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Original article

COMPARATIVE EVALUATION OF SEMINAL PLASMA PROTEINS IN HOLSTEINER AND EAST BULGARIAN HORSE BREEDS IN RELATION TO FUNCTIONAL PARAMETERS OF SPERMATOZOA

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Summary

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The present research was focused on the differentiation of specific proteins in the seminal plasma (SP) of two horse breeds – Holsteiner (n=4) and East Bulgarian (n=4) and their relation with individual or breed characteristics, kinematic parameters of spermatozoa and the sperm head area. After CASA analysis of 8 ejaculates, no statistical differences in the kinematic parameters of the sperms between the two horse breeds were found out with the exception of the sperm head area ($P<0.05$), which can be considered as a morphometric marker of breed affiliation. The values for rapid sperm in East Bulgarian and Holsteiners were 28.1±0.2 μm^2 and 19.9±0.3 μm^2 respectively. The chromatographic analysis demonstrated specific quantitative and qualitative protein content of the individual chromatographic peaks (11 for Holsteiner and 15 for the Eastern Bulgarian breed), with similarity to the basic proteins. Three specific proteins with a molecular mass of 76 kDa, 21.6 kDa and 24.3 kDa, were differentiated by SDS PAGE in the Holsteiner breed, whereas in the Eastern Bulgarian horse breed they had a lower protein mass – 30.1 kDa and 14.2 kDa and 12.6 kDa. In conclusion, differences in the specific protein profile of Holsteiner and Eastern Bulgarian horse breeds are individually and naturally determined without significant effect on sperm kinematics. The sperm head area was a breed-specific difference.

Key words: computer and chromatographic analysis, seminal plasma proteins, stallion semen

INTRODUCTION

Seminal plasma (SP) is the liquid phase in the ejaculate, secreted by the epididymis and the additional sexual glands just before and during ejaculation. It is composed of wide range of compounds, such as proteins, ions, amino acids, monosac-

charides, lipids, polyamines, prostaglandins and steroid hormones. The main function of the SP is to stimulate the movement of sperm cells through the stallion's genital tract during ejaculation. Other functions of the SP include transport, protection, and nutrition of the sperm cells in the female genital tract. Some of the SP factors regulate the energy capacity of the sperms; participate in the acrosome reaction and the interaction between gametes (Töpfer-Petersen *et al.,* 2005). In the recent years, the attention of scientists has been focused on the analysis of seminal plasma proteins and their role in sperm vitality and function in the reproductive process (Rodriges-Martinez *et al*., 2011). It is well known that in stallions, SPP has some similarity with the content of the major protein fractions (Fn-2, CRISP and spermadhesins) with other animal species, but there are still many unexplored breed and individual differences. There are data for horses that some factors in the SP affect the motility, survival and fertility of sperm (Portus *et al.,* 2005). Initially, SP proteins are described as horse sperm plasma proteins (HSP), e.g. HSP-1 to HSP-8. These proteins are predominantly with low molecular weight (14–30 kDa). They are thought to form multi-protein aggregates (without HSP-4) which are attached to the surface of the plasma membrane (PM) of the sperm (Töpfer-Petersen *et al.,* 2005). Two of the major proteins – heparin-binding HSP-1 and HSP-2 – have been shown to account for 70–80% of the total protein in the SP. It is believed that these proteins are the main modulators of capacitation process. Both HSP-1 and HSP-2 (also called SP-1 and SP-2) are short Fn-2 proteins and are similar to the major bovine heparin-binding proteins. They all were suggested to be connected with capacitation (Töpfer-Petersen *et al.,* 2005) and their action is based on the binding of these proteins to phospholipids – phosphatidylcholine or sphingomyelin on the surface of PM of the ejaculated sperm, which resulted in structural changes in PM and sperm behaviour (Greube *et al.,* 2004; Ekhtasi-Hundrieser *et al.,* 2005). There is evidence that HSP-3 protein (or horse CRISP-3) related with gamete's fertilising potential (Hamann *et al.,* 2007). Some authors consider that this protein is a selective protector against polymorphonuclear cell binding (Troedson *et al.,* 2010). Conducted experiments reported that the first semen fractions contain an acrosin and PSA inhibitor and are similar to kallikrein proteins (such as HSP-6 and HSP-8, which are isoforms), but it has been shown that HSPs are present in all semen fractions. HSP-1 is thought to be a major protein presented in all semen fractions (Kareskoski *et al*., 2011). HSP-7 is the only member of the spermhedins family and similar to its homologue AWN-1, which shows zona pellucida-binding activity (Reinert *et al.,* 1996). Studies in horses reveal that one of the proteins associated with fertility and determining a high or low fertilising ability of sperm is osteopontin. The higher concentrations of three proteins (SP-2, SP-3 and SP-4) in stallion SP have been found to result in low fertility of sperm. In this regard some authors suggest that SP-1 positively correlates with fertility and is a homologue to osteopontin (Kilian *et al.,* 1993; Brandon *et al.,* 1999).

There is currently conflicting information on the presence of seminal plasma proteins and their relationship with sperm motility and fertility in horses. It is not clear whether there is any connection between the presence of some seminal plasma proteins and the breed or whether

these proteins are associated with individual characteristics. Also, there is no information on the presence of breed-specific proteins in seminal plasma in Holsteiner and Eastern Bulgarian horse breeds in Bulgaria. In this connection our efforts were focused to analyse species-specific proteins in the seminal plasma of Holsteiner and East Bulgarian horse breeds and the evaluate their relationship to individual or breed characteristics affecting motility, progressive motility, speed parameters and the sperm head area.

MATERIALS AND METHODS

Animals

For the purpose of the experiment, 8 ejaculates of Holsteiner (n=4) and East Bulgarian (n=4) stallions were obtained and evaluated. Horses were owned by private farms, aged 17 years. Ejaculates were obtained from December 2018 to April 2019. An artificial vagina was used to obtain the semen. Each ejaculate was collected in double-walled collecting cups. The experiment has been done with whole ejaculates, extended with Minitube Equi Plus, 100 mL.

Computer-assisted sperm analysis (CASA)

Semen was analysed by the CASA System Sperm Class Analyzer® (Microptic®, Spain), with software analytical module "Motility and concentration". The investigations were carried out using 8 μL semen volume and 18 × 18 mm cover glass was carefully placed on semen sample on the slide. The analysis was performed on a minimum 1000 spermatozoa in 5 fields. The CASA parameters assessed in this study were: sperm concentration ($\times 10^6$/mL); motility and progressive movement of spermatozoa (%) (static, progressive, non-progressive); sperm velocity (%) (fast, medium and slow velocity); sperm head area (μm^2) – (total and in individual sperm populations: static, slow, medium and fast); kinematic parameters (total and in individual sperm populations: static, slow, medium and fast): VCL – curvilinear velocity (μm/s) – mean velocity of the sperm head on the actual route; VSL – straight line velocity (μm/s) – the speed along the straight line connecting the starting point and the end point of the travelled way; VAP – average path velocity (μm/s) – the average velocity of the sperm head in an averaged trajectory; LIN – linearity (%); LIN=(VSL/VCL)×100; STR – straightness (%); STR=(VSL/VAP)×100; WOB – wobble (%); WOB=(VAP/VCL)×100; ALH (μm) – the amplitude of the lateral variation of the sperm head (generally and in the populations of moderately progressive and fast progressive sperm); BCF – beat/cross frequency (Hz) – the frequency with which the sperm head crosses the median plane of the upright trajectory (generally in the populations of moderately progressive and fast progressive sperm).

Seminal plasma proteins' chromatography separation

Separated seminal plasma was centrifuged twice at 2000 rpm for 10 min at 4° C and again at 12,000 rpm for 5 min. The supernatant was filtered through a 0.22 μm membrane (Milipore) and stored at −80 °C for subsequent analyses.

The separation and analysis of the proteins from the SP was performed by a Binary HPLC Pump 1525 chromatographic system with a UV/VIS detector 2489 (Waters Company®) with simultaneous reading at two wavelengths (λ=220 nm and λ=280 nm). TSK gel® Size Exclusion SW-Type HPLC column, 30 mm × 7.5

mm, 10 μm particle size, and 10 to 500 kDa was used. The protein marker was Protein Standard Mix 15–600 kDa (Sigma-Aldrich®). The assay was performed on all SP of the obtained ejaculates using an injection volume of 50 μL, containing 1 μg of protein per injection. The run time of the sample was 20 min at 1.2 mL/min flow rate. Chromatograms containing the protein profiles of the SP were obtained.

Protein content determination

The total protein concentration in the SP was measured spectrophotometrically by Ultrospec 2000 UV/VIS Spectrophotometer, Pharmacia Biotech®. Each assayed SP sample was diluted 1:100 with PBS, equilibrated against PBS. Analysis of protein samples was performed at λ=280.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The characterisation of the proteins was made by electrophoresis under reducing conditions using TV 100 (Bio-Rad®) apparatus with 10×10 cm glasses. Seminal plasma samples were diluted 1:3 with 4× Laemmli Sample buffer (Bio-Rad®) supplemented with 10% β-mercaptoethanol and heated for 1 min at 100 °C for establishing the reducing conditions. From each sample, 40 μL with protein concentration of 5 μg in the pocket were used. Electrophoretic separation initially started at a voltage of 80 V which after entering in the separating gel was increased to 120 V. SDS-PAGE was done at 12% and 15% gels to provide good separation of bands of high and low molecular proteins. Following the electrophoresis, the gels were stained with Coomasy Brilliant Blue for the visualisation of the protein bands. The exact molecular weight determination was made with Sigma Marker™ Wide Range 6,500–200,000 Da (Sigma-Aldrich®) and a standard molecular weight curve – measured according to the start.

Statistical analysis

Statistical analyses were performed on MS Excel and through the CASA software product. All data are presented as means ± standard deviation. Statistical significance was determined by Student's *t*-test.

RESULTS

The ejaculate volumes of the two horse breeds varied from 40.00±15.3 to 75.0±18.77 mL. The sperm concentration was from 148.9×10^6 to 324.6×10^6 sperm cells/mL. There were no statistically significant differences between the parameters of the two horses, but specific tendencies, depending on the breed and the individuals were shown.

CASA

CASA data showed a specific profile of the main sperm parameters in both breeds of horses. The analysis of sperm progression showed statistically significant differences in the percentage of rapid sperm whose values were higher in the Eastern Bulgarian breed ($P<0.05$), as well as higher values for medium fast sperm which were in favour of Holsteiners ($P<0.05$, Fig. 1).

The analysis of the kinematic parameters showed specific trends without relevant differences between Holsteiner and East Bulgarian horses (Fig. 2).

An important result was the analysis of the head area of the spermatozoa in relation to the individual populations studied – static, slow, medium and rapid. There were statistically highly significant differences between the East Bulgarian and Holsteiner horse breeds for medium and rapid sperm head area values ($P<0.001$).

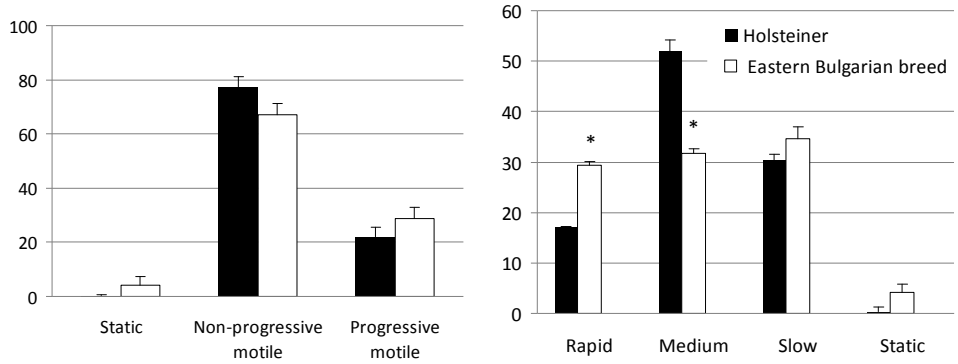

Fig. 1. Comparative analysis of sperm motility and progression (%) in Holsteiner and East Bulgarian horse breed (mean±SD); * P<0.05 between breeds.

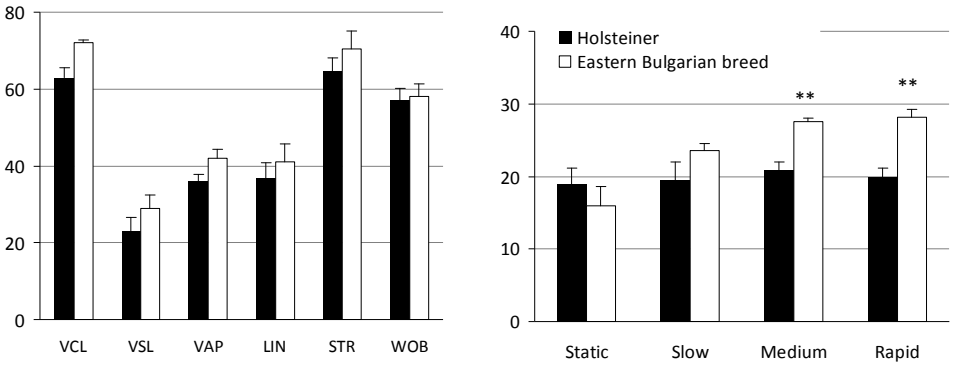

Fig. 2. Comparative analysis of kinematic parameters (VCL: curvilinear velocity (μm/s); VSL: straight line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity (%); STR: straightness (%); WOB: wobble (%)) and sperm head area of static, slow, medium and fast spermatozoa (μm^2) of Holsteiner and East Bulgarian horse breeds (mean±SD); ** P<0.001 between breeds.

HPLC and SDS-PAGE

The results of the spectrophotometric analysis of total protein SP content indicated differences with specific trends in horses of both breeds. In Holsteiner, the proteins present in the SP were 25.44±0.76 g/L, while for the Eastern Bulgarian breed – 29.12±0.36 g/L.

Chromatographic analysis of the content and quantity of seminal plasma proteins in both breeds of horses showed differences and specificities. Chromatograms demonstrated a different number of protein peaks – 11 for the Holsteinier breed and 15 for the Eastern Bulgarian breed under the same analytical conditions. The molecular mass of the proteins in the separate peaks ranged from 670 kDa to 137.14 Da, according to the protein marker (Fig. 3, 4 and 5). Both breeds of horses had proteins with high molecular weight. These were the proteins in the first fractions separated between the the 4^{th} and 6^{th} minutes.

It should be noted that in both chromatograms, there were proteins present at a high proportion in the Holsteinier breed

but in a little one in East Bulgarian horses and vice versa. Such proteins were contained in the peaks separated between the 6th and 9th min, corresponding to the molecular masses of gamma-globulin and ovalbumin. In Holsteiners, there were no peaks with a molecular weight of 150 kDa and 44.3 kDa while in East Bulgarian horses there was a protein peak with a molecular weight 44.3 kDa.

In the evaluation of the peaks containing proteins of lower molecular weight, it was found that in the seminal plasma of all tested horses contained proteins with almost the same quantity and quality. It should also be noted that in both breeds of horses there were protein fractions containing a wide range of unidentified proteins – they were separated between the 9th and 11th minutes.

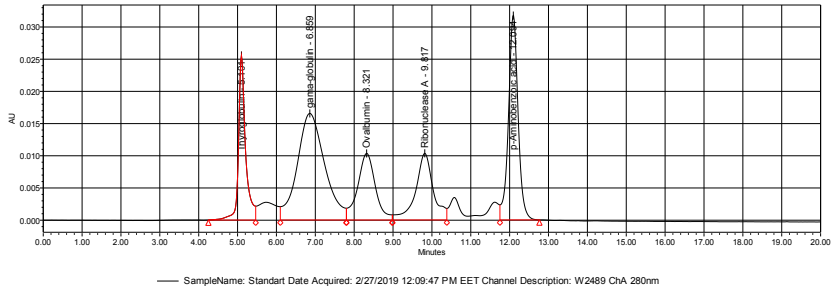

Fig. 3. Standard curve of the protein marker for protein assay by HPLC.

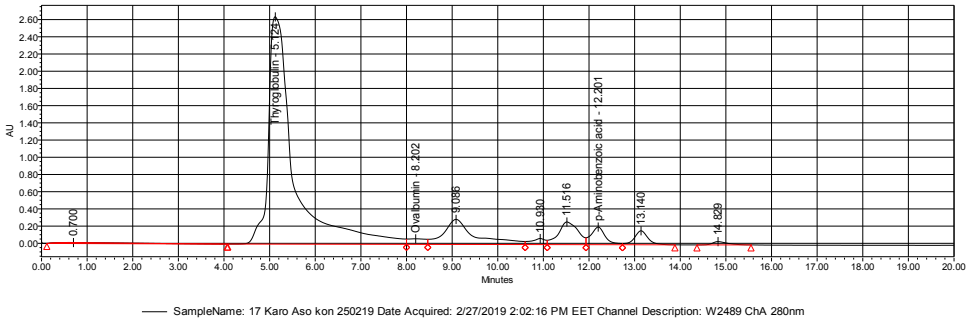

Fig. 4. Chromatogram of seminal plasma proteins of the Holsteiner horse breed.

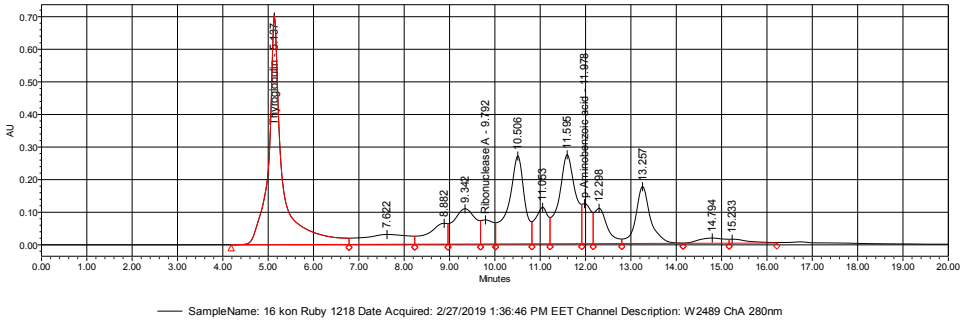

Fig. 5. Chromatogram of seminal plasma proteins of East Bulgarian horse breed.

Table 1. Relative quantity of the main seminal plasma proteins of investigated horses, calculated on the base of peak height and the HPLC protein marker. The lowest value is accepted as (+), missing marker as (–), on the base of 100%.

Peaks	Elution time (min)	Molecular weight	Holsteiner horse breed	East Bulgarian horse breed
Thyroglobulin	5.101	670 kDa	+++++	+++
Gamma-globulin	6.859	150 kDa	–	–
Ovalbumin	8.321	44.3 kDa	++	–
Ribonuclease A	9.817	13.7 kDa	–	+
p-aminobenzoic acid	12.094	137.14 Da	++	++

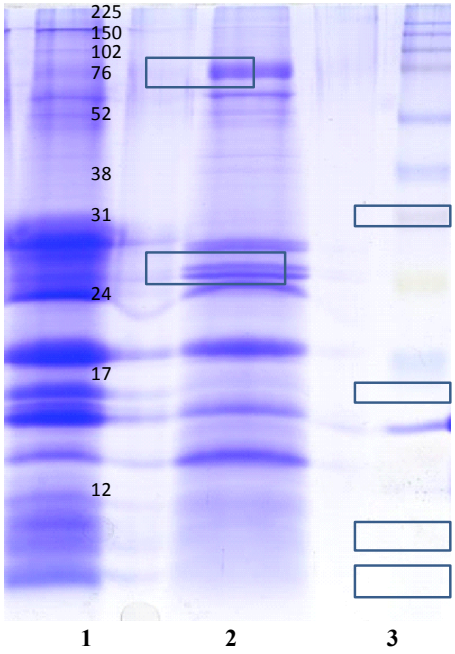

Fig. 6. SDS PAGE electrophoretic assay of seminal plasma, 15% gel; 1–protein marker, 2–Holsteiner breed, 3–East Bulgarian breed.

The data obtained from the HPLC analysis showed that the seminal plasma of the two studied horse breeds contained similar basic proteins from qualitative and quantitative point of view, which were characteristic and typical of the equine species. At the same time, our results demonstrated convincing presence or absence of proteins, as evidenced by the individual chromatograms of each tested subject (Table 1).

The SDS-PAGE analysis (Fig. 6), allowed identifying about 29 proteins with a molecular weight range of 225 to 12 kDa in the two horse breeds. These proteins corresponded to the protein peaks registered by HPLC, separated between minutes 6–12.

Obviously, most of the proteins were identical or similar for both breeds. At the same time, there were clearly distinct groups of specific proteins, present only in the ejaculates of a given breed. The confirmation of the HPLC results by SDS PAGE analysis revealed a great diversity in some proteins, creating specific protein profiles due to quantitative presentation, absence or presence of individual groups of proteins. For the Holsteiner breed, there were 3 proteins that can be assumed as specific in this breed – the proteins with a molecular weight of about 76 kDa, present at a high percentage, and 21.6 kDa and 24.2 kDa proteins. For East Bulgarian horse breed, specific proteins were those with a molecular weight of 30.1 kDa and 14.2 kDa, as well as low molecular weight proteins with a molecular weight of 12.6 kDa and lower. These proteins were almost lacking in Holsteiners (Fig. 6, Table 2).

Table 2. Specific proteins present in the seminal plasma of Holsteiner and East Bulgarian horse breeds presented on a base of standard curve and visualisation of the electrophoretic bands.

Horse breed	Specific proteins	Highly prevalent proteins	Less prevalent or missing proteins
Holsteiner	76 kDa, 24.2 kDa, 21.6 kDa	65.1 kDa, 20.6 kDa, 27.0 kDa, 14.2 kDa	26–52 kDa, 15–17 kDa, <12 kDa
East Bulgarian	30.1 kDa, 14.2 kDa, 12.6 kDa, 7–9 kDa	150 kDa, 65.1 kDa, 20.6 kDa 27.0 kDa, 14.2 kDa	31–52 kDa, 15–16 kDa

DISCUSSION

The present CASA studies showed no distinctive and significant differences in the basic parameters of sperm in the two studied breeds of horses – Holsteiner and Eastern Bulgarian breed. Novel and interesting data were obtained for the sperm head area. We believe that these data can be used as a morphometric marker for the breed. The chromatographic analysis for the separation of seminal plasma proteins by molecular weight resulted in separation of 15 protein peaks for the Eastern Bulgarian breed and 11 protein peaks for the Holsteiner breed. The presumed presence and characterisation of SP proteins for comparison of data for the two breeds was made by SDS-PAGE. This method has been demonstrated four proteins, specific to the Eastern Bulgarian breed that were not present in the Holsteiner SP. For Holsteiner breed, 3 specific proteins were differentiated.

When comparing our results with those of other authors, there were some variations in spermograms due to the season, volume of the ejaculate that may affect the sperm concentration and the total number of normal and functional spermatozoa (Heckenbichler *et al.,* 2010). We assume that variations in the composition and amount of certain proteins from the SP may also occur (Abou-Ahmed *et al.,* 1993). The assertions by some authors that the main SPPs were detected in ejaculates with good or low quality parameters without any difference in the number of peaks in a RP-HPLC analysis (Koskinen *et al.,* 2002) confirms our HPLC analysis data. The chromatographic analysis of the SP proteins showed that the content and the quantity of proteins in the two breeds of horses had differences and specificities, but the major groups of SP proteins were identical. The variations of proteins with different molecular weights in the SP, found in the current SDS-PAGE studies, which were specific to the individuals studied, were attributed by other authors to the origin of the SP in the formation of the ejaculate (Weber & Woods, 1993; Magistrini *et al*., 2000, Ekholmi-Hundrieser *et al.,* 2005). At the same time there is a deficiency of data, whether such specific differences in seminal plasma protein profile may be related to the breed affiliation. We suggest that the difference in the quantity of certain proteins may be related to the age, habitat and feeding of the horses, ejaculation regime, season, and

other factors, but we also assume that the presence of specific proteins was probably a trait of the breed. These data give us reason to assert that the specific proteins found in this study may be an individual breed characteristic. The proteins identified in this study, did not exist in the Proteome-*pl*: Proteome Isoelectric Point Database and they were marked as uncharacterised and new. In this case there is a need on their further characterisation by 2D and mass spectrometry.

The results of the current studies on the specificity of the SP proteins and CASA of two horse breeds allowed concluding that:

The data on motility, progression and kinematic parameters in both tested breeds did not show statistically significant differences, which suggests that regardless of breed and individual differences, spermatological parameters were similar. The only important difference was found in the sperm head area, which can serve as a morphometric breed marker.

The major seminal plasma proteins that are characteristic for horses had a substantial quantitative and qualitative similarity, determined by chromatographic analysis and confirmed by SDS-PAGE studies. The specific individual horse protein profiles determined by HPLC included 11 protein peaks in the Holsteiner breed, and 15 peaks in East Bulgarian breed.

Three specific proteins (76 kDa, 21.6 kDa, and 24.3 kDa) were differentiated for the Holsteiner horse breed. Proteins with molecular weight 30.1 kDa, 14.2 kDa, 12.6 kDa and proteins with molecular weight lower than 12 kDa, specific for the East Bulgarian horse breed, were identified.

The established breed-specific differences in the protein profile of seminal plasma of Holsteiner and East Bulgarian horses, had no significant effect on the basic sperm parameters – volume, sperm concentration, protein content of seminal plasma, motility and kinematic parameters. A specific morphometric marker for the breed was the sperm head area, the values for rapid sperm in East Bulgarian horse and Holsteiner were 28.1 ± 0.2 μm^2 and 19.9 ± 0.3 μm^2 respectively.

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