Journal of Reproduction and Development, Vol. 66, No 3, 2020

—Original Article—

Season does not have a deleterious effect on proportions of stallion seminal plasma proteins

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Abstract. The mechanism by which the content of the major groups of seminal plasma proteins in stallion semen changes between the breeding and non-breeding seasons remains unknown. Here, we investigated the proportions of non-heparin-binding, phosphorylcholine-binding, and heparin-binding proteins in seminal plasma with the aim of relating them to sperm quality and testosterone levels in good and bad freezer stallions. Only minor variations in the major protein groups were found between the breeding and non-breeding seasons. In the non-breeding season, a higher content of a subset of non-heparin binding proteins as well as of heparin-binding proteins was found. Analysis of semen characteristics revealed a somewhat contrasting picture. While only minor variations in sperm kinematics and sperm morphology were found between seasons, the flow-cytometric measurements of mitochondrial membrane potential and also, to some extent, reactive oxygen species production indicated lower sperm quality in the breeding season. Chromatin integrity and testosterone levels were unchanged between seasons. The results suggest that stallion ejaculates could be used year-round for freezing, since only minor differences in protein composition exist between the breeding and non-breeding seasons, as well as between good and bad freezers. In addition, sperm quality is not impaired during the non-breeding season.
Key words: Chromatography, Cytometry, Protein fractions, Seasonal changes, Stallion

(**J. Reprod. Dev. 66:** 215–221, 2020)

The seminal plasma (SP) of stallions is a secretion from the epididymis and the accessory glands of the male reproductive tract. It has an important role in sperm maturation, as well as functioning as a vehicle for ejaculated sperm. It contains proteins, lipids, carbohydrates, and ions; its composition and effects on sperm longevity vary between stallions and fractions of the ejaculate, as reviewed by Kareskoski & Katila [1]. The protein contents of SP [2, 3] as well as those of different fractions of the SP have been investigated [4]. The most abundant proteins in stallion SP are HSP-1 and HSP-2, which are phosphorylcholine-binding proteins [4]. Addition of SP to sperm samples has shown that while DNA integrity is reduced by the addition of SP, motility is affected in a more varied way [5]. Similar results were obtained in other studies [6, 7]. Freezability of stallion semen shows individual variation, in that about one-third of the stallions produce ejaculates that freeze well, one-third produce ejaculates that can sometimes be frozen successfully, and one-third produce ejaculates that cannot be frozen [8], thus prompting studies to identify the cause of this variability.

The influence of the different components of SP on freezability has been reported by several authors. Vitamin E levels have been found to be lower in ejaculates from good freezers [9], a finding contradicted by Usuga *et al*. [10]. Usuga *et al*. [11] found the highest motility in frozen-thawed samples with high levels of cysteine-rich secretory protein-3 (CRISP-3) compared to samples with low or medium levels of CRISP-3. While studies of how sperm quality of stallion samples changes with the season have been performed [12, 13], to our knowledge, no studies of how the composition of equine SP changes with the season have been undertaken. The aim of the present study was to investigate the differences in major protein groups between the breeding and non-breeding season, in relation to semen characteristics and testosterone levels between good and bad freezers. The breeding season in Germany, where the stallions

Received: June 20, 2019
Accepted: January 20, 2020
Advanced Epub: Februay 13, 2020

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were kept, begins at the February/March transition and reaches its peak at the April/May transition [14].

Materials and Methods

Animals and husbandry

Eight warmblood stallions and one draught horse (4–12 years of age) of proven fertility and known freezability were available for semen collection. They were categorized as good freezers (GF, n = 5) or bad freezers (BF, n = 4), according to both the stud's historical records for semen freezability and a test freezing done immediately prior to the start of the experiment to confirm the classification. According to the stud evaluation, the stallion was classified as a "good freezer" if total motility was ≥ 60% and progressive motility was ≥ 40%, and as a bad freezer if total motility was ≤ 50% and progressive motility was ≤ 30% [15]. The stallions were housed under standard husbandry conditions at Brandenburg State Stud Neustadt/Dosse, Germany. Ethical permission is not required for semen collection from stallions.

Semen collection and preparation

One ejaculate was obtained from each stallion during early February (non-breeding season) and May (breeding season). For semen collection, the stallions mounted a phantom and ejaculated into a lubricated Hannover artificial vagina at 42–44ºC. The semen was collected in a warm glass bottle and was then filtered through gauze into a measuring cylinder to remove the gel fraction. Immediately after semen collection, the volume of gel-free portion was estimated. An aliquot (5 ml) of each ejaculate was used to extract the SP as described below. The remainder of the ejaculate was extended 1:1 (v/v) using EquiPlus extender (Minitube, Tiefenbach, Germany) at 38ºC. Sperm concentration was determined by photometry (SpermaCue; Minitube). Subsequently, sperm motility was subjectively estimated in 5 µl of extended semen placed on a warm slide under an 18 × 18 mm coverslip at 400 × magnification, using a phase contrast light microscope. Afterward, the ejaculates were processed to prepare artificial insemination doses according to the stud´s usual procedure, i.e., the dose should contain 500 × 10^6 progressively motile spermatozoa/ml in a volume not exceeding 13 ml. EquiPlus extender warmed to 38ºC was used for this purpose. One artificial insemination dose from each ejaculate was transported to the laboratory at the Swedish University of Agricultural Sciences (SLU) in an insulated box containing a cold pack (average storage temperature 5 to 7°C); sperm kinematics, sperm chromatin integrity, and mitochondrial membrane potential were evaluated after overnight storage within 24 h of semen collection.

Preparation of seminal plasma

Samples of SP were harvested in Germany from raw gel-free semen immediately after collection by centrifugation at 2000 × *g* for 10 min. After aspiration of the supernatant into sterile tubes, it was checked for the presence of spermatozoa. If spermatozoa were detected, the samples were centrifuged again at 3500 × *g* until the SP became sperm-free. The SP was filtered into 4 ml tubes using a membrane syringe filter of 0.2 µm size, frozen at –80ºC, transported to Sweden, and stored until analyzed by affinity liquid chromatography and assessment of testosterone concentration.

Affinity liquid chromatography of seminal plasma

Aliquots (350 µl) of SP protein samples were thawed, the pH of the protein sample was adjusted to 7.5 with a 0.5 M tris buffer to a final concentration of 25 mM, and the sample was cleared by centrifugation. Protein concentrations were estimated by UV absorption at 280 nm (NanoDrop ND-2000 Spectrophotometer). Using an ÄKTA Explorer™ system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 1 ml of the prepared protein sample was loaded on a HiPrep Heparin FF 16/10 column, which had previously been equilibrated with 0.02 M Tris-HCl pH 7.5. The non-heparin-binding proteins, peak 1 and 2 (F1 + F2), were eluted with 0.02 M Tris-HCl buffer containing 0.156 M NaCl, pH 7.5. The phosphorylcholine-binding proteins, peak 3 (F3), were eluted with 0.02 M Tris-HCl buffer containing 0.156 M NaCl and 0.05 M phosphorylcholine, pH 7.5. The proteins adsorbed on heparin, peak 4 (F4), were eluted using a 0.02 M Tris-HCl buffer, 2 M NaCl pH 7.5. Peak height (mAU), peak area (mAU*ml), and percentage of area for each peak in relation to the total area were recorded for each fraction as well as the ratios of the different fractions [16, 17].

Sperm evaluation

Sperm kinematics: Sperm motility was determined by computer assisted sperm analysis (CASA; Sperm Vision® version 3.5; Minitube), which was connected to a microscope equipped with warm stage and phase contrast optics (20 objective, Optiphot-2; Nikon, Tokyo, Japan).

An aliquot (5 µl) of each sperm sample was placed on a pre-warmed glass slide and covered with a coverslip. For each sample, ≈ 200 spermatozoa in each of eight fields were tracked and analyzed to determine the kinematics, using a frame rate of 60 per second. The following parameters were measured: total and progressive motility (%), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/sec), straight line velocity (VSL; µm/sec), straightness (STR; VSL/VAP), linearity (LIN; VSL/VCL), wobble (WOB; VAP/VCL), amplitude of lateral head deviation (ALH; µm), and beat cross frequency (BCF; Hz). The spermatozoa were considered to be immotile if VAP < 20 and locally motile if VAP > 20 and <30, STR < 0.5, and VCL < 9.

Sperm morphology: In Germany, aliquots of the raw ejaculates were used to prepare air-dried slides for later assessment of the shape of the sperm head of 500 spermatozoa at 1000 × magnification, following staining. Additional aliquots were fixed in a buffered formaldehyde solution for subsequent evaluation of 200 spermatozoa in wet smears. The slides and formaldehyde-fixed samples were then transported to Sweden for evaluation [18]. The morphological examinations were performed by skilled staff from the sperm laboratory at the Division of Reproduction, SLU, which acts as a national reference laboratory in Sweden. The mean proportion of morphologically normal spermatozoa was estimated as the remaining proportion after subtraction of the total number of abnormal spermatozoa. Spermatozoa with distal cytoplasmic droplets were included in the fraction of normal spermatozoa, since these are considered to be normal in stallion spermatozoa.

Assessment of sperm chromatin integrity (SCSA): The evaluation was performed according to a previously reported methodology

[19, 20]. Briefly, the sperm aliquots were extended 1:1 (v/v) with a Tris-sodium chloride-ethylenediamine tetraacetic acid (TNE) buffer (0.15 mol/l NaCl, 0.01 mol/l Tris-HCl, 1 mmol/l EDTA, pH 7.4), and then immediately snap-frozen in liquid nitrogen (LN_2). The samples were stored at –80°C until further processing and analysis. The samples were thawed on crushed ice, partially denatured and stained with an acridine orange (AO) staining solution. The stained samples were analyzed within 3–5 min of AO staining. Measurements were conducted on a BD LSR flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with standard optics. The AO was excited at 488 nm. Emitted fluorescence was detected using green (FL1) and red (FL3) filters. Data were collected from 10,000 events for each sample and presented as DNA fragmentation index (%DFI), calculated as the ratio of the percentage of spermatozoa with denatured, single-stranded DNA to total spermatozoa acquired.

Mitochondrial membrane potential

Sperm mitochondrial membrane potential (MMP) was measured using the reagent 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, (JC-1; Thermo Fisher Scientific, Waltham, MA, USA). Approximately 1000 µl CellWASH™ (BD Biosciences), containing one million spermatozoa, and 0.5 µl of 3 mM JC-1 were added and the samples were incubated at 37ºC for 40 min in the dark. The stained samples were analyzed using a BD LSR flow cytometer (BD Biosciences). Excitation of JC-1 in stained cells was obtained by an argon-ion laser (488 nm). Emitted fluorescence was detected using bandpass filters 530/28 nm (FL1, green fluorescence) and 575/26 nm (FL2, orange fluorescence). A total of 30,000 events was evaluated and classified in 2 distinct groups: spermatozoa with high respiratory activity MMP-H (orange fluorescence) and those with low respiratory activity MMP-L (green fluorescence).

Reactive oxygen species (ROS)

An aliquot of each sperm suspension (2 million spermatozoa in 300 µl CellWASH) was stained with 9 µl of 40 µM Hoechst 33258 (HO; Sigma-Aldrich, St. Louis, MO, USA), 9 µl of 40 µM hydroethidine (HE; Thermo Fisher), and 9 µl of 2 mM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA; Thermo Fisher) in the dark, for 30 min at 38°C. The content of ROS was detected using a BD LSR flow cytometer (BD Biosciences). Excitation was achieved with an argon-ion laser (488 nm) and a HeCd laser (325 nm). Green fluorescence (FL1) was detected with a bandpass filter (530/28 nm), blue fluorescence (FL5) was detected with a long-pass filter (380 nm), and red fluorescence (FL3) was detected through a long-pass filter (> 670 nm). A total of 30,000 events was analyzed and classified as follows (%): viable, SO-negative; viable, SO-positive; non-viable, SO-positive; viable, H_2O_2-negative; viable, H_2O_2-positive; non-viable, H_2O_2-negative; and non-viable, H_2O_2-positive.

Assessment of testosterone concentration in stallion seminal plasma

Stallion SP testosterone concentration (ng/ml) was measured using a competitive horse testosterone ELISA Kit (catalogue no. CSB-E13193Hs; Cusabio Biotech, Wuhan, China) with LOD 0.05 ng/ml, according to the manufacturer's instructions. Briefly, aliquots (50 µl) of SP, control, or calibrator (4 × diluted, 0 to 20 ng/ml) were added in duplicate to the corresponding wells on a microtiter plate pre-coated with primary antibody (goat-anti-rabbit) against horse testosterone. Subsequently, 50 µl of horseradish-peroxidase conjugated testosterone (HRP) were loaded (excluding blank wells), and a secondary antitestosterone antibody (50 µl) was added to each well. The plate was covered with an adhesive sealing sheet and mounted for mixing on a plate shaker (700 rpm). Then it was incubated for 60 min at 37°C on a plate reader (Infinite M200Pro; Tecan, Männedorf, Switzerland). The wells were decanted and washed three times with the kit wash buffer (200 µl using multichannel pipette), after which complete removal of the wash buffer was achieved by vigorously tapping the plate several times against tissue paper. The detection step was performed by adding 50 µl of substrates A and B to each well, mixing on a plate shaker and incubating for 15 min at 37°C on a plate reader away from direct light. After incubation, a stop solution (50 µl) was added and mixed gently on the plate shaker. Optical density (at 450 nm) was measured within 10 min. Testosterone concentrations were calculated using a four parameter logistic curve-fit (https://www.myassays.com/). Based on the values obtained from the standard curve, the higher optical density measured represents lower (opposite) testosterone concentration in the sample. The intra-assay CV was 9.2% (manufacturer recommendation < 15%) and the spiked sample percent recovery was 128%.

Statistical analysis

Statistical analyses were carried out using SAS software (SAS® 9.3, Cary, NC, USA), after checking the normal distribution of the residuals. A mixed model (PROC MIXED) was used to analyses the data. The statistical model included the fixed effects of seasons (n = 2, breeding and non-breeding) and stallion status ("good" or "bad" freezer) in addition to seasons*status interaction and the random effects of stallions (n = 9). All values were reported as Least Squares Means ± Standard Error (LSMEAN ± SEM). Differences were taken to be statistically significant when $P \leq 0.05$.

Results

Proportions of seminal plasma proteins

A typical chromatogram with four peaks obtained, (peak 1 and peak 2, non-heparin-binding proteins; peak 3, phosphorylcholine-binding proteins; and peak 4, heparin-binding proteins), is shown in Fig. 1. In all samples, the phosphorylcholine-binding proteins constituted the most abundant group. The concentration of non-heparin binding proteins found in peak 2, as indicated by both peak height ($P = 0.04$) and area ($P = 0.02$), was higher in the non-breeding season than in the breeding season (Table 1). However, there was no significant difference between seasons when adding together the two peaks for non-heparin binding proteins. Also, the heparin-binding proteins in peak 4 were more abundant in the non-breeding season than in the breeding season (Area: $P = 0.03$; Height: $P = 0.01$). The ratio of heparin-binding proteins to total proteins tended to be higher in the non-breeding season ($P = 0.07$). When comparing good and bad freezers in the different seasons, the only difference found was that the peak height for the heparin-binding proteins was higher in the non-breeding season for bad freezers ($P = 0.05$).

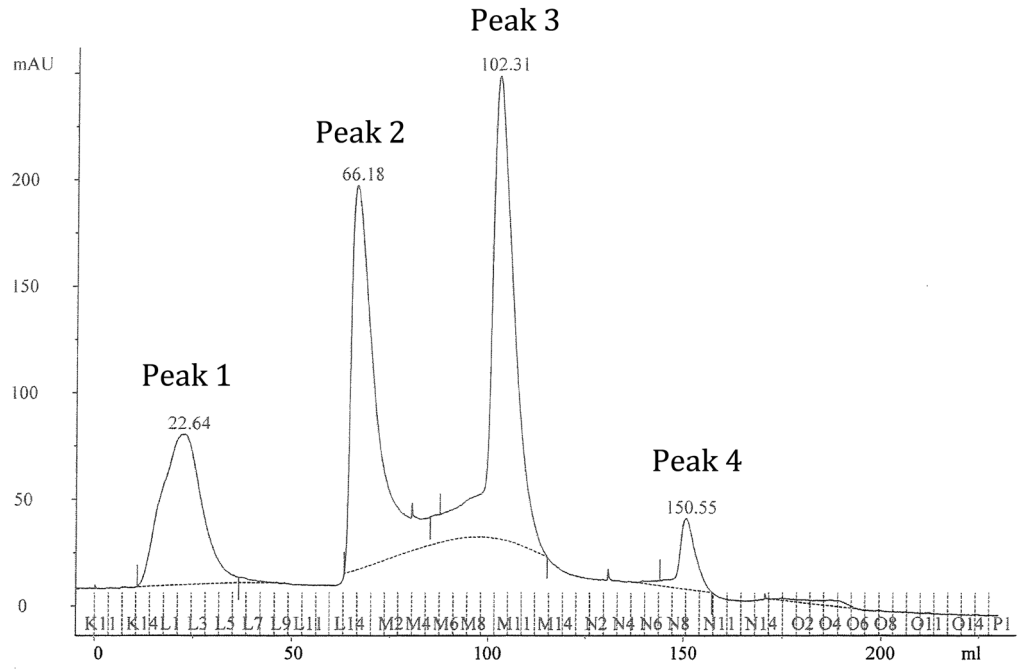

Fig. 1. Typical FPLC chromatogram of stallion seminal plasma proteins (Non-heparin-, phosphorylcholine-, and heparin-binding). Peak 1 and 2 contain proteins not interacting with heparin (Fraction F1), while peak 3 (Fraction F2) and peak 4 (Fraction F3) contain phosphorylcholine-binding and heparin-binding proteins, respectively.

Table 1. Proportions and ratios for the different peaks of seminal plasma (SP) proteins

FPLC results	All stallions		Good freezer stallions		Bad freezer stallions	
	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
Peak 1 Area	192 ± 31	138 ± 31	229 ± 41	150 ± 41	156 ± 46	126 ± 46
Peak 1 Height	34.2 ± 7.6	17.1 ± 7.6	43.3 ± 10.1	19.0 ± 10.1	25.0 ± 11.3	15.3 ± 11.3
Peak 2 Area	128 ± 25 [a]	229 ± 25 [b]	119 ± 33	212 ± 33	137 ± 37	246 ± 37
Peak 2 Height	24.8 ± 4.2 [a]	40.2 ± 4.2 [b]	25.2 ± 5.7	38.6 ± 5.7	24.4 ± 6.3	41.8 ± 6.3
Peak 3 Area	358 ± 39	386 ± 39	336 ± 52	386 ± 52	380 ± 58	385 ± 58
Peak 3 Height	62.8 ± 6.8	69.1 ± 6.8	59.5 ± 9.0	67.0 ± 9.0	66.1 ± 10.1	71.2 ± 10.1
Peak 4 Area	16.3 ±3.0 [a]	25.1 ± 3.0 [b]	13.2 ± 4.1	21.5 ± 4.1	19.4 ± 4.6	28.8 ± 4.6
Peak 4 Height	4.34 ± 0.76 [a]	6.06 ± 0.76 [b]	3.57 ± 1.01	4.54 ± 1.01	5.12 ± 1.13 [a]	7.6 ± 1.1 [b]
Total Area	734 ± 63	823 ± 63	743 ± 84	795 ± 84	724 ± 93	850 ± 93
Area 1+2	320 ± 35	367 ± 35	348 ± 46	362 ± 46	293 ± 52	372 ± 52
Area3/Area4	374 ± 39	411 ± 39	349 ± 52	407 ± 52	400 ± 58	414 ± 58
Area 1+2/total area	43.8 ± 2.6	44.7 ± 2.6	47.3 ± 3.5	46.2 ± 3.5	40.3 ± 3.9	43.2 ± 3.9
Area 3/total area	48.6 ± 3.0	46.78 ± 3.0	44.8 ± 4.0	47.7 ± 4.0	52.4 ± 4.4	45.9 ± 4.4
Area 4/total area	2.40 ± 0.46	3.14 ± 0.46	1.91 ± 0.61	2.81 ± 0.61	2.89 ± 0.68	3.47 ± 0.68

Values are represented as LSMEAN ± SEM. Different superscript letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($P \leq 0.05$). For all stallions P-values were as follows: Peak 2 Area, P = 0.02; Peak 2 Height, P = 0.04; Peak 4 Area, P = 0.03; Peak 4 Height, P = 0.01. For bad freezers P-value was as follows: Peak 4 Height, (BF), P = 0.05.

Sperm quality parameters

Sperm concentration was higher in the non-breeding season than in the breeding season (P = 0.01), whereas the proportion of spermatozoa with normal morphology was higher in the breeding season (P = 0.05; Table 2). In addition, in bad freezers, there was a higher proportion of spermatozoa with normal morphology in the breeding season than in the non-breeding season (P = 0.04), while there were no differences in morphology between seasons in good freezers.

No differences were found for testosterone concentration and ejaculate volume. However, in the non-breeding season, good freezers

Table 2. Results from analysis of semen characteristics

Semen values	All stallions		Good freezer stallions		Bad freezer stallions	
	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
Concentration (10^6/ml)	240 ± 23 [a]	348 ± 23 [b]	273 ± 30	395 ± 30	208 ± 34	301 ± 34
Ejaculate volume (ml)	22.2 ± 2.2	20.8 ± 2.2	21.2 ± 3.0	20.0 ± 3.0	23.2 ± 3.3	21.5 ± 3.3
Normal morphology (%)	71.4 ± 3.0 [a]	68.38 ± 3.0 [b]	78.4 ± 4.0	79.0 ± 4.0 *	64.4 ± 4.4 [a]	57.8 ± 4.4 [b]
Testosterone (ng/ml)	1.46 ± 0.34	0.81 ± 0.34	1.41 ± 0.46	0.55 ± 0.46	1.51 ± 0.51	1.06 ± 0.51

Values are represented as LSMEAN ± SEM. Different superscript letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($P \leq 0.05$). For all stallions, P-values were as follows: Concentration, P = 0.01; Morphology, P = 0.05. For bad freezer stallions, P-value was as follows: Morphology P = 0.04. An asterisk (*) indicates a difference in morphology between good freezer stallions and bad freezer stallions in the non-breeding season (P = 0.04).

Table 3. Results of the analysis of spermatozoa kinetics

Kinematics	All stallions		Good freezer stallions		Bad freezer stallions	
	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
MOT (%)	68.2 ± 4.8	77.3 ± 4.8	67.2 ± 6.4	80.8 ± 6.4	69.1 ± 7.2	73.8 ± 7.2
PM (%)	40.0 ± 5.9	55.1 ± 5.9	43.1 ± 7.8	60.0 ± 7.8	36.9 ± 8.7	50.3 ± 8.7
VAP (μm/sec)	76.6 ± 3.8	74.1 ± 3.8	78.9 ± 5.0	77.1 ± 5.0	74.3 ± 5.6	71.2 ± 5.6
VCL (μm/sec)	137 ± 6.2	135 ± 6.2	142 ± 8.3	140 ± 8.3	133 ± 9.3	129 ± 9.3
VSL (μm/sec)	56.7 ± 3.5	58.5 ± 3.5	59.4 ± 4.7	61.2 ± 4.7	54.1 ± 5.3	55.8 ± 5.3
STR (%)	0.73 ± 0.01 [a]	0.78 ± 0.01 [b]	0.75 ± 0.02	0.78 ± 0.02	0.72 ± 0.02	0.78 ± 0.02
LIN (%)	0.41 ± 0.01	0.43 ± 0.01	0.41 ± 0.02	0.42 ± 0.02	0.40 ± 0.02	0.43 ± 0.02
WOB (%)	0.55 ± 0.01	0.54 ± 0.01	0.55 ± 0.01	0.54 ± 0.01	0.56 ± 0.02	0.55 ± 0.02
ALH (μm)	4.31 ± 0.13 [a]	3.44 ± 0.13 [b]	4.30 ± 0.17 [a]	3.48 ± 0.17 [b]	4.33 ± 0.19 [a]	3.41 ± 0.19 [b]
BCF (Hz)	30.0 ± 0.63	31.7 ± 0.63	30.6 ± 0.84	32.2 ± 0.84	29.3 ± 0.94	31.2 ± 0.94

Values are represented as LSMEAN ± SEM. MOT, total motility (%); PM, progressive motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VSL, straight line velocity (μm/sec); STR, straightness (VSL/VAP); LIN, linearity (VSL/VCL); WOB, wobble (VAP/VCL); ALH, amplitude of lateral head deviation (μm); BCF, beat cross frequency (Hz). Different superscript letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($P \leq 0.05$). For all stallions, P-values were as follows: STR, P = 0.04; ALH, P = 0.002. For good freezers stallions, P-value was as follows: ALH, P = 0.05. For bad freezer stallions, P-value was as follows: ALH, P = 0.04.

had better morphology than bad freezers (P = 0.04). Regarding kinematics (Table 3), higher values for STR were seen in the non-breeding season (P = 0.04), while ALH was higher in the breeding season (P = 0.002). As shown in Table 3, ALH was higher in the breeding season for good freezer stallions (P = 0.05), as well as for bad freezer stallions (P = 0.04). Sperm chromatin integrity did not differ (Table 4). There was a higher proportion of spermatozoa with high MMP in the non-breeding season than in the breeding season (P = 0.01), although no differences were found when comparing good and bad freezers in different seasons. For ROS, there was a higher proportion of viable SO- ($P < 0.0001$), a lower proportion of viable SO+ ($P < 0.001$), a lower proportion of non-viable SO+ spermatozoa ($P < 0.0001$), and a lower percentage of non-viable H_2O_2-spermatozoa ($P < 0.0001$) in the non-breeding season than in the breeding season. These differences also occurred when samples were split into good and bad freezers.

Discussion

We studied the major protein groups in SP samples collected from good and bad freezer stallions in different seasons and found only minor differences. These findings, coupled with an indication that sperm quality is better in the non-breeding season than in the breeding season, suggest that it should be possible to freeze stallion semen at any time of the year without affecting its freezability.

In the present study, we found an increase in one peak for non-heparin, non-phosphorylcholine-binding proteins in the non-breeding season. However, this finding should be interpreted with caution, since when all non-heparin, non-phosphorylcholine-binding proteins were considered, there was no difference between seasons. Previous studies [11, 21] have shown higher motility in samples with high levels of CRISP-3 (a non-heparin binding protein [22]) and have detected differences in sperm quality parameters correlated to the CRISP-3 genotype. Hence, it is possible that CRISP-3 is the protein causing the above-mentioned increase in one of the non-heparin-binding protein peaks. Other studies found that CRISP3 was positively correlated to first cycle conception rate [23].

The apparent shift toward more heparin-binding proteins in the non-breeding season could indicate higher sperm quality in the non-breeding season. A positive correlation between the heparin-binding protein, osteopontin, and fertility has previously been described [24], while negative correlation to fertility for three other proteins has

Table 4. Results of flow-cytometric measurements

Sperm parameter	All stallions		Good freezer stallions		Bad freezer stallions	
	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
%DFI	9.06 ± 1.12	8.56 ± 1.12	7.44 ± 1.49	7.20 ± 1.49	10.7 ± 1.7	9.93 ± 1.66
MMP-L	60.7 ± 5.6 [a]	44.5 ± 5.6 [b]	59.8 ± 7.5	46.8 ± 7.5	61.6 ± 8.4	42.1 ± 8.4
MMP-H	35.4 ± 5.5 [a]	52.2 ± 5.5 [b]	35.8 ± 7.4	49.4 ± 7.4	35.0 ± 8.2	55.0 ± 8.2
Viable SO-	19.3 ± 2.6 [a]	55.3 ± 2.6 [b]	18.8 ± 3.5 [a]	57.4 ± 3.5 [b]	19.9 ± 3.9 [a]	53.2 ± 3.9 [b]
Viable SO+	12.2 ± 1.4 [a]	4.48 ± 1.41 [b]	13.2 ± 1.9 [a]	4.70 ± 1.89 [b]	11.1 ± 2.1 [a]	4.26 ± 2.12 [b]
Non-viable SO+	68.5 ± 3.2 [a]	39.8 ± 3.2 [b]	68.0 ± 4.2 [a]	37.4 ± 4.2 [b]	69.0 ± 4.7 [a]	42.1 ± 4.7 [b]
Viable H_2O_2-	30.0 ± 5.8	38.6 ± 5.8	29.2 ± 7.8	37.9 ± 7.8	30.8 ± 8.7	39.4 ± 8.7
Viable H_2O_2+	1.52 ± 5.61	20.0 ± 5.6	2.7 ± 7.5	22.7 ± 7.5	0.36 ± 8.37	17.4 ± 8.4
Non-viable H_2O_2-	63.4 ± 5.8 [a]	23.3 ± 5.8 [b]	59.4 ± 7.8 [a]	17.3 ± 7.8 [b]	67.4 ± 8.7 [a]	29.3 ± 8.7 [b]
Non-viable H_2O_2+	3.72 ± 4.39	15.6 ± 4.4	6.67 ± 5.85	19.2 ± 5.8	0.77 ± 6.54	12.1 ± 6.5

Values are LSMEAN ± SEM. Different letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($P \leq 0.05$). %DFI, DNA fragmentation index; MMP-L, low-mitochondrial membrane potential (%); MMP-H, high-mitochondrial membrane potential (%). For all stallions, P-values were as follows: MMP-L, P = 0.02; MMP-H, P = 0.01; viable SO-, $P < 0.0001$; viable SO+, $P < 0.001$; non-viable SO+, $P < 0.0001$; non-viable H_2O_2-, $P < 0.0001$. For good freezer stallions, P-values were as follows: viable SO-, $P < 0.0001$; viable SO+, P = 0.03; non-viable SO+, P = 0.0002; non-viable H_2O_2-, P = 0.006. For bad freezer stallions, P-values were as follows: viable SO-, $P < 0.0001$; non-viable SO+, P = 0.004; non-viable H_2O_2-, P = 0.04.

been observed. In addition, a higher quantity of the heparin-binding protein, fibronectin, has been reported in boar semen samples with high freezability than in those with poor freezability [25]. In agreement with previous results [4], phosphorylcholine-binding proteins were found to be abundant in the present study, although no difference between seasons was found.

Seminal plasma also affects the mare at insemination. It has been shown that the heparin-binding SP component, lactoferrin, suppresses the mRNA expression of tumour necrosis factor (TNF)-alpha in inseminated mares susceptible to persistent mating-induced endometritis in a similar way to SP [26].

Other components of the SP, apart from those investigated in the present study, might also influence fertility parameters and freezability. Usuga *et al*. [10], focussing on other SP components and total protein content, found that a high level of vitamin A in the SP was associated with the best sperm quality, while low levels of vitamin E were associated with poor motility. In contrast, Mráčková *et al*. [9] encountered lower vitamin E levels in ejaculates from good freezers.

Our results might indicate that in some aspects sperm quality is lower in the breeding season than in the non-breeding season. This could be due to more frequent sampling during the breeding season. That sperm concentration was higher in the non-breeding season than in the breeding season is in accordance with earlier studies [12]. However, in contrast to those studies, we did not note a seasonal variation in testosterone concentration in SP. This may be due to the low number of stallions included in the present study. However, the finding is in agreement with results from a previous study where testosterone concentration in blood was not affected in stallions frequently used for semen collection [27]. In contrast, based on sperm concentration, the available testosterone per spermatozoon was found to be less in the non-breeding season in the present study. Our finding that chromatin integrity is unaffected by season is in accordance with an earlier study [28]. Janett *et al*. [13] showed that in warmblood stallions in Switzerland, sperm viability and morphology were at their lowest in the summer. This is somewhat in contrast to our study which found that the bad freezers had better morphology in the breeding season than in the non-breeding season. The same group demonstrated that in Franches-Montagnes stallions, sperm quality was best in the autumn [29]. This might point to differences between breeds in their seasonal variations in sperm quality, or to differences due to the stallion´s location during semen collection. In bulls, breed was shown to affect which parameters are useful for prediction of fertility [30]. Aurich concluded that because of small variations in semen quality between seasons, the choice of stallion is more important than the season of collection, and our results support this conclusion [14].

In the future, the fractions of seminal plasma proteins collected in the present study could be investigated to see how they affect survival of spermatozoa and/or epithelial cells. The fractions could also be investigated for their content of possible markers of fertility and freezability, such as CRISP-3, SP1, and SP2.

Taken together, the results suggest that stallion ejaculates could be used year-round for freezing, since only minor differences in protein composition exist between breeding and non-breeding seasons, as well as between good and bad freezers. In addition, sperm quality is not impaired during the non-breeding season.

Conflict of interest: The authors do not have any conflicts of interest to declare.

Acknowledgements

We wish to thank the personnel at Graf Lehndorff-Institute for Equine Sciences, Neustadt/Dosse, Germany, for their help during semen collection and freezing. We are grateful to the laboratory personnel at the Department of Clinical Sciences, SLU, and especially to Karin Selin-Wretling, for performing the morphology analyses, and to Professor Ulf Olsson for his suggestions and com-

ments on the statistical analyses. We are also grateful to Professor Andrzej Madej and Dr Malgorzata Madej for suggestions and comments regarding the FPLC analyses. EA and AA were funded by the Iraqi Ministry of Higher Education and Scientific Research, Baghdad. This study was funded by the Swedish Foundation for Equine Research (H-14-47-008). The authors would like to acknowledge the Cells for Life Platform at SLU for providing the facilities for this project.

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