, thus affecting the traits of the mare population. If the first cycle PR is less than 50%, as the FH in our study had, the mare would need more than three cycles to achieve the goal of a seasonal PR of 80%. In Finland, the FH mares start to cycle later in spring than the SB mares [28], and therefore their breeding season tends to be shorter than that of SB mares. Both the number of inseminated cycles and inseminations per cycle came out as significant factors, emphasizing the importance of giving enough opportunities for the mare to conceive. Two or three inseminations during estrus resulted in a higher PR in FH than one insemination only or four inseminations or more, but in SB all repeated inseminations were better than only one. Similarly, Sieme et al. [29 showed that multiple inseminations (from 2 to ≥4) resulted in a higher PR compared to one insemination only. In the study of Batellier et al. [30], a second AI was carried out post-ovulation and resulted in higher PR than one insemination. The beneficial effect of repeated inseminations was not related to the added numbers of spermatozoa but presumably to inseminating close to ovulation or to some unknown factor. In the cooled semen study of Squires et al. [31], mares inseminated twice with 1 x 10^9 progressively motile sperm on two consecutive days (24 and 48 h after semen collection) had a higher pregnancy rate (64%) than mares inseminated once with 1 x 10^9 progressively motile sperm (PR 30%) or those inseminated once with 2 x 10^9 progressively motile spermatozoa (PR 41%). However, the mares inseminated only once had 48 h between AI and ovulation as compared to 22 h for the mares inseminated on two consecutive days because hCG was given at the time of the first

AI. When the time between AI and ovulation was standardized, there was no longer any benefit of holding half of the insemination dose for rebreeding on the following day [32]. It is very likely that the advantage of multiple inseminations during the heat is due to a shortened interval from the last insemination to ovulation.

Although high pregnancy rates are achieved with fresh semen up to three days before ovulation [33], inseminations with cooled semen should be done within 24 h before ovulation or within 12 to 16 h after ovulation [29,34]. Heiskanen et al. [35] reported high pregnancy rates with cooled semen stored for 70 or 80 h (77% and 57%, respectively) when the mares were inseminated within 12 h after ovulation with 2 x 10^9 spermatozoa. Mare management and AI close to ovulation play a major role in equine fertility when inseminating with cooled semen.

Semen and insemination dose

The type of AV did not affect PR. Unexpectedly, semen collected by open-ended AV did not have significantly higher sperm concentration than semen collected by a Missouri-type AV. Open collection may have been chosen for stallions producing semen with low sperm concentration, as recommended.

The ideal dilution ratio of semen for shipments is 1:4 (semen:extender) to decrease the proportion of SP to less than 20% [8]. Also, the optimal sperm concentration must be considered, and therefore less extender is added if the sperm concentration of the ejaculate is low [16]. In this material, the mean sperm concentration of the AI dose was between 51 and 54 x 10^6/mL, so in many cases more extender could have been added and still be within the minimal concentration of 25 x 10^6/mL. One

315 explanation for the low dilution rate is the practice to dilute to 40 mL which is the
316 maximum volume that can be accommodated in the transport box. If motility and
317 concentration are low, the total volume with the extender will readily become too
318 high. Because adequate dilution of semen was seldom done, 82% of the AI doses
319 contained more than the recommended 20% SP.
320
321 Several studies have shown that SP decreases sperm survival during storage [7,9,11,
322 36-38]. In the present study, the proportion of SP included in the insemination dose
323 did not seem to affect the outcome of the AI, probably because semen was stored for
324 a short period of time. The choice of AV did not affect the results either, showing
325 that sperm survival during a short-term cooled storage is not different when the
326 whole ejaculate is used instead of the sperm-rich part of the ejaculate. Previously,
327 Varner et al. [5] have demonstrated higher motility for the sperm-rich part compared
328 to the whole ejaculate at 12 and 24 h of storage. According to Kareskoski et al. [36],
329 sperm-rich fractions with low levels of SP maintained a higher level of DNA
330 integrity during 24-h storage than sperm-poor fractions. Although sperm-rich
331 fractions have higher motility and lower DNA fragmentation index, it was not
332 reflected in fertility in our study.
333
334 Despite suboptimal processing of AI doses, the PR (both breeds included) is
335 satisfactory for cooled semen with a per cycle PR of 47% and a foaling rate of 70%.
336 However, the median time from collection to AI was only 10 h which is shorter than
337 in many other countries where it is commonly around 24 h [4].
338

The total number of spermatozoa in the studied insemination doses was unexpectedly high (from 1.7 to 1.9 x 10^9), which shows that most stallions did not have a large book of mares. The high numbers of spermatozoa could have partly compensated for possible negative effects of high SP content and decrease in progressive motility. On the other hand, damaged and abnormal spermatozoa generate large amounts of reactive oxygen species, and therefore low total sperm count with high PMOT may result in better fertility than high sperm numbers with low PMOT [4]. The material also contained some stallions with semen that had decreased tolerance of storage, as shown by the low progressive motility at the time of AI and by a low PR. These stallions could have benefited from a higher dilution rate.

Progressive sperm motility both before and after shipment and the number of progressively motile sperm in the AI dose at the time of insemination significantly affected the PR. In the Austrian study including 102 doses of transported semen, total motility, PMOT and percentage of morphological sperm defects (all these evaluated at the time of AI) significantly influenced the outcome of AI [4]. In our study, PMOT at the time of semen processing was most often reported as 70 or 80%, with only minimal variation. Cooled shipped semen is not a preferred method to use with subfertile stallions, and this may partly account for the lack of variation in PMOT values. Stud farm staff may also overestimate motility in an attempt to increase the number of doses per ejaculate. It is not easy to evaluate PMOT of stallion sperm, particularly with the microscopes at the stud farms which in our experience do not provide the best visualization. Inadequate dilution of concentrated semen before microscopy results in visualization of higher motility because individual spermatozoa cannot be seen. Microscopic evaluation of progressive motility is subjective and

varies considerably among readers [39]. Some studies have shown correlations with fertility whereas some others have failed to do so [8, 39]. Despite these limitations, PMOT is still used routinely as a measure of semen quality in field conditions, and the number of progressively motile sperm at the time of AI shows the ability of semen withstand cooling and storage. In addition, it also reflects the quality of semen processing which affects the results with cooled-shipped semen [4]. The common use of this parameter in practice warrants its inclusion in the analyses in this study.

Conclusions

In conclusion, breed and breed-related mare population characteristics, adequate breeding opportunity, and more than one insemination/estrus in addition to adequate sperm numbers with good progressive motility significantly affect the fertility of inseminations with cooled semen stored for 10 h. Any possible harmful effects of a suboptimal dilution rate and subsequent high proportion of seminal plasma may have been partly compensated by the use of high numbers of spermatozoa in the insemination doses. In stallions whose sperm have poor tolerance of cooling and storage as well as in longer lasting transports, optimal proportion of SP is probably more vital.

Declaration of interests: None.

Funding. This work was supported by Makera funding from the Ministry of Agriculture in Finland.

Acknowledgements. Suomen Hippos and Terttu Peltonen are thanked for providing us the breeding data and semen transport forms. Special thanks to Tero Wahlberg for performing the statistical analysis of the data.

Authorship. MK contributed to analysis and interpretation of the data, drafted the article and approved the final manuscript. HV contributed to acquisition, analysis and interpretation of data, revised the article and approved the final manuscript. A-M V participated in the conception and design of the study and in the analysis and interpretation of data; she also revised the article and approved the final version. TK was responsible for the conception and design of the study, contributed to writing and correction of the manuscript and approved the final version.

References

[1] Katila T, Reilas T, Nivola K, Peltonen T, Virtala A-M. A 15-year survey of reproductive efficiency of Standardbred and Finnhorse trotters in Finland - descriptive results. Acta Vet Scand 2010;52:40.

[2] Pickett BW, Amann RP. Extension and storage of stallion spermatozoa: a review. Equine Vet Sci 1987;7:289-302.

[3] Katila T, Combes GB, Varner DD, Blanchard TL. Comparison of three containers used for the transport of cooled stallion semen. Theriogenology 1997;48:1085-92.

[4] Heckenbichler S, Deichsel K, Peters P, Aurich C. Quality and fertility of cooled shipped stallion semen at the time of insemination. Theriogenology 2011;75:849-56.

[5] Varner DD, Blanchard TL, Love CL, Garcia MC, Kenney RM. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. Theriogenology 1987;28:709-23.

[6] Webb GW, Arns MJ, Pool KC. Sperm concentration influences recovery of progressively motile spermatozoa and number of inseminations shipped in conventional containers. J Equine Vet Sci 1993;13:487-9.

[7] Pruitt JA, Arns MJ, Pool KC. Seminal plasma influences recovery of equine spermatozoa following in vitro culture (37 ºC) and cold-storage (5 ºC). Theriogenology 1993;39:291-6.

[8] Jasko DJ, Hathaway VL, Schaltenbrand VL, Simper WD, Squires EL. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. Theriogenology 1992;37:1241-52.

[9] Todd P, Arns MJ, Chenoweth P, Schultz B. Influence of seminal plasma and processing on cold-stored stallion spermatozoa. Anim Reprod Sci 2001;68:335-6.

[10] Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 2005;63:1584-91.

[11] Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 2000;54:129-36.

[12] Tischner M, Kosiniak K, Bielanski W. Analysis of the pattern of ejaculation in stallions. J Reprod Fert 1974;41:329-35.

[13] Tischner M, Kosiniak K. Bacterial contamination of stallion semen collected by "open" artificial vaginas. Vlaams Diergeneeskundig Tijdschrift 1986;55:90-4.

[14] Lindeberg H, Karjalainen H, Koskinen E, Katila T. Quality of stallion semen obtained by a new semen collection phantom (Equidame) versus a Missouri artificial vagina. Theriogenology 1999;51:1157-73.

[15] Brinkerhoff JM, Love CC, Thompson JA, Blodgett G, Teague SR, Varner DD. Influence of mare age, pre-breeding mare status, breeding method, and stallion on the first cycle pregnancy rates on a large commercial breeding farm. Anim Reprod Sci 2010;121S:S159.

[16] Loomis P. Factors affecting the success of artificial insemination with cooled, transported semen. Proc 38th Ann Conv Am Ass Equine Practitioners 1993;629-47.

462

463 [17] Parlevliet JA, Kemp B, Colenbrander B. Reproductive characteristics and semen
464 quality in maiden Dutch Warmblood stallions. J Reprod Fert 1994;101:183-7.

465

466 [18] Dowsett KF, Knott LM. The influence of age and breed on stallion semen.
467 Theriogenology 1996;46:397-412.

468

469 [19] Brown LD, Cai TT, Das Gupta A. Interval estimation for a binomial proportion. Stat
470 Sci 2001;16:101-33.

471

472 [20] Sergeant ESG. Epitools epidemiological calculators. Aus Vet Animal Health Services
473 and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease,
474 2017. Available at: http://epitools.ausvet.com.au.

475

476 [21] Langlois B, Blouin C. Statistical analysis of some factors affecting the number of
477 horse births in France. Reprod Nutr Dev 2004;44:583-95.

478

479 [22] Carnevale EM, Ginther OJ. Relationship of age to uterine function and reproductive
480 efficiency in the mare**.** Theriogenology 1992;37:231-46.

481

482 [23] Allen WR, Brown L, Wright M, Wilsher S. Reproductive efficiency of Flatrace and
483 National Hunt Thoroughbred mares and stallions in England**.** Equine Vet J 2007;39:438-
484 45.

485

[24] Bosh KA, Powell D, Shelton B, Zent W. Reproductive performance measures among Thoroughbred mares in central Kentucky during the 2004 mating season**.** Equine Vet J 2009;41:883-8.

[25] Hanlon DW, Stevenson M, Evans MJ, Firth EC. Reproductive performance of Thoroughbred mares in the Waikato region of New Zealand: 2. Multivariable analyses and sources of variation at the mare, stallion and stud farm level. N Z Vet J 2012;60:335-43.

[26] Haadem C, Nødtvedt A, Farstad W, Thomassen R. A retrospective cohort study on fertility in the Norwegian Coldblooded trotter after artificial insemination with cooled, shipped versus fresh extended semen. Acta Vet Scand 2015;57:77.

[27] Lane EA, Bijnen MLJ, Osborne M, More SJ, Henderson ISF, Duffy P, Crowe M. Key factors affecting reproductive success of Thoroughbred mares and stallions on a commercial stud farm. Reprod Domest Anim 2016;51:181–7.

[28] Koskinen E, Katila T. Onset of luteal activity in non-foaling mares during the early breeding season in Finland. Acta Vet Scand 1991;32:313-8.

[29] Sieme H, Schäfer T, Stout TAE, Klug E, Waberski D. The effects of different insemination regimes on fertility in mares. Theriogenology 2003;60:1153-64.

[30] Batellier F, Duchamp G, Vidament M, Arnaud G, Palmer E, Magistrini M. Delayed insemination is successful with a new extender for storing fresh equine semen at 15ºC under aerobic conditions. Theriogenology 1998;50:229-36.

[31] Squires EL, Brubaker JK, McCue PM, Pickett BW. Effect of sperm number and frequency of insemination on fertility of mares inseminated with cooled semen. Theriogenology 1998;49:743-9.

[32] Shore MD, Macpherson ML, Combes GB, Varner DD, Blanchard TL. Fertility comparison between breeding at 24 hours or at 24 and 48 hours after collection with cooled equine semen. Theriogenology 1998;50:693-8.

[33] Woods J, Bergfelt DR, Ginther OJ. Effects of time of insemination relative to ovulation on pregnancy rate and embryonic-loss rate in mares. Eq Vet J 1990;22:410-5.

[34] Newcombe JR, Cuervo-Arango J. The effect of time of insemination with fresh cooled transported semen and natural mating relative to ovulation on pregnancy and embryo loss rates in the mare. Reprod Domest Anim 2011;46:678-81.

[35] Heiskanen M-L, Huhtinen M, Pirhonen A, Mäenpää PH. Insemination results with slow-cooled stallion semen stored for 70 or 80 hours. Theriogenology 1994;42:1043-51.

[36] Kareskoski M, Sankari S, Johannisson A, Kindahl H, Andersson M, Katila T. The association of the presence of seminal plasma and its components with stallion fertility and sperm longevity in fractionated ejaculates. Reprod Domest Anim 2011;46:1073-81.

[37] Jasko DJ, Moran DM, Farlin ME, Squires EL. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 1991;35:1059-67.

[38] Braun J, Sakai M, Hochi S, Oguri N. Preservation of ejaculated and epididymal stallion spermatozoa by cooling and freezing. Theriogenology 1994;41:809-18.

[39] Love CC. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76:547-57.

545 Table 1. Pregnancy rate per cycle of mares (mean, 95% confidence interval (CI) in different categories).
546 N = number of inseminated cycles.
547

Variable	Category	N	Pregnancy rate per cycle (95% CI)
Breed	Finnhorse	889	42.2 (39.0-45.5)
	Standardbred	745	52.9 (49.3-56.4)
Artificial vagina	Open-ended	563	47.8 (43.7-51.9)
	Closed	1003	47.4 (44.3-50.5)
	Unknown	68	36.8 (26.3-48.6)
Insemination place	Stud farm/clinic	771	46.7 (43.2-50.2)
	Home stable	640	46.4 (42.6-50.3)
Insemination performed by	Veterinarian	935	47.0 (43.8-50.2)
	Technician	364	46.7 (41.6-51.8)
Number of inseminated estrus cycles	1	1069	46.2 (43.2-49.2)
	≥2	565	48.7 (44.6-52.8)
Number of inseminations/estrus	1	1133	46.0 (43.1-48.9)
	≥2	501	49.5 (45.1-53.9)
Seminal plasma in the insemination dose (% of total volume)	≤25%	662	48.8 (45.0-52.6)
	25.1%-33.9%	382	42.9 (38.1-47.9)
	≥34%	578	47.6 (43.5-51.7)
Month	March-May	403	47.6 (42.8-52.5)
	June	640	46.7 (42.9-50.6)
	July	445	49.2 (44.6-53.8)
	August-September	146	40.4 (32.8-48.5)
Age of mare (years)	2 - 9	561	50.5 (46.3-54.6)
	10 - 13	505	46.3 (42.0-50.7)
	14 - 16	325	46.8 (41.4-52.2)
	17 - 22	203	39.9 (33.4-46.8)
Age of stallion (years)	3 - 6	516	45.2 (40.9-49.5)
	7 - 9	320	51.9 (46.4-57.3)
	10 - 13	459	44.4 (40.0-49.0)
	14 - 26	339	49.0 (43.7-54.3)

548

*Table 2a. Descriptive statistics (mean, standard deviation (SD), median, minimum, and maximum) of cycles resulting in pregnancy or non-*pregnancy for Finnhorses. Number of horses per variable is not necessarily the same as the total number of horses in the group because of missing data.

	Cycles resulting in pregnancy (n=375)					Cycles not resulting in pregnancy (n=514)				
Variable	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max
Age of stallion (years)	11.8	5.3	11.0	5.0	26.0	10.7	4.9	10.5	5.0	26.0
Age of mare (years)	11.8	4.1	12.0	3.0	21.0	12.3	4.2	13.0	3.0	22.0
Semen transport time (hours)	9.4	3.2	9.7	0.8	37.0	9.8	3.6	10.1	0.5	39.0
Semen quality: ejaculate										
Volume (mL)	64.8	33.2	60.0	10.0	180.0	59.3	29.9	55.0	5.0	150.0
Sperm concentration (10^6/mL)	163.7	88.2	134.0	50.0	466.0	159.8	86.1	131.5	25.0	466.0
Total number of spermatozoa (10^9)	9.1	4.3	7.9	1.3	19.9	8.1	3.7	7.5	1.3	19.7
Progressive sperm motility (%) *	75.3	8.9	80.0	20.0	80.0	73.2	11.4	80.0	20.0	80.0
Number of progressively motile spermatozoa (10^9)	6.8	3.3	6.2	0.5	15.9	5.9	2.9	5.7	0.7	15.8
Semen quality: insemination dose										
Volume (mL)	35.4	11.9	40.0	16.0	77.0	35.5	12.3	40.0	15.0	100.0
Seminal plasma (proportion of volume)	0.4	0.1	0.4	0.1	0.7	0.4	0.1	0.3	0.1	0.6
Sperm concentration (10^6/mL)	53.7	17.7	52.8	17.0	105.3	51.1	17.5	52.0	12.5	133.3
Total number of spermatozoa (10^9)	1.8	0.7	1.7	0.7	5.0	1.7	0.7	1.5	0.6	6.0
Number of progressively motile spermatozoa (10^9)	1.3	0.5	1.2	0.5	3.4	1.2	0.4	1.1	0.5	4.8
Progressive sperm motility (%) at insemination	44.3	16.8	40.0	2.0	80.0	42.9	17.8	40.0	3.0	80.0
Number of progressively motile spermatozoa (10^9) at insemination	0.8	0.4	0.7	0.0	2.8	0.8	0.4	0.7	0.0	2.4
Number of inseminations/season	2.0	1.2	2.0	1.0	7.0	2.0	1.3	2.0	1.0	8.0
Number of inseminated estrus cycles	1.5	0.7	1.0	1.0	4.0	1.5	0.8	1.0	1.0	5.0
Number of inseminations/estrus	1.4	0.5	1.0	1.0	3.0	1.4	0.7	1.0	1.0	5.0
Number of insemination doses sent/stud farm/season	121.9	64.0	114.0	10.0	210.0	120.9	63.3	123.0	10.0	210.0

549 *is also progressive motility of the insemination dose at the time of sending

Table 2b. Descriptive statistics (mean, standard deviation (SD), median, minimum, and maximum) of cycles resulting in pregnancy or non-pregnancy for Standardbreds. Number of horses per variable is not necessarily the total number of horses in the group.

	Cycles resulting in pregnancy (n=394)					Cycles not resulting in pregnancy (n=351)				
Variable	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max
Age of stallion (years)	9.0	4.7	7.0	3.0	24.0	9.0	4.5	7.0	3.0	24.0
Age of mare (years)	10.7	3.9	10.0	2.0	21.0	11.0	3.8	10.0	2.0	21.0
Semen transport time (hours)	10.4	3.0	10.4	2.3	27.0	9.9	3.4	10.0	1.5	29.8
Semen quality: ejaculate										
Volume (mL)	53.9	23.9	53.0	10.0	175.0	49.9	24.9	50.0	10.0	136.0
Sperm concentration (10^6/mL)	217.7	106.7	186.0	11.0	612.0	231.6	118.1	195.0	29.0	613.0
Total number of spermatozoa (10^9)	8.9	3.2	8.8	0.9	19.7	9.1	3.7	8.9	1.7	19.0
Progressive sperm motility (%)*	72.8	8.4	70.0	40.0	80.0	71.4	10.0	70.0	40.0	80.0
Number of progressively motile spermatozoa (10^9)	6.4	2.6	6.4	0.8	15.7	6.5	2.9	6.3	0.8	14.8
Semen quality: insemination dose										
Volume (mL)	36.6	10.0	40.0	18.0	120.0	36.0	10.9	40.0	20.0	120.0
Seminal plasma (proportion of volume)	0.3	0.1	0.3	0.1	0.6	0.3	0.1	0.3	0.1	0.7
Sperm concentration (10^6/mL)	52.5	16.1	50.5	3.1	104.8	54.5	15.7	51.5	19.0	1115.3
Total number of spermatozoa (10^9)	1.8	0.5	1.8	0.1	4.2	1.9	0.5	1.8	0.8	4.1
Number of progressively motile spermatozoa (10^9)	1.3	0.4	1.3	0.1	5.4	1.3	0.3	1.3	0.6	2.8
Progressive sperm motility (%) at insemination	50.7	14.5	50.0	10.0	80.0	45.6	17.1	45.0	2.0	80.0
Number of progressively motile spermatozoa (10^9) at insemination	0.9	0.3	0.9	0.2	2.0	0.8	0.4	0.8	0.0	2.2
Number of inseminations/season	1.9	1.2	2.0	1.0	7.0	1.9	1.3	1.0	1.0	9.0
Number of inseminated estrus cycles	1.4	0.7	1.0	1.0	4.0	1.4	0.7	1.0	1.0	4.0
Number of inseminations/estrus	1.4	0.6	1.0	1.0	5.0	1.3	0.6	1.0	1.0	5.0
Number of insemination doses sent/stud farm/season	139.3	62.0	132.0	10.0	210.0	134.9	61.9	132.0	10.0	210.0

*is also progressive motility of the insemination dose at the time of sending

553 Table 3. Crude logistic regressions for pregnancy per cycle: odds ratio (OR), 95% confidence interval (CI)
554 and Wald's p-value (with stud farm, stallion and mare as random effects). N = number of cycles.
555

Variable	N	OR	95% CI	p
Breed: Standardbred vs. Finnhorse	1634	1.51	1.11 - 2.08	0.010
Insemination place: stud farm/clinic vs. home stable	1411	1.01	0.81 - 1.26	0.917
Insemination performed by veterinarian vs. technician	1299	1.01	0.78 - 1.30	0.954
Number of inseminated estrus cycles: ≥2 vs. 1	1634	1.57	1.28 - 1.93	<0.0001
Number of inseminations/estrus: ≥2 vs. 1	1634	1.22	0.99 - 1.51	0.069
Seminal plasma in the insemination dose (% of total volume):	1057			0.177
≤25% vs. ≥34%		1.05	0.84 - 1.31	0.670
25.1-33.9% vs. ≥34%		0.83	0.64 - 1.08	0.157
Weekday: Saturday-Thursday vs. Friday	1634	1.03	0.84 - 1.26	0.793
Month:	1634			0.386
June vs. March-May		1.05	0.82 - 1.36	0.689
July vs. March-May		1.19	0.90 - 1.57	0.226
August-September vs. March-May		0.87	0.59 - 1.30	0.494
Number of insemination doses sent/stud farm/season	1634			0.750
100-149 vs. <100		1.17	0.68 - 2.00	0.574
≥150 vs. <100		1.20	0.69- 2.10	0.524
Age of mare:	1594			0.106
2-9 vs. 17-22 years		1.48	1.06 - 2.06	0.022
10-13 vs. 17-22 years		1.21	0.86 - 1.69	0.280
14-16 vs. 17-22 years		1.34	0.93 - 1.92	0.117
Age of stallion:	1634			0.742
3-6 vs. 14-26 years		0.96	0.63 - 1.46	0.838
7-9 vs. 14-26 years		1.19	0.76 - 1.84	0.447
10-13 vs. 14-26 years		1.01	0.68 - 1.50	0.963
Semen transport time (hours)	1474	1.00	0.97 - 1.04	0.836
Semen quality: ejaculate				
Volume (mL)	1097	1.00	1.00 - 1.01	0.140
Sperm concentration (10^6/mL)	1634	1.00	1.00 - 1.00	0.893
Total number of spermatozoa (10^9)	1097	1.00	1.00 - 1.01	0.164
Progressive sperm motility (%)*	1611	5.33	1.30 - 21.8	0.020
Number of progressively motile spermatozoa (10^9)	1083	1.04	0.99 - 1.10	0.097
Semen quality: insemination dose				
Volume (mL)	1629	1.01	1.00 - 1.02	0.175
Seminal plasma (proportion of volume)	1622	0.93	0.31 - 2.83	0.901
Sperm concentration (10^6/mL)	1622	1.00	0.99 - 1.01	0.784
Total number of spermatozoa (10^9)	1623	1.17	0.97 - 1.42	0.108
Progressive sperm motility (%) at insemination	787	3.53	1.46 - 8.51	0.005
Number of progressively motile sperm (10^9) at insemination	781	1.63	1.10 - 2.41	0.015

556 *is also progressive motility of the insemination dose at the time of sending

Table 4. Multivariable logistic regression models for pregnancy per cycle: odds ratio (OR), 95% confidence interval (CI), and Wald's p-value (with mare, stallion, and stud farm as random effects), n = number of cycles.

Multivariable logistic regression models I - III			
Model I (n=1571)			
Variables	OR	95% CI	p
Breed (Standardbred vs. Finnhorse)	1.56	1.13 - 2.16	0.007
Number of inseminated estrus cycles (≥2 vs. 1 cycle)	1.54	1.25 - 1.90	<0.0001
Age of mare:			0.163
2-9 vs. 17-22 years	1.41	0.99 - 2.01	0.057
10-13 vs. 17-22 years	1.15	0.80 - 1.65	0.440
14-16 vs. 17-22 years	1.38	0.94 - 2.02	0.104
Progressive sperm motility in the ejaculate/insemination dose (%)	6.20	1.56 - 24.66	0.010
Model II (n=758)			
Variables	OR	95% CI	p
Breed (Standardbred vs. Finnhorse)	1.43	0.97-1.22	0.073
Number of inseminated estrus cycles (≥2 vs. 1 cycle)	1.56	1.15-2.12	0.004
Age of mare:			0.260
2-9 vs. 17-22 years	1.57	0.92-2.68	0.097
10-13 vs. 17-22 years	1.29	0.75-2.21	0.355
14-16 vs. 17-22 years	1.66	0.93-2.98	0.089
Progressive sperm motility in the ejaculate/insemination dose (%)	14.52	2.58-81.75	0.003
Number of progressively motile spermatozoa in the insemination dose (10^9) at insemination	1.6	1.05-2.42	0.028

Table 5. Multivariable logistic regression models for pregnancy rates per cycle in Finnhorses and Standardbred stallions: odds ratio (OR), 95% confidence interval (CI), and Wald's p-value (with mare, stallion, and stud farm as random effects), n = number of cycles.

Model for Finnhorses (n=602); stud farm and mare as random effects			
Variables	OR	95% CI	p
Number of inseminations/cycle:			0.141
2 vs. 1	1.18	0.78-1.79	0.431
3 vs. 1	1.66	1.04-2.66	0.035
≥4 vs. 1	0.89	0.51-1.57	0.690
Total number of spermatozoa in the ejaculate (10^9)	1.01	1.00-1.01	0.013
Progressive sperm motility in the ejaculate/ insemination dose (%)	38.00	3.38-427.21	0.003
Total number of spermatozoa (10^9) in the insemination dose	1.73	1.21-2.48	0.003
Model for Standardbreds (n=350); mare as the random effect			
Variables	OR	95% CI	p
Number of inseminations/cycle			0.017
2 vs. 1	2.14	1.27-3.62	0.005
3 vs. 1	1.95	1.02-3.72	0.044
≥4 vs. 1	2.03	0.97-4.26	0.062
Progressive sperm motility (%) at insemination	10.14	2.31-44.47	0.002