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Fast protein liquid chromatography profiles of seminal plasma proteins in young bulls: A biomarker of sperm maturity?

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HIGHLIGHTS

- 10-month old bulls can serve as semen donors but sperm quality may be poor or cryosurvival low.
- Seminal plasma was collected from 10-month old bulls and again later.
- Fertility-associated proteins were evaluated by fast protein liquid chromatography.
- Heparin-binding proteins were more abundant in Sample II than in Sample I.

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ABSTRACT

Breeding companies want to use semen from bulls as soon as possible to take advantage of their desirable genetics. It takes several weeks for the sperm quality of young bulls to stabilize and for post-thaw sperm quality to become acceptable for artificial insemination. Seminal plasma proteins protect spermatozoa during cryopreservation; it may take some time for the seminal plasma protein profile to stabilize. The purpose of this study was to determine if the seminal plasma protein profile can be used as a marker of likely seminal maturity in young bulls. A comparison was made of the seminal plasma protein profile in the ejaculates of 10 bulls of 9-10 months old (Sample I), with the profiles from ejaculates taken from the same bulls at 13-16 months old (Sample II) using fast protein liquid chromatography. This is a method for separating classes of proteins according to their binding ability. The peak area and peak height of different classes of proteins did not differ significantly between the two samples for each bull, except for peak 5 (heparin-binding proteins) and total peak area ($p<0.05$). The heparin-binding protein peak height and area were significantly higher ($p<0.05$) in Sample II than in Sample I. In conclusion, levels of fertility associated heparin-binding proteins increase with age in young bulls and might serve as a biomarker of sperm maturity. .

1. Introduction

Once a bull calf with a desirable genome has been identified, there is considerable pressure from geneticists in the breeding company to be able to use the semen for artificial insemination (AI) as soon as possible. However, there is individual variation in sperm quality of semen from young bulls and little is known about the freezability of such samples or their potential fertility when used for AI. The spermiogram tends to show a high proportion of spermatozoa with abnormal morphology, retained cytoplasmic droplets and defective acrosomes, which are associated with poor fertility (Karabinus et al., 1990). However, it is not known whether the seminal plasma protein profile changes along with sperm maturation in these young animals. Seminal plasma contains many proteins that play a role in reproduction by influencing sperm survival and functionality e.g. heparin-binding proteins and phosphorylcholine-binding proteins facilitate capacitation by inducing cholesterol efflux, and phosphorylcholine-binding proteins bind to spermatozoa, which may have a protective function in the female reproductive tract (Druart & de Graaf, 2018).

Identifying individual proteins in seminal plasma may not be particularly helpful in predicting fertility since one protein may compensate for another; therefore, analysis of different classes of

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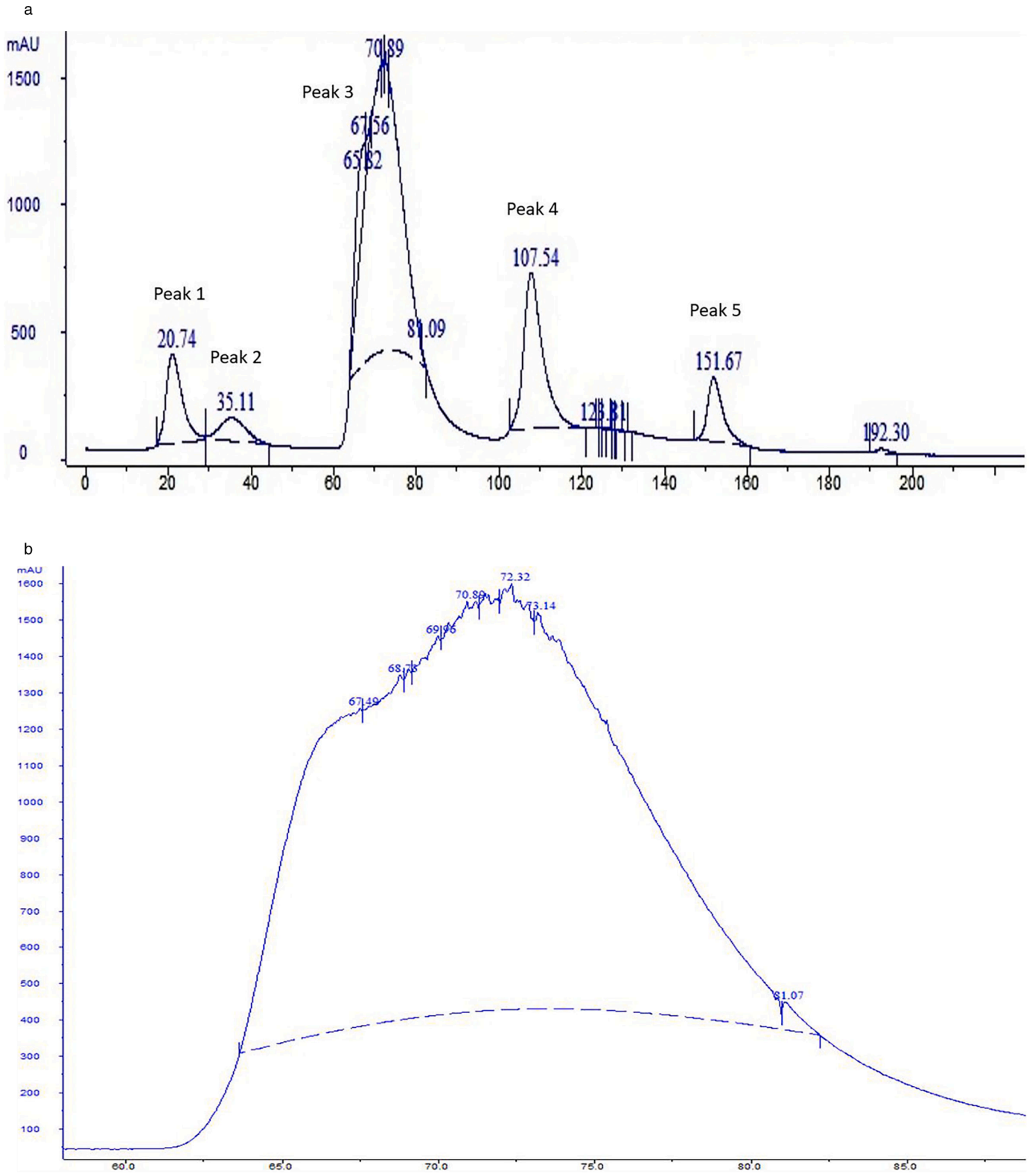

Fig. 1. Chromatogram from a young Swedish Red bull, 298 days old (Sample I): a) profile showing 5 peaks; b) enlargement of peak 3. ote: peaks 4 and 5 are the phosphatidylcholine- and heparin-binding peaks, respectively.

proteins might be more informative (Lima-Verde et al., 2020). Previous studies in our laboratory focused on the use of Fast Protein Liquid Chromatography (FPLC) to separate different categories of seminal plasma proteins in stallion (Johannisson et al., 2020) and bull semen (Lima-Verde et al., 2020). This technique was used to investigate the fertility-associated proteins in seminal plasma, particularly phosphorylcholine–binding and heparin-binding proteins. Therefore, the purpose of the present study was to compare the seminal plasma protein profiles in semen samples from young bulls at approximately 9-10 months old and further samples from the same bulls several weeks later when the

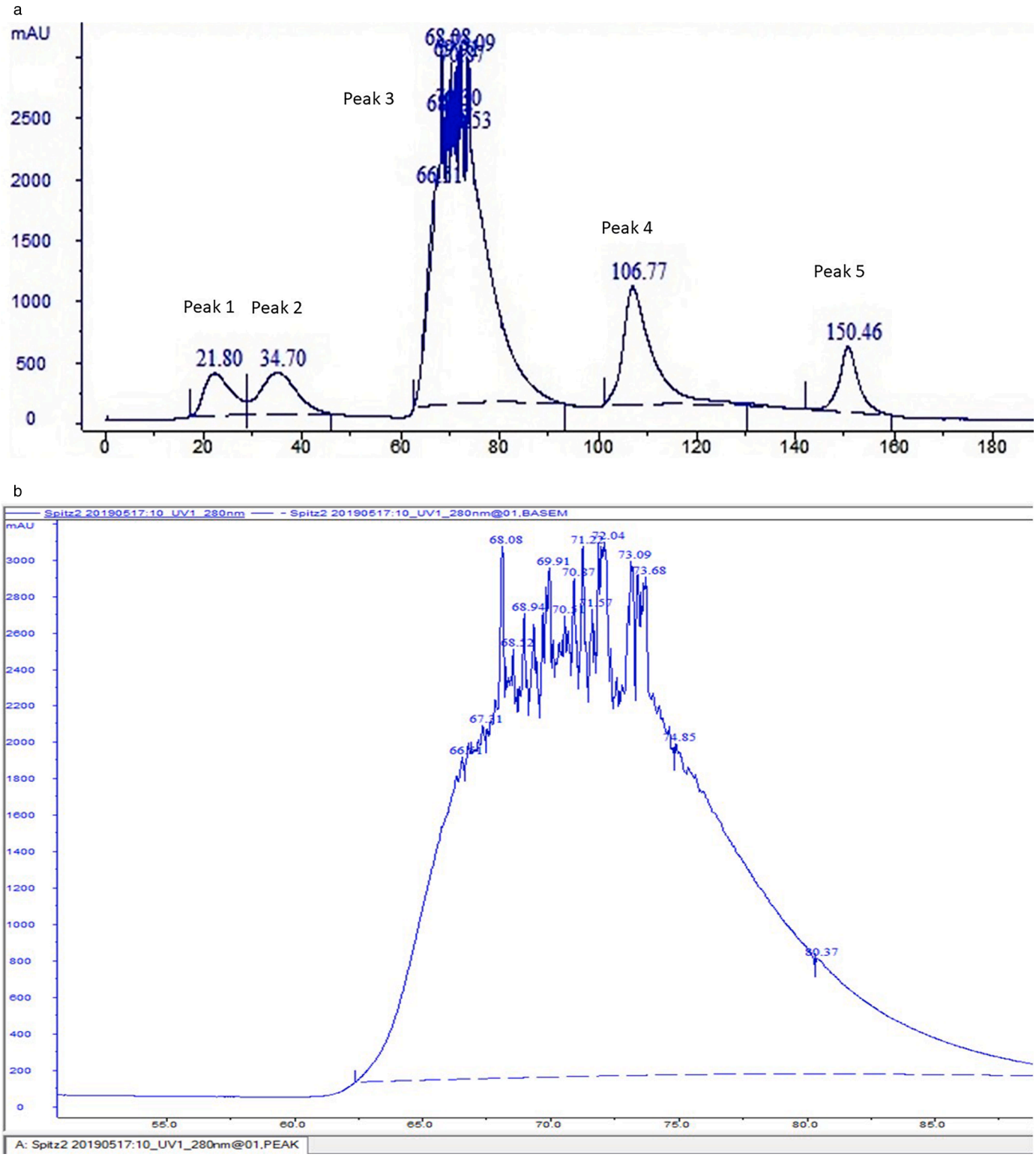

Fig. 2. Chromatogram from a young Swedish Red bull, 377 days old (Sample II); a) profile showing 5 peaks; b) enlargement of peak 3. Note: peaks 4 and 5 are the phosphatidylcholine- and heparin-binding peaks, respectively.

semen collections that were required for commercial freezing by the breeding company were completed.

2. Materials and methods

2.1. Semen samples

No ethical approval was required for this study in Sweden since the animals themselves were not part of the experiment. The bulls were owned by Viking Genetics, who gave permission to use the ejaculates for

Table 1
Proportions and ratios of different protein peaks in seminal plasma of young bulls at two different ages (means ±SD).

FPLC results	"Young" sample (I; n=10)	"Mature" sample (II; n=10)
Peak 1 Area (mAU*ml)	1266.62 ± 1049.43	1674.41 ± 803.42
Peak 1 Height (mAU)	235.94 ± 218.63	280.24 ± 128.43
Peak 2 Area (mAU*ml)	581.89 ± 310.83	1077.62 ± 909.25
Peak 2 Height (mAU)	85.42 ± 45.18	153.21 ± 112.77
Peak 3 Area (mAU*ml)	5008.10 ± 2677.08	5640.89 ± 1539.78
Peak 3 Height (mAU)	876.21 ± 382.41	1148.02 ± 668.66
Peak 4 Area (mAU*ml)	4210.55 ± 3114.56	5680.18 ± 2269.49
Peak 4 Height (mAU)	926.21 ± 824.62	1520.71 ± 912.87
Peak 5 Area (mAU*ml)	1086.86^{a} ± 263.30	1723.02^{b} ± 530.41
Peak 5 Height (mAU)	237.11^{a} ± 59.89	360.42^{b} ± 123.92
Total Area (mAU*ml)	2430.80^{a} ± 2583.74	3159^{b} ± 2452.06
Area 1+2+3/Total Area	2.82	2.65
Area 4/Total Area	1.73	1.79
Area 5/Total Area	0.44	0.54

Note: Different superscripts in a row indicate significant difference ($p<0.05$)

research purposes. The animals were maintained in accordance with national and international guidelines.

Samples from 10 bulls (9 Swedish Red and 1 Holstein) were available for this study. The bulls were owned by a breeding company (Viking-Genetics, Skara, Sweden) and were housed and handled according to national and international regulations. No ethical approval was required in Sweden for the collection of semen by artificial vagina at the time of the study. The bulls were brought into the semen collection barn once a week from the age of eight months and were allowed to mount teaser animals. If an ejaculate was obtained, the sample was examined for the presence of spermatozoa. Ejaculates containing $>100 \times 10^6$ spermatozoa/mL were used for other experiments to characterise the semen characteristics of young bulls (Hurri et al., in preparation). Samples with $<100 \times 10^6$ spermatozoa/mL were frozen at -20°C until required for FPLC (Sample I).

Once the ejaculates contained $>500 \times 10^6$ spermatozoa/mL and had 70% normal morphology, they were frozen according to the breeding company's internal protocols. A post-thaw evaluation of sperm quality was carried out and acceptable ejaculates were approved for commercial artificial insemination. Once sufficient semen doses had been collected from an individual bull, he was removed from the collection routine. At this stage, an additional sample of seminal plasma was obtained (Sample II).

Fast protein liquid chromatography

For FPLC, SP was thawed, keeping all samples separate, and aliquots of each sample (350 μL) were mixed with 40 μL of 0.05 M tris buffer and 630 μL H_2O. The mixture was centrifuged at 1000 rpm for 20 min. The supernatant (1000 μL) was decanted into a fresh tube and the protein concentration was measured using a Nanodrop 2000 spectrophotometer (Thermofisher Scientific). The SP proteins were separated on column HiPrep 16/10 Heparin FF, 20 mL (GE Healthcare bio-Sciences AB, Uppsala Sweden). One mL of the sample was injected through a valve with a 1 mL sampling loop. The flow rate was 1 mL/min. The non-heparin-binding proteins (peaks 1, 2 and 3) were eluted with 0.02 M Tris HCl buffer containing 0.156 M HCl, pH 7.5. The phosphorylcholine-binding proteins (peak 4) were eluted with 0.02 M Tris-HCl buffer containing 0.156 M HCl and 0.05 M phosphorylcholine, pH 7.5. The proteins binding to heparin (peak 5) were eluted using 50% of 0.02M Tris-HCL buffer containing 0.156 M HCl. Peak height (mAU), peak areas (mAU*mL) and ratio peak area to total area were recorded.

2.2. Statistical analysis

The data were checked for normality and then subjected to two-way ANOVA (Snedecor and Cochran, 1994), considering bull and age (Sample 1, Sample II) as the two main effects.

3. Results

The median age of first and second samplings was 285 days and 423 days, respectively (range 243-302 days and 377-491 days). The median interval between the two sample collections was 143.5 days, (range 75 to 231 days).

A typical chromatogram contains five peaks (peaks 1, 2 and 3: non-heparin and non-phosphorylcholine-binding proteins; peak 4: phosphorylcholine-binding proteins; and peak 5: heparin binding proteins). The chromatogram from one of the young bulls is shown in Fig. 1, with the corresponding chromatogram from the same bull several weeks later shown in Fig. 2. Peak 3 from each chromatogram is enlarged in Figs. 1b and 2b, to show the detail of this part of the chromatogram.

The peak heights and peak areas of different protein groups in the two sets of samples are presented in Table 1. The peak area and peak height of different proteins did not differ significantly between the very young and young bulls, except in peak 5 (heparin-binding proteins) and total peak area ($p<0.05$). The heparin-binding protein peak height and area were significantly higher ($p<0.05$) in sample II than in Sample I. However, none of the other parameters showed statistical differences between groups. Peak 5 area (Fig. 3a) and height (Fig. 3b) for samples I and II are shown for individual bulls.

4. Discussion

Very few studies have been done in young bulls but the results of the present study are in overall agreement with those of (Vince et al., 2018) who showed that young bulls had less albumin in their seminal plasma than older bulls, particularly during warm periods. Insulin levels in seminal plasma of peripubertal Zebu bulls were associated with proportion of abnormal spermatozoa (Souza et al., 2012). It was not possible to evaluate sperm morphology in our study since Sample I for each bull was oligozoospermic or azoospermic due to their young age. However, other samples from the same bulls taken for another study, when sperm concentration was 100-500 $\times 10^6$/ml, showed that there was a high proportion of abnormal spermatozoa and retained proximal cytoplasmic droplets in the initial ejaculates from each bull, which improved with age (Hurri, et al., in preparation). There are few other studies on seminal plasma profiles in young bulls. The findings of (Souza et al., 2012) in Zebu bulls are in agreement with studies on semen from adult human patients, where low levels of insulin in seminal plasma were associated with abnormal morphology (Baccetti et al., 2002).

In a study on heparin-binding proteins in ram seminal plasma, clusterin, Bodhesin 2, matrix metalloproteinase 2, beta mannosidase and dentin matrix acidic phosphoprotein 1 were identified, among others (Martins et al., 2013). Clusterin has been negatively associated with protamine deficiency, sperm DNA fragmentation, and abnormal morphology (Salehi et al., 2013). Therefore, it could be speculated that the increase in heparin-binding proteins in the samples at the second time point in the present study (Sample II) might be due to an increase in clusterin (or, indeed, other similar proteins), associated with better sperm morphology and motility as bull age increases. It would be interesting to see if the levels of heparin-binding proteins are associated with the type of spermiogram desirable in a fertile adult bull.

The dominant phosphorylcholine-binding protein in bovine seminal plasma is the so-called PDC-109 (Protein with N-terminus aspartic acid, D, and carboxy terminus Cystine, having 109 amino acids) (Calvete et al., 1996; Gasset et al., 1997). Since this protein induces cholesterol efflux, it is essential for capacitation and the acrosome reaction, and is therefore crucial for fertilization (Anbazhagan & Swamy, 2005). This protein also has chaperone- activity, protecting other proteins from degradation (Sankhala & Swarmy, 2010). Future studies should focus on whether the levels of this protein change during maturation.

Regarding the association between seminal plasma proteins and sperm morphology, a study on the proteins in human semen samples (Sharma et al., 2013) grouped them according to sperm count and

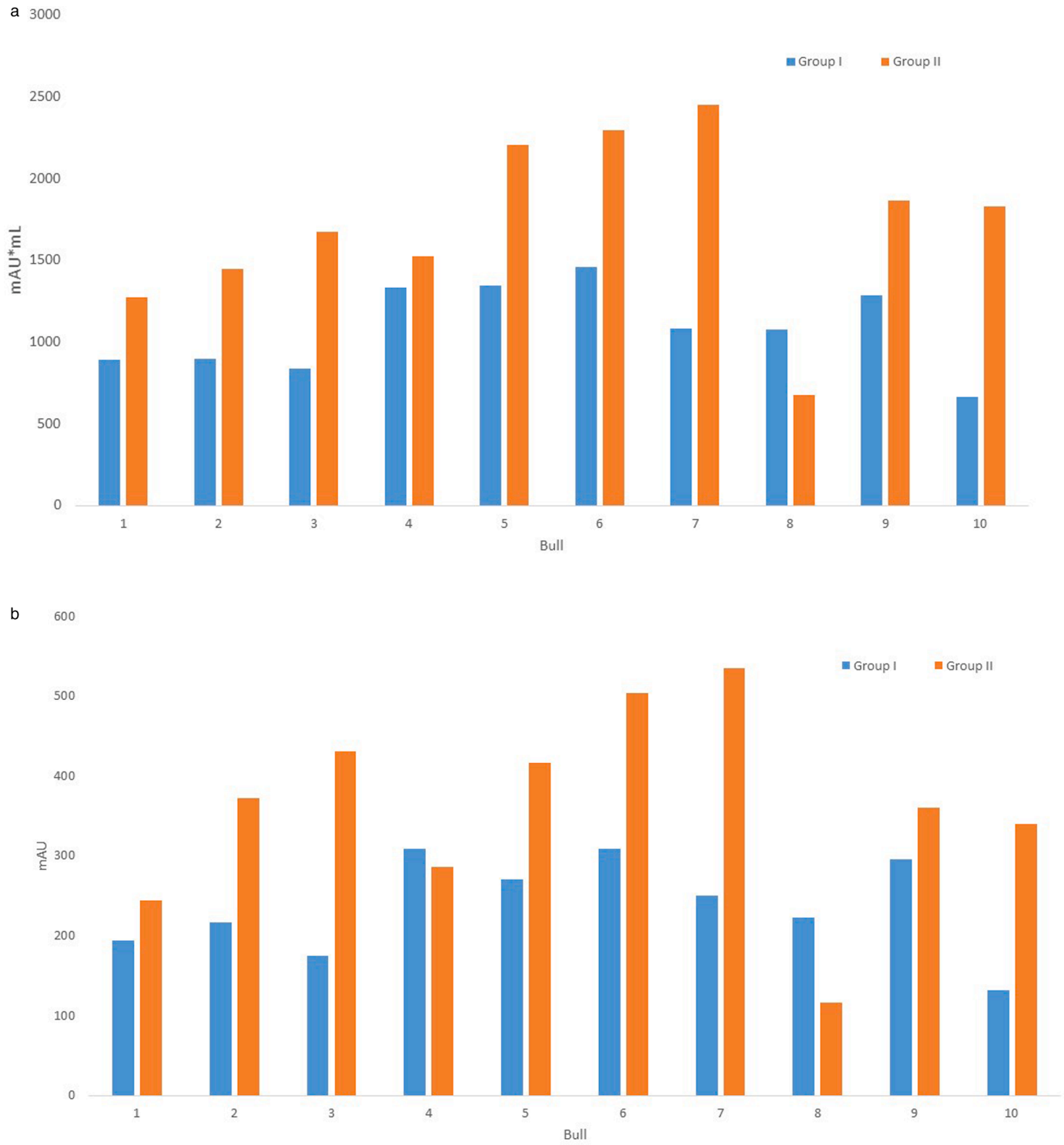

Fig. 3. Differences between bulls in characteristics of Peak 5: a) Peak Area; b) Peak Height. Note: a) difference among bulls in Group I; $p<0.003$: Difference among bulls in Group II, Not Significant. Note: b) difference among bulls in Group I; $p<0.012$: Difference among bulls in Group II, Not Significant.

morphology (normal sperm count and normal morphology; normal sperm count and abnormal morphology; oligozoospermia and normal morphology; oligozoospermia and abnormal morphology). Some proteins were found to be under-expressed whereas others were over-expressed in the groups containing spermatozoa with abnormal morphology. Eleven proteins were found that were common to all groups, and 20 that were differentially expressed among the groups. Among the latter, 5 proteins were down-regulated in the abnormal groups (neither normal sperm count nor normal morphology) and 2 were up-regulated. Semenogelin I isoform b preprotein was up-regulated in the oligospermia + abnormal morphology group; this protein may contribute to low sperm motility (Sharma et al., 2013). Clusterin was down-regulated in the oligozoospermic normal morphology group. However, these findings may not be directly comparable with bull seminal proteins since there are considerable differences between species, e.g. human seminal plasma did not contain phosphorylcholine-binding proteins, unlike bull and boar seminal plasma (Liberda et al., 2002).

In conclusion, heparin-binding proteins are low in the seminal plasma of peripubertal young bulls and are higher several weeks later, when post-thaw sperm quality is suitable for commercial semen doses for AI.Therefore, the level of heparin-binding proteins might serve as a biomarker of sperm maturity in these animals.

5. Implications

Breeding companies want to use semen from young bulls as soon as possible but there is considerable variation in the age of bull at which spermatozoa will survive freezing and be capable of fertilization after artificial insemination. Seminal plasma proteins protect spermatozoa during freezing. We examined the proportions of different types of fertility-associated seminal plasma proteins in semen from very young bulls and again several weeks later when their semen was being frozen for artificial insemination. The proportion of heparin-binding proteins had increased significantly in all bulls between the two sampling points. Therefore, these proteins could serve as an indicator of maturity in young bulls.

Author statement

Sourabh Deori, Conceptualisation, formal analysis, investigation, writing editing, visualisation, funding acquisition. Emma Hurri, Investigation, writing editing. Saeid Karkehabadi, Methodology, formal analysis, resources, writing editing, visualisation. Jane Morrell, Conceptualisation, methodology, resources, writing original draft, supervision, project administration, funding acquisition.

Author contributions

JMM and EH designed the study. EH collected the samples, SD performed the FPLC under the supervision of KS; SD and KS interpreted the data. SD performed the statistical analysis. SD and JMM drafted the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

EH was an employee of VikingGenetics at the time of the study. None of the other authors have any competing interests to declare.

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References

Anbazhagan, V., Swamy, M.J., 2005. Thermodynamics of phosphorylcholine and lysophosphatidylcholine binding to the major protein of bovine seminal plasma, PDC-109. FEBS Lett. 579, 2933–2938.

Baccetti, B., La Marca, A., Piombioni, P., Capitani, S., Bruni, E., Petraglai, F., de Leo, V., 2002. Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. Hum. Reprod. 17, 2673–2677.

Calvete, J.J., Varela, P.F., Sanz, L., Romero, A., Mann, K., Topfer-Petterson, E., 1996. A procedure for the large-scale isolation of major bovine seminal plasma proteins. Protein Expres. Purif. 8, 48–56.

Druart, X., de Graaf, S., 2018. Seminal plasma proteomes and sperm fertility. Anim. Reprod. Sci. 194, 33–40.

Gasset, M., Saiz, J.L., Laynez, J., Sanz, L., Gentze, M., Töpfer-Petersen, E., Calvete, J.J., 1997. Conformational features and thermal stability of bovine seminal plasma protein PDC-109 oligomers and phosphorylcholine-bound complexes. Eur. J. Biochem. 250, 735–744.

Johannisson, A., Al-Essawe, E., Al-Saffar, A.Kh., Karehabadim, S., Lima-Verde, I., Wulf, M., Aurich, C., Morrell, J.M, 2020. Season does not have a deleterious effect on proportions of stallion seminal plasma proteins. Reprod. Devel. 66, 215–221.

Karabinus, D., Evenson, D.P., Jost, L.K., Baer, R.K., Kaproth, M.T., 1990. Comparison of Semen Quality in Young and Mature Holstein Bulls Measured by Light Microscopy and Flow Cytometry. J Dairy Sci 73, 2364–2371.

Liberda, J., Maňásková, P., Švesták, M., Jonákova, V., Tichá, M., 2002. Immobilization of L-glyceryl phosphorylcholine: isolation of phosphorylcholine-binding proteins from seminal plasma. Journal of Chromatography B 770, 101–110.

Lima-Verde, I., Nongbua, T., Karkehabadi, S., Johannisson, A., Morrell, J.M., 2020. Effect of season on bovine seminal plasma proteins in Thailand. J. Therm. Biol. 90, 102576. doi.org/10.1016/j.jtherbio.2020.102576.

Martins, J.A.M., Souza, C.E.A., Silva, F.D.A., Cadavid, V.G., Nogueira, F.C., Domont, G. B., de Oliveira, J.T.A., Moura, A.A., 2013. Major heparin-binding proteins of the seminal plasma from Morada Nova rams. Small Rum. Res. 113, 115–127.

Salehi, M., Akbari, H., Heidari, M.H., Molouki, A., Murulitharan, K., Moeini, H., Novin, M.G., Aabed, F., Taheri, H., Fadaei, F., Mohsenzadeh, M., Jafari, M., Pirouzi, A., Heidari, R., 2013. Correlation between human clusterin in seminal plasma with sperm protamine deficiency and DNA fragmentation. Mol. Reprod. Dev. 80, 718–724.

Sankhala, R.S., Swarmy, M.J., 2010. The major protein of bovine seminal plasma, pDC-109, is a molecular chaperone. Biochemistry 49, 3908–3918. https://doi.org/10.1021/bi100051d.

Sharma, R., Agarwal, A., Mohanty, G., Jesudasan, R., Goplan, B., Willard, B., Yadav, S.P., Sabanegh, E., 2013. Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. Reprod. Biol. Endocrin. 11, 38–57.

Snedecor, G.W., Cochran, W.G., 1994. Statistical Methods. Oxford and IBH Publishing Company, New Delhi.

Souza, F.A., Martins, J.A.M., do Vale Filho, V.R., de Andrare, V.J., Brandao, M., Ferreira, D., Emerick, L.L., Pinot, P.F.B., Leite, T.G., 2012. Leptin and insulin in the seminal plasma of zebu bulls in peripuberty. R. Bras. Zootec. 41, 923–928.

Vince, S., Žura Zaja, I., Samardžija, M., Majić Balić, I., Vilić, M., Đuričić, D., Valpotić, H., Marković, F., Milinković-Tur, S., 2018. Age-related differences of semen quality, seminal plasma, and spermatozoa antioxidative and oxidative stress variables in bulls during cold and warm periods of the year. Animal 12, 559–568.