Semen quality evaluation in young stallions – feasibility and comparison of two different protocols

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Summary: Semen analysis is a valuable approach to assess male fertility in the horse. The aim of this study was to evaluate the effects of two different semen collection protocols, stallion's age, season and previous rearing conditions on sperm parameters of young stallions. Semen samples were obtained from 23 stallions (age range: 30–42 months old) every six weeks. Stallions were reared under different conditions prior to the start of the study. Semen collection was either performed twice with an interval of one hour on a given day (protocol A) or once daily for seven days (protocol B). The ejaculates were examined for total sperm number (TSN), progressive motility (PM), plasma membrane integrity (PMI), DNA fragmentation index (DFI) and semen quality after storage at $5\,°C$ for 24 hours. TSN and DFI were higher for protocol A as compared to protocol B. TSN in stallions submitted to protocol A (1st ejaculate) was influenced by season, whereas TSN remained unchanged in stallions submitted to protocol B. PM exceeded 70% in every ejaculate and the protocol did not significantly affect motility. PMI was lower in ejaculates of 33.4 months old stallions on average as compared to older individuals. No other semen parameter was affected by the stallions' age. In conclusion, breeding soundness examination in 33.4 months old stallions is feasible and semen analysis after daily collection for 7 days provides less variable results throughout the year than semen analysis with protocol A. Therefore, semen analysis after daily collection for 7 days is recommended for young stallions.

Keywords: breeding soundness examination, semen collection protocol, age, spermatological examination

Introduction

Breeding soundness examination in stallions involves a clinical and a spermatological examination (*Götze* 1942, *Kenney* et al. 1983, *Merkt* and *Klug* 1989, Hurtgen, 1992, *Varner* 2016). The examination aims to gather information concerning the health status (general, hereditary, genital), the ability of the stallion to mount a mare and the fertilization capacity of the stallion's semen. The stallion's ejaculate has to meet certain requirements of volume, color, sperm density, total sperm number, progressive motility, morphology, pH and plasma membrane integrity. In order to be classified as a fertile individual, the ejaculate has to contain at least 1×10^9 morphologically normal, progressively motile sperm in total (*Kenney* et al. 1983).

The value of semen collection and sperm analysis in young stallions (30–36 months old) is controversially discussed. In Germany, the breeding soundness examination of stallions presented for licensing is limited to the evaluation of general, hereditary and genital health. However, stallions are not examined for mounting ability and fertilization capacity. Their

breeding soundness is guaranteed by the seller for a warranty period. In contrast, the Netherlands demand a complete breeding soundness examination for every stallion presented for licensing (*Colenbrander* et al. 2003). Failure to meet the minimum requirements leads to exclusion from licensing. As sub- or infertile stallions are thus successfully excluded from submission to an auction, the Dutch approach is favorable from an economic point of view. On the other hand, critics of an early-age breeding soundness examination prior to licensing point to the lacking comparability of stallions from different rearing conditions.

Different protocols for breeding soundness examination have been described, with differences primarily in regard to frequency of semen collection. The gold standard, as described by *Kenney* et al. (1983), includes two semen collections at a one-hour interval on a given day. Another protocol introduced by *Love* et al. (1991) promotes semen collection once daily for 10 days, reaching the most reproducible and representative values on day 7. Based on the determination of the testicular volume, the comparison of predicted and actual daily sperm output (DSO) is possible using this protocol (*Thompson* et al. 2004).

The aim of this study was a) to investigate whether semen quality evaluation is possible in stallions at the age of 30 to 36 months and b) to evaluate the effects of two different semen collection protocols, age, season and previous rearing conditions on measured sperm parameters (total sperm number (TSN), progressive motility (0 and 24h), percentage of plasma membrane intact sperm (PMI, 0 h and 24 h), and DNA fragmentation index (DFI)) of young stallions.

Material and Methods

Trial 1 (breeding season): animals, study design and examination period

From March to September, 15 healthy young Warmblood stallions currently undergoing the stallion performance test were included in the study. All stallions were owned by the Lower Saxony State Stud Celle. Seven stallions were purchased at the Hanoverian stallion's licensing and auction in Verden (Germany) in Autumn at an age of 30.0 months on average from private breeders. Eight stallions were purchased as weanlings and reared at the upbringing station of the Lower Saxony State Stud Celle in Hunnesrück. They were kept in groups of 15–20 colts until the age of approximately 30 months. These colts were kept on pasture during the summer months and had no special training before the licensing.

At the first examination, stallions were aged 36.8 months on average. The stallions were assigned randomly for examination according to two different semen collection protocols. For protocol A, semen collection was performed at a one-hour interval on a given day (n = 8). For protocol B, semen was collected once daily for seven days in a row and the ejaculate of the seventh day was included into the analysis (n = 7). Both protocols were repeated five times each at a six-week interval in March, May, June, August and September (breeding season). Stallions were not involved in a breeding or semen collection program during the experimental trial. Stallions from both rearing conditions were distributed equally among the two test groups. All stallions were housed in individual straw bedded stalls, they were fed hay, oats and concentrate thrice daily and always had access to water. Experiments were performed in agreement with German animal welfare legislation and approved by the Lower Saxony State Office for Consumer Protection and Food Safety.

Trial 2 (non-breeding season): animals and examination period

The second experimental trial was conducted from December until January (non-breeding season) and included eight stallions belonging to the Lower Saxony State Stud Celle. At the first examination, stallions were aged 33.4 months on average. Distribution of stallions from different rearing conditions was equal among test groups (protocol A: n = 4; protocol B: n = 4). Experimental conditions and procedures did not differ from the first experimental trial, both protocols were repeated once each at six-week intervals and housing and feeding of stallions was as described for the first experimental trial.

Semen collection

All stallions were accustomed to semen collection on a phantom six weeks before the start of the experiments until they were able to mount the breeding phantom, insert the penis into the artificial vagina, thrust and ejaculate completely. Before beginning the experiments all stallions were sexually rested for six weeks. After stimulation by a mare in heat, semen collection was performed on a phantom and with an artificial vagina (model Hanover, Minitüb, Tiefenbach, Germany). Prior to every semen collection, a new inner disposable liner (Minitüb, Tiefenbach, Germany) was inserted into the artificial vagina. The ejaculates were collected in a sterile, clean and scaled glass bottle with an inline filter for separation of debris and gel from the ejaculate. Stallions were handled and collected by the same well-trained operators for both experimental trials.

Semen processing

All semen samples were processed and examined by one well-trained operator. Immediately after semen collection, an aliquote of 2 ml raw semen was filled into cryovials (Eppendorf Hamburg, Germany), shock frozen in liquid nitrogen (–196 °C) and stored until determination of sperm chromatin integrity. The gel-free part of the ejaculate was extended to a concentration of 25 million motile sperms per ml using skim milk extender (INRA 82). INRA-82 was prepared by mixing equal volumes of commercial 0.3 % ultra-heat-treated skim milk and glucose saline solution (prepared by dissolving the following components in 500 mL water: 25 g glucose monohydrate, 1.5 g lactose monohydrate, 1.5 g raffinose pentahydrate, 0.25 g sodium citrate dihydrate, 0.41 g potassium citrate monohydrate, 4.76 g HEPES, 0.5 g penicillin, 0.5 g gentamycin). The pH of INRA-82 was 6.8–7.0 and the osmolality 300–330 mOsm kg-1. Extended semen samples were incubated in a warm water bath (37 °C, 2 min) and subsequently submitted to computer-assisted semen analysis (CASA), evaluation plasma membrane integrity. The measurements (CASA and plasma membrane integrity) were repeated after storage of samples at 5 °C for 24 hours.

Semen evaluation

Spermatological examination

Macroscopic parameters (volume, color, consistency, absence of contamination) of the gel-free fraction were assessed. Sperm concentration (per ml) was determined using a SpermaCue™ (Minitüb, Tiefenbach, Germany). By multiplication of volume and sperm concentration, total sperm number (TSN) was calculated.

Semen motility analysis was performed using a phase-contrast microscope (Olympus, Hamburg, Germany) with a stage heater (HT 200, Minitüb, Landshut, Germany) at a temperature of 37 °C combined with a CASA system (MIKA Motion Analyser, Windows Version 1.1 Stroemberg, Mika, Montreux, Switzerland). After extension of semen samples and incubation, five fields per chamber (MIKA measuring chamber) were

analyzed. Cells moving slower than $10\,\mu m/s$ were considered immotile, whereas cells moving $\geq 25\,\mu m/s$ were considered to be progressively motile.

Plasma membrane integrity

The percentages of plasma membrane-intact sperm (PMI) and propidium iodide (PI) negative were flow cytometrically estimated using the Syto-PI-assay. Semen samples were diluted with Tyrode's media to 5×10^6 sperm/ml. After staining $500\,\mu l$ extended semen sample with $2\,\mu l$ SYTO 17 (0.5 mM; moBitec GmbH Göttingen S 7579) and $3\,\mu l$ PI (2.99 mM; Sigma-Aldrich, Munich P 4170) as well as 15 minutes incubation in the dark at $37\,°C$, the samples were evaluated with a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany), equipped with an 488 nm argon ion laser. A total of 10,000 events were accumulated for each measurement. According to their dotplot staining pattern, two sperm populations were distinguished: PMI, vital sperm (PMI; SYTO 17 positive, PI negative) and plasma membrane-defect, non-vital sperm (SYTO 17 negative, PI positive; membrane-defect).

DNA fragmentation index

DNA fragmentation index was determined as described by *Evenson* and *Jost* (2000). Briefly, snap frozen native semen samples were thawed (37 °C water bath) and concentration was set at 1–2 Million/ml by dilution in tris borate ethylene-diaminetetraacetic acid (TNE) buffer. After addition of 0.4 ml acidic detergent, the suspension was mixed thoroughly for 30 seconds. Consecutively, 1.2 ml acridine orange staining solution was added and incubated for 3 min in ice-cold water. Finally, the sample was analyzed by flow cytometry. After the measurement of 10 samples, accuracy was controlled by analysis of a reference sample. Differences in DNA denaturation (relation of red fluorescence to total fluorescence) were expressed as DNA fragmentation index (DFI %). Data analysis was performed using DAS Version 4.40 software (*Beisker*, 1994).

Statistical analysis

Statistical analysis of the obtained data was performed using the SAS program (Statistical Analysis Systems, SAS Institute, Iowa, Cary, USA). Statistical advice was given by the Institute for Biometry and Epidemiology, University for Veterinary Medicine, Hanover, Germany. Multifactorial analysis of variances showed significant effects of semen collection protocol, trial week, ejaculate and rearing conditions. Average values were compared using the Tukey-Kramer-Test. Differences between the experimental trials were determined via a Wilcoxon-Test. A p-value of $p < 0.05$ was considered significant.

Results

No significant difference between trial 1 and 2 in TSN ($p > 0.05$) and motility (0 h & 24 h: $p > 0.05$) was detected. Nevertheless, PMI and DFI differed significantly between the two experimental trials.

Total sperm number (TSN)

A significant effect of the week of the experimental trial and of the ejaculate was found in both experimental runs ($p \leq 0.05$, Fig. 1). In experimental trial 1, the semen collection protocol showed a significant influence on TSN. Rearing conditions and stallion's age did not affect TSN ($p > 0.05$). TSN was highest in the respective first ejaculate of both experimental trials. In trial 1, TSN varied significantly over the course of the trial. TSN of the second ejaculate typically reached 65.6 % of the first ejaculate (protocol A; mean = 58.2–69.7 %). With protocol B, TSN tended to be constant in all ejaculates during a given week over the experimental trial ($p = 0.09$).

Progressive motility

Progressive motility on the day of semen collection was neither significantly affected by the semen collection protocol, nor by ejaculate ($p > 0.05$, Fig. 2), rearing conditions or age. Progressive motility did not decrease below 70 % during the experiment and tended to be higher in protocol B as compared to protocol A in both trials ($p = 0.06$). Significant differences were only observed in June.

Progressive motility after 24 h storage

After storage for 24 hours, progressive motility was significantly affected by the semen collection protocol in trial 2 and by the week of the experimental run as well as ejaculate in trial 1 ($p < 0.05$, Fig. 3). No significant influence of rearing conditions and age was detected. Progressive motility decreased significantly after storage for 24 hours in almost every ejaculate. Except for the examinations in January, March and May, all ejaculates exceeded 35 % progressively motile sperms.

Plasma membrane integrity (PMI)

PMI on the day of semen collection was significantly affected by the week of the trial (trial 1 and 2) and the ejaculate (trial 2, Fig. 4). There was no significant effect of the rearing conditions ($p > 0.8$). The age of the stallion influenced PMI significantly ($p < 0.05$). Stallions in trial 1 showed higher plasma membrane integrity values compared than animals in trial 2. Furthermore, values were higher in March, May and June than in August and September in trial 2.

Plasma membrane integrity after 24 h storage

After storage for 24 hours, PMI was only affected by the ejaculate in trial 2 ($p < 0.05$). None of the other factors showed a significant influence (Fig. 5).

DNA fragmentation index

In trial 1, the semen collection protocol affected DFI values significantly ($p < 0.05$; Fig. 6). No significant effects were observed for the week of the experimental run, rearing condi-

tions and age. DFI values were higher for protocol A than for protocol B, but significance was only observed in trial 1 ($p < 0.05$). For protocol A, DFI values showed a significant variation during the course of the experiment, reaching the highest values in June ($p < 0.05$). No variation was observed for protocol B.
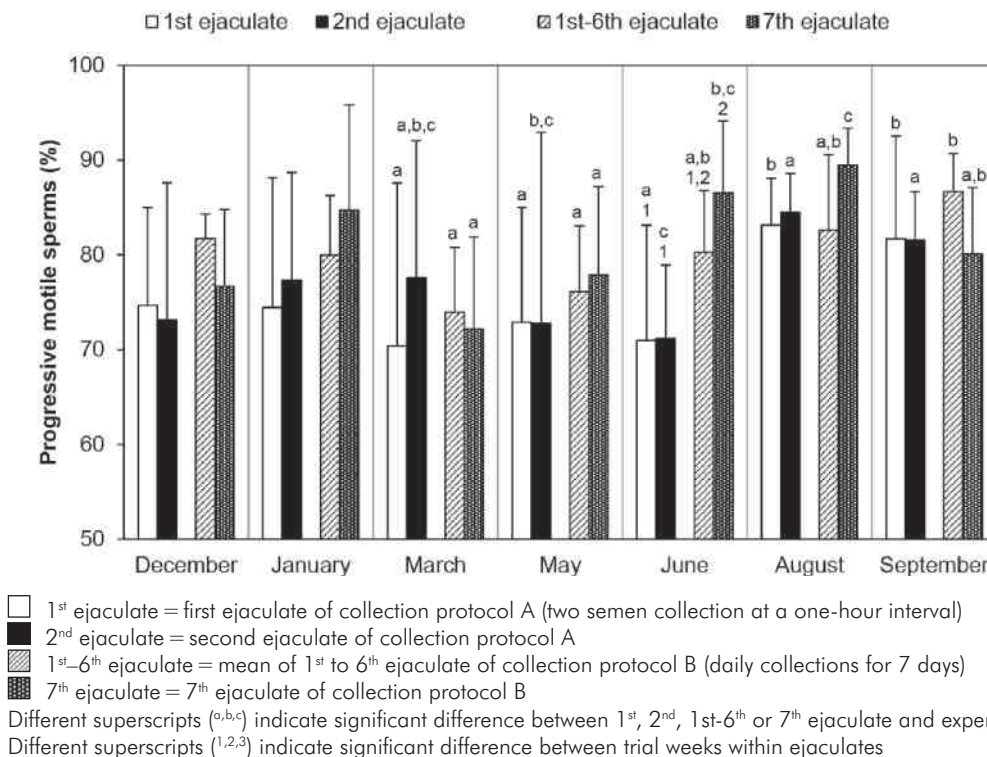
Discussion

In the present study, the influence of two different semen collection protocols, season, age and rearing conditions on spermatological parameters (TSN, progressive motility, PMI, DFI and sperm quality after storage for 24 h at 5 °C) in young Hanoverian stallions was examined.

The semen collection protocol A (two ejaculates at one-hour interval) was in line with the standard for breeding soundness examination as described by the Society for Theriogenology (*Kenney* et al. 1983, *Turner* 2005). The protocol had been developed for sexually rested stallions and can be performed without a long preparation period. For a fertile stallion, both ejaculates have to contain one billion morphologically normal, progressively motile sperm regardless of the season (*Kenney* et al. 1983, *Turner* 2005). Another commonly used protocol for breeding soundness examination aims to evaluate semen quality at the daily sperm output (DSO) level. The DSO is defined as the number of sperm produced by a stallion on a daily basis, obtained at a regular collection frequency (≤ 1 ejaculate/ day). For the determination of the DSO, semen is collected daily either for a period of 10 days to empty all sperm reservoirs
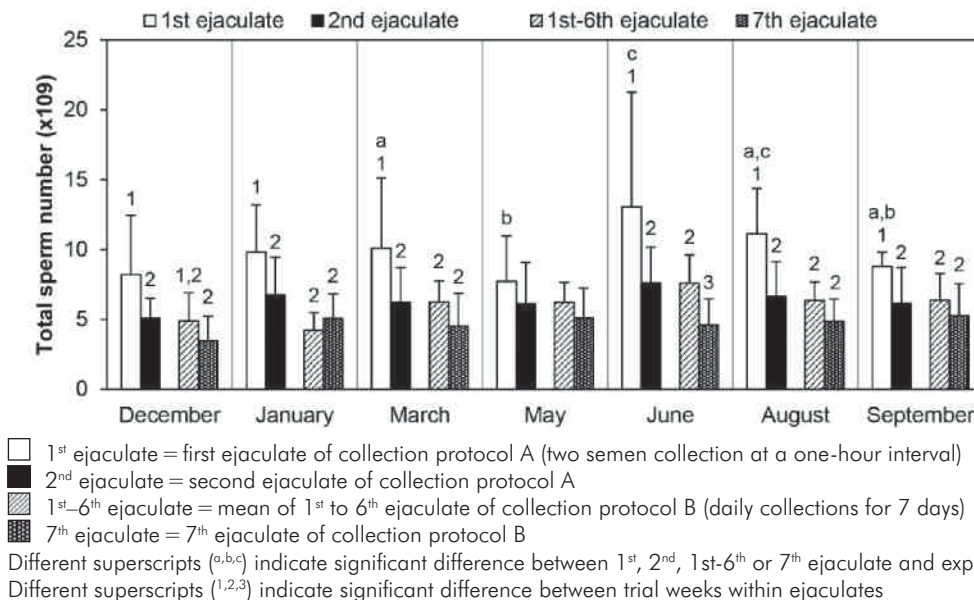
Fig. 1 Total sperm number (mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).

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■ 2nd ejaculate = second ejaculate of collection protocol A
▨ 1st–6th ejaculate = mean of 1st to 6th ejaculate of collection protocol B (daily collections for 7 days)
▩ 7th ejaculate = 7th ejaculate of collection protocol B
Different superscripts ([a,b,c]) indicate significant difference between 1st, 2nd, 1st-6th or 7th ejaculate and experimental trial (1,2) within trial weeks
Different superscripts ([1,2,3]) indicate significant difference between trial weeks within ejaculates

Fig. 2 Progressive motile sperms (%, mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).
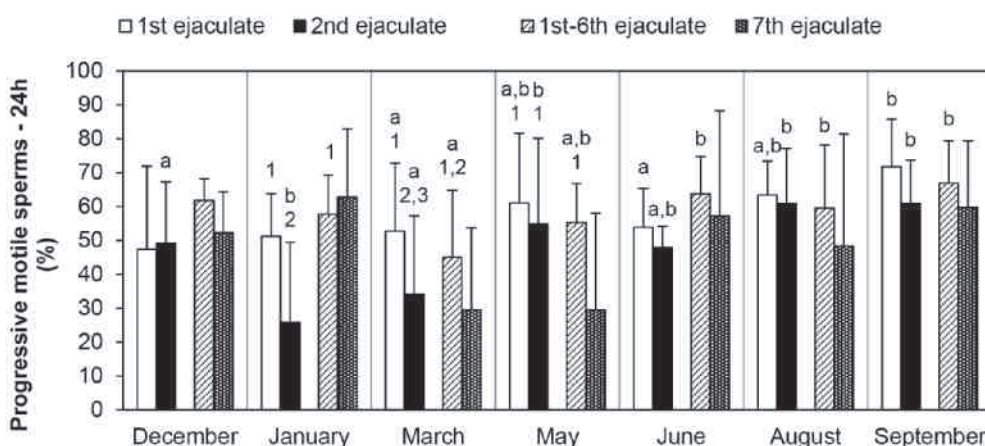
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(*Gebauer* et al. 1974) or for a period of 7 days (*Gamboa* et al. 2011). *Gamboa* et al. (2011) demonstrated for the latter case that the ejaculate collected on the 7th day corresponds to the estimated DSO (calculation on behalf of the testicular dimensions (*Love* et al. 1991)). In the present study, this approach corresponds to protocol B. Protocol A obtained the highest total sperm number in the first ejaculate and a significant reduction of the sperm count in the second ejaculate. This finding is consistent with other authors' results (*Parlevliet* et al. 1994, *Sieme* et al. 2004, *Gamboa* et al. 2011). Compared to the study by *Gamboa* et al. (2011), the difference between total sperm number was significantly less pronounced (48 % vs. 56 %). A possible explanation is the use of three different breeds and a smaller number of stallions in the mentioned study. Another study reported a difference of 45.3 % between the first and the second ejaculate (*Pickett* et al. 1976). *Squires* and *Pickett* (2010) underlined the importance of the relation between the

first and second ejaculate for breeding soundness, since the number of possible semen collections per day for the individual stallion can be estimated on its basis.
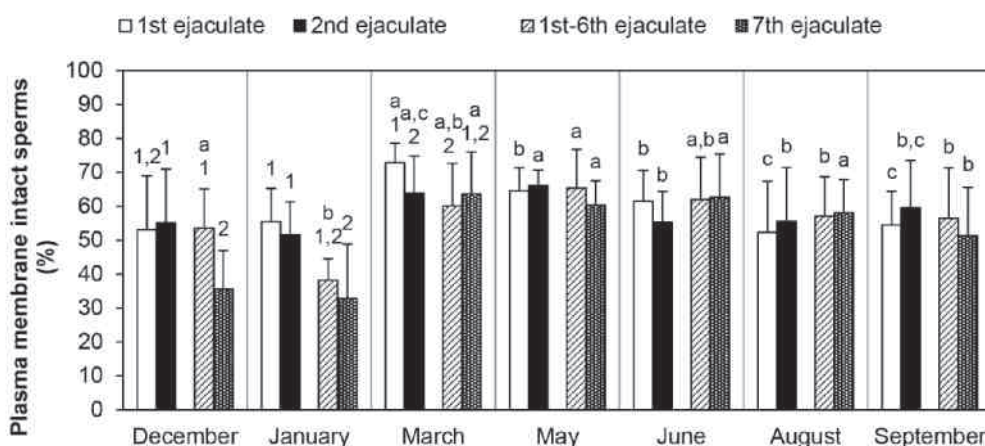
Consistent with results of other authors (*Love* et al. 1991), the ejaculate collected on the 7th day after daily semen collection for 7 days (protocol B) contained the lowest total sperm number. In contrast to results for protocol B, a seasonal influence on total sperm number was observed for the first collected ejaculate (protocol A). This finding confirms the results of *Pickett* et al. (1976) who found a seasonal influence on total sperm number in 5-year-old Quarter Horse stallions after two collections at a one-hour interval. Total sperm number was highest in July and lowest in December and January. These results correspond to findings by *Jasko* et al. (1991), who reported total sperm number to be highest in summer (August) and lowest in winter (December).

Fig. 3 Progressively motile sperms after storage for 24 h at 5 °C (%, mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).

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Different superscripts ([1,2,3]) indicate significant difference between trial weeks within ejaculates

Fig. 4 Plasma membrane integrity of sperms (%, mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Stallions from two different rearing conditions were included: Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).
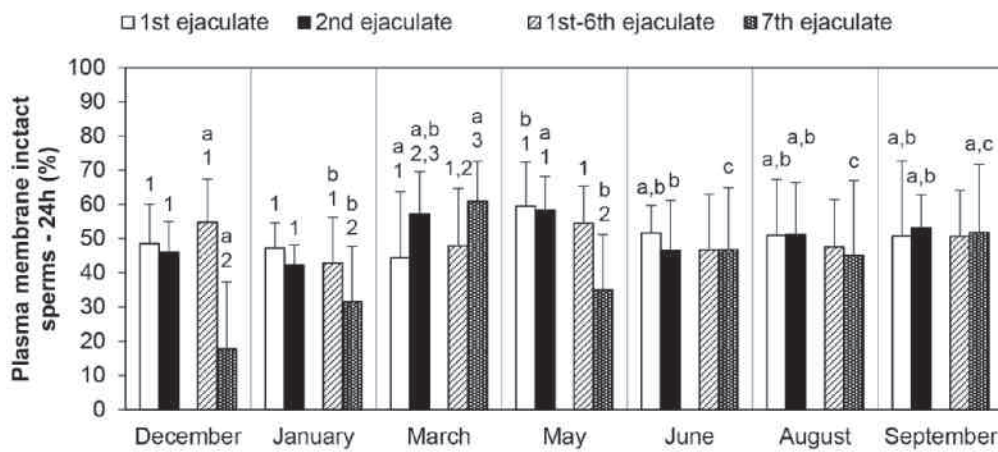
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Different superscripts ([1,2,3]) indicate significant difference between trial weeks within ejaculates

The progressive motility of semen in this study fulfilled the minimum requirement of 50% progressively motile sperms on the day of collection. Similarly, *Parlevliet* et al. (1994) observed a progressive motility of at least 50% in Dutch Warmblood stallions (average age 34 months). A study by *Amann* et al. (1979) did not find any significant difference between the motility of semen in 2 to 3 year old stallions as compared to 4 to 17 year old stallions. Furthermore, a seasonal effect on plasma membrane integrity was detected in our experiments (breeding season > non-breeding season). In younger stallions (trial 2), plasma membrane integrity was significantly lower than in older stallions (trial 1). However, this parameter has to be regarded with caution, since there are to our knowledge no established protocols for a correlation between plasma membrane integrity and fertility prognosis in fresh or cool-stored semen (*Barrier-Battut* et al.

2016). According to *Turner* (2005) the minimum requirement for PMI is set at 70% plasma membrane intact sperms. According to other authors, there is a high correlation between plasma membrane integrity and motility (*Brinsko* et al. 2003, *Love* et al. 2003, *Foster* et al. 2011). In the present study, progressive motility exceeded 70% in every ejaculate whereas plasma membrane integrity showed a large variation throughout the year and between age groups. However, plasma membrane integrity is considered to be especially useful for the examination of frozen-thawed semen (*Salazar* et al. 2011).
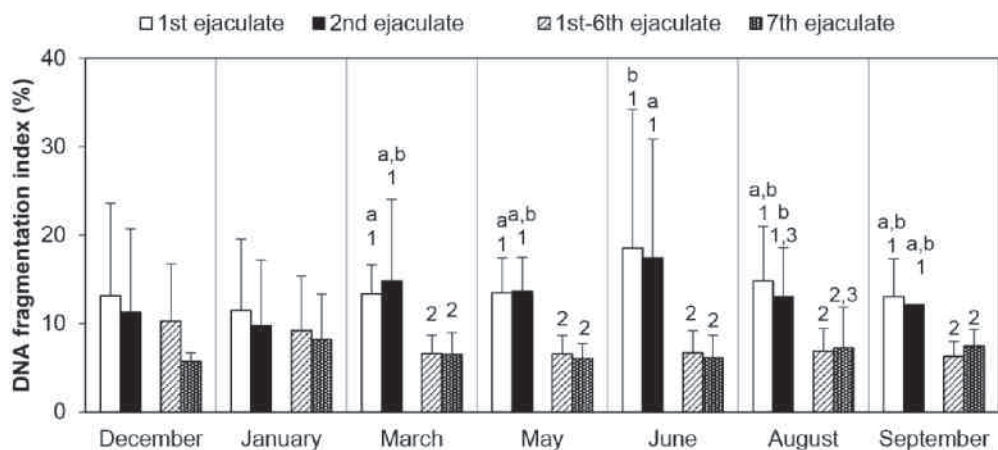
For sperm chromatin integrity, differences in DFI values between the semen collections protocols A and B were most striking. While the values for protocol A (semen collection at a one-hour interval) showed a seasonal influence, no effect

Fig. 5 Plasma membrane integrity of sperms after storage for 24 h at 5°C (%, mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).

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Different superscripts ([1,2,3]) indicate significant difference between trial weeks within ejaculates

Fig. 6 DNA fragmentation index of sperms (%, mean ± SD) of ejaculates collected with two different protocols (protocol A: two semen collections at a one-hour interval, protocol B: daily collections for 7 days). Older stallions from March to September (experimental trial 1: 36.8 months old, group A n = 8, group B n = 7), younger stallions in December and January (experimental trial 2: 33.4 months old, group A n = 4, group B n = 4).

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of the season on DFI was detected in protocol B (daily semen collection for 7 days, examination of the 7th ejaculate). A semen collection protocol emptying the epididymal reserves consequently appears to be beneficial for DFI determination. Although there are no minimum requirements for DFI, several authors found a relation with fertility (*Kenney* et al. 1995, *Love* et al. 1999). DFI-values around 12 are expected for highly fertile semen (*Love* 2005). *Wach-Gygax* et al. (2017) found lower DFI values from August to November than in June and July and concluded that semen quality is impaired in midsummer. In our study, DFI values varied over the year, showing the highest values (resp. lowest sperm chromatin integrity) in June.

After storage at 5 °C for 24 hours, plasma membrane integrity and progressive motility were reduced in nearly every ejaculate in the present study. A study by *Sieme* et al. (2004) compared progressive motility in ejaculates collected either once daily or twice at a one-hour interval. They reported progressive motility to be significantly higher in the first ejaculate than in the second ejaculate collected an hour later as well as in ejaculates collected daily. This observation was confirmed in trial 1, whereas other authors did not find an influence of semen collection frequency on progressive motility (*Pickett* et al. 1975 and 1985, *Squires* et al. 1979).

Another interesting finding of this study addresses the rearing conditions of the colts included in the study. One group had been reared extensively in groups since weaning until they were approximately 30 months old. The other group of stallions was purchased at the Hanoverian stallion licensing auction and had been intensively prepared for this event for several months. Unfortunately, the exact conditions of their individual rearing protocols are beyond our knowledge. However, no significant effect of rearing condition on sperm parameters was observed in this study.

At the start of trial 1, stallions were aged 36.8 months on average and in trial 2 their mean age was 33.4 months. As shown in the results section, only one significant effect of age on the semen quality parameters could be observed (plasma membrane integrity on day of collection). In consequence, semen analysis appears to be an adequate method to verify that semen produced by a stallion meet the minimum requirements. Stallions could thus be diagnosed as in- or subfertile prior to licensing. This approach would result in economic benefits for stallion breeders and prevent legal disputes concerning warranty claims. Unfortunately, an approach to predict future fertility at a prepubertal age based on Anti-Müllerian-Hormone plasma concentration in colts failed (*Scarlet* et al. 2018).

In conclusion, the semen analyses results of the 33.4 months old stallions on average met the minimum requirements for passing the breeding soundness examination. Hence, breeding soundness examination at this age is possible and recommendable. Furthermore, daily semen collection for 7 days and examination of the 7th ejaculate is advantageous since the obtained values are more consistent, particularly for total sperm number and chromatin integrity. Rearing conditions of the animals had no influence on semen quality parameters.

Author contributions

Martin Köhne, Pamela Brüning, Gesa Stuhtmann, Anna Tönissen, Gunilla Martinsson, Dominik Burger and Harald Sieme.

Harald Sieme, Pamela Brüning and Gunilla Martinsson designed the study. Pamela Brüning and Gesa Stuhtmann performed the experiments and semen analysis. Gesa Stuhtmann, Anna Tönissen and Martin Köhne performed data analysis. Martin Köhne, Dominik Burger and Gesa Stuhtmann wrote the manuscript. All authors read, amended and approved the submitted version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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