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A retrospective study on semen quality parameters from four different Dutch horse breeds with different levels of inbreeding

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ABSTRACT

A high degree of inbreeding has been reported to negatively impact semen quality in Friesian horses and Shetland ponies. Both breeds are characterized by a closed studbook, small population size, and high incidence of inbreeding. The Dutch Warmblood studbook (KWPN: Koninklijk Warmblood Paardenstamboek Nederland) is a much larger studbook with two distinct populations: the KWPN-Riding horses, managed as an 'open' studbook, and the KWPN-Harness horses, representing a much smaller subpopulation within the KWPN breed and managed as an 'almost closed' studbook. It was recently reported that the degree of inbreeding in KWPN-Harness horses has increased in recent decades due to the small gene pool; however, the degree of inbreeding is still lower than that of Friesian horses and Shetland ponies. We hypothesized that a high or rising degree of inbreeding might negatively impact semen quality. In the present study, we retrospectively compared semen quality parameters of stallions from four different breeds or types (Friesian Horses, Shetland Ponies, KWPN-Riding horses, and KWPN-Harness horses), each reported with different degrees of inbreeding. Semen concentration, and percentages of motile, morphologically normal and live spermatozoa, and the total number of morphologically normal, progressive motile spermatozoa per ejaculate (TNM) were analyzed for 2832 semen evaluations performed over a 15-year period. KWPN-Harness horses had a significantly lower sperm concentration, % motile spermatozoa and % live spermatozoa than KWPN-Riding horses but the % motile and % morphologically normal spermatozoa and TNM in both KWPN-Harness and KWPN-Riding horses were significantly higher than in Friesian horses and Shetland ponies. These results suggest a lower semen quality in KWPN-Harness than KWPN-Riding horses, potentially as a result of a higher coefficient of inbreeding. The negative trend observed in the KWPN-Harness horses may be a warning sign, and breeders or stud books should monitor the degree of inbreeding carefully to avoid a further reduction in semen quality, to the levels observed in Friesian horses and Shetland ponies.

1. Introduction

Inbreeding is the process of mating genetically similar animals, producing offspring with a higher level of homozygosity. The selective breeding of animals has led to new breeds of domestic animals, genetically suited to specific desirable tasks or traits. In domestic animals, inbreeding often results in a trade-off where a desirable trait is heightened or fixed but at the risk of inadvertently selecting undesirable traits or reducing overall fitness [1]. Inbreeding reduces the number of heterozygous assemblies and increasing the prevalence of genetic defects [1].

The process of domestication and progress of inbreeding can be traced for the Dutch warmblood horses [2]. Warmblood horses were initially used for transportation (Riding and carriage driving) and for agricultural work in the field [2]. The increased focus on performance in sporting competition (dressage and show jumping) brought a substantial improvement in athletic abilities and created what is now commonly referred to as the KWPN warmblood. This was in part accomplished through the selective import of exceptional stallions and mares from other European Warmblood Studbooks, Thoroughbreds and occasional Trotting horses. Within the KWPN studbook, a second type emerged, which is bred for carriage

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pulling and called a harness horse (also known as “Tuigpaard” in Dutch). Harnessing in the Netherlands is a show sport where a very specific gait, typical attitude and conformation are expected from the horse. In only a few generations, a distinct type has thus developed within the Dutch warmblood horse population by defining specialized breeding goals and introgression of specific features from other breeds (e.g. spectacular knee action in the harness horse from Hackney and American Saddlebred horse populations). Within the harness type horses, breeding of harness horses that looked alike was practiced and only limited exchange of genetic material between types or introduction of other breeds took place. This led to a divergence in type from the KWPN riding horse over only a few generations and has reduced the genetic diversity within the Harness horse population, mainly as a result of the extensive use of a few popular sires that complied with the breeding goal [3,4].

In horses in general, genetic diversity is considerable between breeds or types, whereas much less variation is present within each breed or type [5]. Many (European) breeds have developed under more or less isolated conditions, sometimes due to geographic limits, sometimes because specific breed characteristics were prioritized (such as conformation, color, or gait) and in other breeds simply because of the creation of closed studbooks in which horses from other studbooks were no longer allowed for breeding purposes, for example the Friesian horse. In a recent study in the Netherlands, genotype data of horses from nine Dutch or common (European) horse populations were analyzed [6]. The populations examined included the Friesian horse, Shetland ponies, KWPN-Riding horse and the KWPN-Harness horse. In that study, clear differences were observed among the populations in their genetic diversities. In general, populations that allow the introduction of horses from other studbooks/breeds (KWPN-Riding horses) showed less inbreeding and homozygosity than breeds that used very narrow selection criteria (KWPN-Harness horses and Shetland ponies) and breeds with a closed studbook (Friesian horse).

Conserving genetic diversity within a population is important because it contributes to maintenance of a healthy population [7]. For example, in the highly inbred Friesian horse population, a number of single gene heritable disorders such as dwarfism and hydrocephalus have arisen due to inbreeding [3,8–11]. A negative impact of inbreeding on fertility has also been documented, yet remain inconclusive [12]. In dairy cows, intensive breeding programs to increase milk production have led to lower reproductive performance [13–15]. In a study in the Black Forest Draught horses, inbreeding coefficients of the stallions and mares were not associated with the foaling rate [16]. In contrast, in Norwegian Trotters and Standardbreds, inbreeding has been proposed to explain the high incidence of early pregnancy loss and low conception rate [17,18]. However, it is not clear whether these adverse effects are primarily due to mare or stallion subfertility. It has been suggested that a decrease in male fertility is one of the earliest consequences of an increased level of inbreeding because mutations occur much more frequently in the male germline [19,20]. Therefore, it is likely that the reported reduction in reproductive performance in a highly inbred population is due primarily to lower male fertility which may be reflected in reduced semen quality. In a study in dogs, it was reported that a more inbred group had a lower sperm concentration in their ejaculate than outbred groups [21]. In bulls, inbreeding has a negative effect on scrotum size, sperm motility, sperm concentration and the percentage of morphologically normal sperm [22–24]. In endangered gazelles, a species with a high level of inbreeding, the degree of inbreeding is negatively correlated with semen quality, with high coefficients of inbreeding associated with a lower percentage of morphologically normal and motile sperm [19]. In horses, there are apparent discrepancies in the effect of inbreeding on male reproductive capacity. The negative impact of inbreeding on semen quality and on foaling rates per insemination in inbred Standardbred horses has been reported [25,26]. In the Netherlands, Friesian and Shetland stallions score lower on their sperm quality parameters than Warmblood horses, presumably due to a higher degree of inbreeding [25,27,28]. Indeed, as many as 50% of Friesian stallions presented for approval as breeding stallions are excluded due to inadequate sperm quality [28]. Recently, an idiopathic infertility case in a Friesian stallion was suggested to be associated with the high degree of inbreeding, with the presumptive reason for reduced fertility being an impaired acrosomal reaction [29]. Boer et al., reported that acrosomal abnormalities were very common in 1146 Friesian stallions (second ejaculate of two) collected from 1987 to 2002 [28].

Inbreeding is also an issue in Shetland ponies due to the tendency to select a stallion based on height at the withers, color or geographic proximity rather than on pedigree, making it more likely that stallions are selected from a small local group [25]. It has been suggested that this has led to inbreeding accompanied by a decline in the percentage of progressively motile and morphologically normal spermatozoa in Shetland pony stallions [25]. In contrast, Aurich et al., were not able to demonstrate an effect of inbreeding on semen quality in Noriker draught horse stallions [30].

The KWPN warmblood studbook is an ‘open’ studbook that allows other types of (European) warmbloods, Thoroughbred and trotter stallions into the population, thereby reducing the degree of inbreeding [31]. However, the KWPN population consists of two distinct sub-types: the Riding/Sport horses and the Harness horses. The KWPN-Harness horses are a much smaller subpopulation with very strict requirements for specific traits such as gait, attitude and conformation [27,31]. Therefore, there is less genetic diversity among Harness stallions than KWPN-Riding horses, leading to a higher chance of inbreeding in this population. This was recently confirmed in a comprehensive study comparing nine horse breeds in the Netherlands [6]. The potential effects of the increase in the inbreeding indices in KWPN-Harness horses on the sperm quality needs to be considered when planning future breeding programs for this breed. Therefore, the aim of the current study was to compare semen quality in KWPN Harness horses with that of breeds with a lower degree of inbreeding (KPWN-Riding horses) and a higher degree of inbreeding (Friesian horses, and Shetland ponies).

2. Material and methods

2.1. Stallions

Sperm parameters collected from Friesian (n = 1043), Shetland pony (n = 63), KWPN-Riding (n = 1545) and KWPN-Harness (n = 181) stallions between 2 and 5 years old and submitted to the Reproduction Section at Utrecht University for a routine breeding soundness exam (BSE) between 2003 and 2018, were analyzed. The reproductive soundness of stallions is a routine process to evaluate future reproductive potential of a stallion or to determine the cause(s) of its poor reproductive performance [32]. In the present study, for each stallion, the BSE included the collection of two ejaculates at an interval of 1.5–3h. To have an unbiased population, only stallions presented for a routine BSE in the context of evaluation for suitability as a breeding stallion were included; stallions presented for semen evaluation because of suspected or demonstrated fertility problems were excluded from the study. The ejaculates were collected over the entire year. The parameters included in this study are sperm concentration, percentage of motile spermatozoa, percentage of live spermatozoa, percentage of morphologically normal spermatozoa, and the total number of morphologically normal and progressively motile spermatozoa in the ejaculate (TNM).

2.2. Semen evaluation

Evaluation of semen quality was performed using the standard techniques described previously [33,34]. As part of the routine BSE, the volume of gel-free semen, sperm concentration (measured using an Accucell photometer [IMV, L'Aigle, France]) and the percentage of progressively motile spermatozoa was estimated subjectively via a light microscope equipped with a heated stage and performed for each ejaculate immediately after collection. An aniline blue-nigrosin stained smear was prepared for subsequent analysis of the percentage of morphologically normal spermatozoa and the live/dead ratio. A minimum of 200 spermatozoa were categorized as being live (unstained) or dead (stained), and as being morphologically normal. Finally, the total number of morphologically normal, progressively motile spermatozoa per ejaculate (TNM) was calculated. Only the semen parameters obtained for the second ejaculate were used for further statistical analysis [35].

2.3. Data analysis

Statistical analysis was performed using the JMP® Pro 14.0.0 statistical software (SAS Institute, version; Cary, NC). Data were assessed for normality using a Kolmogorov–Smirnov test and for equal variance with Bartlett's test. Normal quantile transformation was performed to normalize the data [36]. The statistical analysis and visualization were performed on the normalized data. To compare the semen parameters among the breeds, we performed the Welch *t*-test for the comparison between the parameters with unequal variances and ANOVA for the parameters with equal variances. The changes in the semen parameters for each breed over the study time was examined using a mixed model with semen parameters as dependent variables, the year as a fixed effect, and stallion and age of stallion as random effects [37]. A regression analysis (linear) was used to evaluate any trends in the semen parameters over the period of semen collection, with the year as a factor and the transformed semen parameters (normal quantile) as the responses [38]. A p-value < 0.05 was considered as significant.

3. Results

3.1. Semen parameters

The semen parameters for 2832 s ejaculates collected during a routine breeding soundness examination over a period of 15 years were included in the study.

Concentration: Sperm concentrations were significantly lower in KWPN-Harness stallions ($110 \times 10^6 \pm 4.3 \times 10^6$) than in KWPN-Riding horses ($141 \times 10^6 \pm 2.7 \times 10^6$). Sperm concentrations were highest in Shetland ponies ($164 \times 10^6 \pm 20.0 \times 10^6$) and similarly high in KWPN-Riding stallions (Fig. 1a). Sperm concentrations were lower in Friesian ($105 \times 10^6 \pm 4.0 \times 10^6$) and KWPN-Harness ($112 \times 10^6 \pm 8.3 \times 10^6$) stallions.

Motility: The percentage of motile sperm was lower in KWPN Harness stallions (~71% ± 0.2%) than in KWPN-Riding stallions (~74% ± 0.2%) (Fig. 1b). The percentage of motile spermatozoa was lower in the Friesian stallions (~67% ± 0.2%) than in either KWPN horse types and was lowest in Shetland ponies (~59% ± 1.4%).

Live cells: The KWPN-Riding horses (~75% ± 0.3%) had the highest and Shetland ponies (~62% ± 1%) the lowest percentages of live cells per ejaculate (Fig. 1c). The percentage of live cells in KWPN-Harness stallions (~73% ± 0.8%) was significantly lower than in KWPN-Riding horses, but similar to Friesian horses (~72% ± 0.4%).

Morphology: The percentages of morphologically normal spermatozoa was similar in KWPN-Riding (~67% ± 0.3%) and Harness stallions (~65% ± 2.1%) and were significantly higher than for Friesian horses (~52% ± 0.4%) and Shetland ponies (~39% ± 2.1%) (Fig. 1d).

TNM: There was no significant difference in TNM values between KWPN-Riding ($2983 \times 10^6 \pm 43 \times 10^6$) and Harness stallions ($2759 \times 10^6 \pm 114 \times 10^6$) (Fig. 1e). TNM values were lower in Friesian stallions ($1567 \times 10^6 \pm 56 \times 10^6$) and lowest in Shetland ponies ($320 \times 10^6 \pm 269 \times 10^6$).

3.2. Changes in semen parameters over the 15-year period

Based on our model, there was an effect of year on the total motility ($P < 0.01$) for all breeds. There was also a significant effect of year on the percentage of sperm with normal morphology in KWPN-Riding horses ($P = 0.02$). No effect of year was observed on concentration, TNM, and percentage of live cells. Next, we performed linear regression to investigate the possibility of increasing or decreasing trends in percentages of motile and morphologically normal sperm over the years of the study. We were not able to detect any significant pattern in these parameters over the years among the studied breeds, and the significant effect of the year (detected by the Mixed model) was due to year-to-year variations among the studied stallions.

4. Discussion

In the current retrospective study, we evaluated semen quality parameters in stallions from four breeds for which different degrees of inbreeding have been reported [6]. Our results indicate that Shetland ponies, a breed with a small population (<1000 registered horses) and a high degree of inbreeding, had the lowest semen quality among the studied breeds. Shetland ponies had the highest average sperm concentration over the 15 years; however, the percentages of live cells and morphologically normal spermatozoa were significantly lower in this breed, leading to a total number of progressively motile and morphologically normal spermatozoa in the ejaculate that was one tenth of that seen in the other breeds. In a previous study, low sperm quality in Shetland ponies was shown to be associated with the degree of inbreeding with a higher percentage of sperm with abnormal acrosomes and a lower percentage with normal morphology among the more inbred stallions [25].

Similar to the Shetland pony, the Friesian breed has a high inbreeding coefficient and is genetically predisposed to a number of single gene recessive or homozygosity related conditions, including dwarfism, immune-mediated disorders, megaesophagus, aortic rupture and orthopedic disorders [10,11]. The population of Friesian horses is limited to 70,000 registered horses with less than 100 approved stallions. Boerma et al., postulated that many reported abnormalities in the Friesian horses are related to the relatively small gene pool of the Friesian horse and/or the high reproduction rate when this breed became very popular in the eighties [11]. An animal with a high degree of inbreeding and serious deleterious genetic defects (e.g. hydrocephalus, dwarfism) will not reach adulthood and the reproductive phase of its life; however, highly inbred offspring that do survive to adulthood may have decreased fertility due to recessive alleles that could influence pathways involved in gametogenesis, endocrine function, sperm transport, ovulation, fertilization, or implantation [12]. For instance, recently an idiopathic infertility case in a Friesian stallion was suggested to be associated with the high degree of inbreeding in this breed, with the presumptive reason for reduced fertility being an impaired

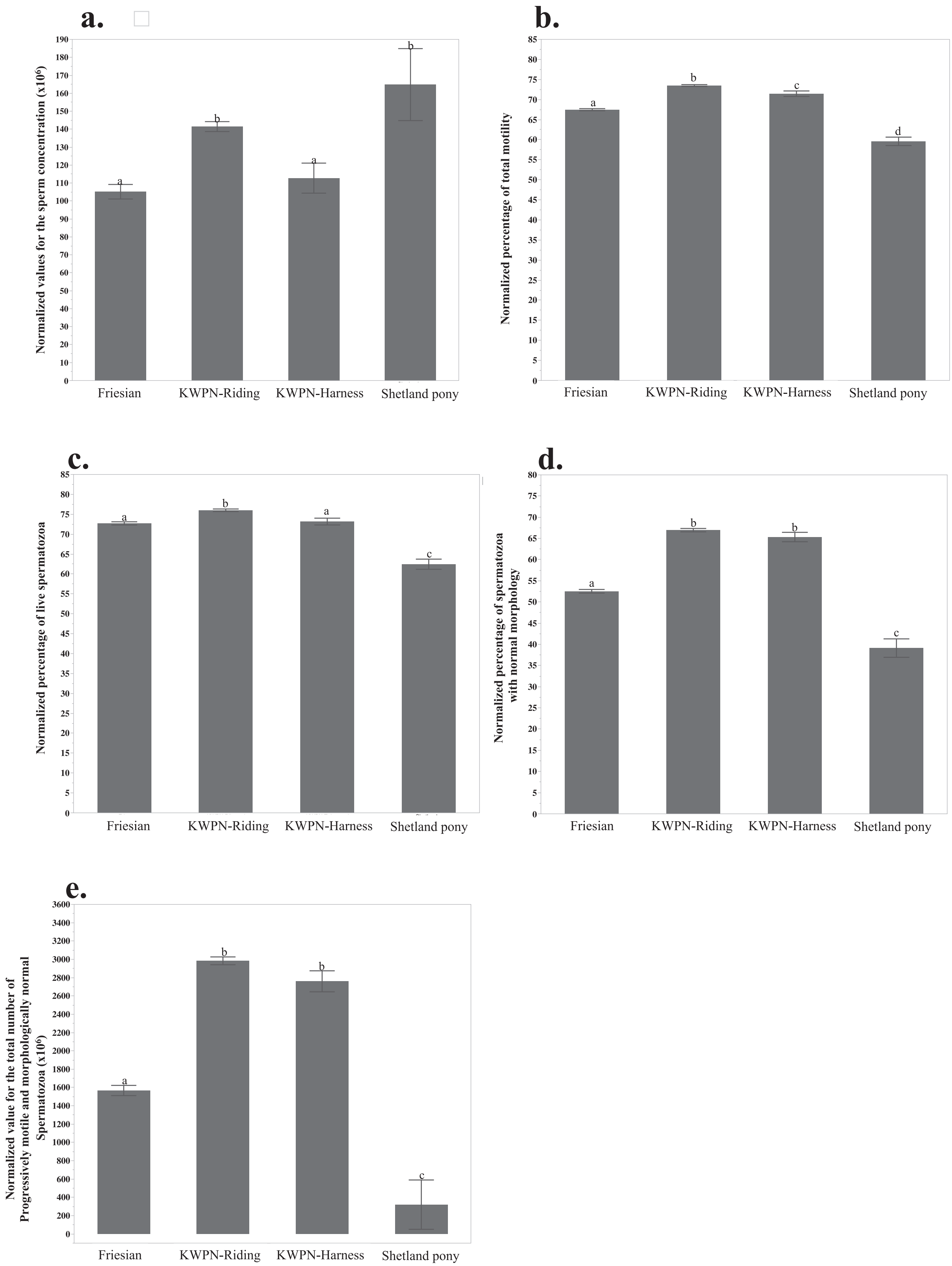

Fig. 1. Semen parameters for 2832 ejaculates over a period of 15 years were included in the study. Sperm concentrations (a), total motility (b), percentage of live cells (c), percentage of spermatozoa with normal morphology (d), and the total number of progressively motile and morphologically normal spermatozoa (e) The percentages of morphologically normal spermatozoa are presented in the figure. Data are presented as normal quantile. Different subscript demonstrated a significant different between the breeds ($P < 0.05$).

Table 1
Summary of the sperm quantity and quality parameters in Friesian stallions over a 30-year period (2003–2018).

	1987–2002[a]		2003–2018	
	Mean ± SD	min-max	Mean ± SD	min-max
Gel-free volume (mL)	53 ± 25	10–210	48 ± 24	10–285
Sperm concentration (x10^6)	109 ± 116	1–1128	116 ± 109	2–864
Total motility (%)	67 ± 13	1–90	68 ± 10	10–90
Morphologically normal sperm (%)	53 ± 16	1–88	53 ± 15	9–86
Total number of progressively motile and morphologically normal spermatozoa (x10^6)	1816 ± 1717	0.1–17028	1709 ± 1479	8–12142

[a] Data was collected from Boer et al. (2007).

acrosome reaction [29]. Boer et al., also reported that acrosomal abnormalities are very common in the sperm of Friesian stallions [28]. In the aforementioned study, semen parameters of 1146 Friesian stallions (second ejaculate) collected from 1987 to 2002 were evaluated. Comparing Boer et al.'s study with the results of the current study, there was no evidence of statistical differences in the semen quality parameters for Friesian stallions over the last 30 years (Table 1). Similarly, we did not find any general trends in semen quality of the studied breeds over a period of 15 years.

In the present study, we further compared the semen quality of two breeds with a high degree of inbreeding with those of KWPN-Riding stallions, a breed with a so-called open studbook. We observed a higher % motile spermatozoa, % live spermatozoa, % morphologically normal spermatozoa and a higher number of progressively motile and morphologically normal spermatozoa (TNM), in KWPN-Riding horses than in Shetland ponies and Friesian stallions. Many factors can influence semen quality, including the breed of stallions [39]. To investigate whether the lower semen quality in Shetland ponies and Friesian stallions were solely due to the breed differences [40], we separated semen parameters for KWPN-Riding and KWPN-Harness stallions in our study. KWPN-Harness horses and KWPN-Riding horses have the same origin; however, KWPN-Harness horses have a more restricted gene pool (more restricted studbook). This restriction is assumed to be the risk factor for a higher degree of inbreeding in this sub-population. Schurink et al. [6] reported that the degree of inbreeding in the KWPN-Harness horses is higher than that in KWPN-Riding horses but is still lower than in Shetland ponies and Friesian horses. In our study, KWPN-Harness stallions had a lower concentration, lower % of motile spermatozoa and a lower % of live spermatozoa in their ejaculates than KWPN-Riding stallions, suggesting that the decrease in semen quality parameters is not breed-dependent but more likely the result of different degrees or rates of change of inbreeding.

In conclusion, this study demonstrates a difference in sperm quality parameters between KWPN-Riding and KWPN-Harness horses. The observed differences may be the result of an increased level of inbreeding in KWPN-Harness horses and suggest that careful planning of breeding or introduction of out-cross animals may be warranted in KWPN-Harness horses to avoid further deterioration of semen quality and fertility within this population. These measures may include a careful selection of stallions based on genetic diversity and on the degree of inbreeding of the product of a specific stallion-mare combination. In addition to the strategic combining of stallions and mares, the studbook may need to look for opportunities to introduce new, genetically divergent stallions from other studbooks. Moreover, implementing strategies such as optimal contributions method and minimum co-ancestry mating for maintaining variation should be assessed in the breeding programs. It is likely that a compromise between genetic diversity and breeding the desired phenotype will be required to maintain an acceptable fertility and reproductive efficiency.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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