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Original article

EFFECT OF DIFFERENT BREEDS ON THE PROTEIN PROFILE IN RAM SEMINAL PLASMA

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Summary

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Summary

In this study the individual profiles of seminal plasma proteins (SPP) in rams of three breeds – Merino, Pleven Blackhead and Ile de France – were analysed. The study was carried out with three rams at 3, 6 and 10 years of age, grown and fed under similar conditions. Eighteen ejaculates (6 ejaculates from each ram) were evaluated by Sperm Class Analyzer. The total SPP concentration was measured spectrophotometrically. The separation and characterisation of SPP was performed by HPLC and one dimensional polyacrylamide gel electrophoresis (SDS-PAGE). There were no significant differences between the characteristics of ejaculates and the main kinematic parameters of the sperm in the breeds studied. Chromatograms showed specific profiles with 9, 10 and 11 protein peaks for Merino, Pleven Blackhead and Ile de France breeds, respectively. The total SPP concentration was the highest in the Pleven Blackhead breed and the lowest in Ile de France breed. The major parts of SPP in the three breeds were identical. The seminal plasma of Merino breed contained proteins with molecular mass of 30.3 kDa, 15.7 kDa and 15.2 kDa that were not present in the other two breeds. In the Ile de France and Pleven Blackhead samples only, two proteins with molecular masses of 39.7 kDa and 21.1 kDa, were observed. In conclusion, the detection of specific proteins can be used as a biological marker for sheep breed identification.

Key words: ram, semen parameters, seminal plasma proteins

INTRODUCTION

Seminal plasma (SP) is a product of the accessory sex glands containing wide variety of biological and biochemical components (Druart & de Graaf, 2018). Despite numerous investigations many questions related to the physiological role of the seminal plasma and its effect on the sperm fertilising ability are still unclear

(Bergeron *et al.,* 2005; Bedford, 2015). Epididymal spermatozoa without influence of SP factors can fertilise ova *in vitro* and *in vivo* (Davis *et al.,* 1991; Rickard *et al.,* 2014). At the same time these spermatozoa show low survivability and *in vivo* fertilising capacity, after their deposition at the entrance of the female reproductive tract, because they have to migrate to the infundibulum (Peitz, 1988; Rickard *et al.*, 2014). The seminal plasma proteins (SPP) stimulate sperm motility (Maxwell *et al.*, 2007), regulate their resistance (Manjunath *et al.,* 2008; Muino-Blanco *et al.*, 2008) and influence the survivability of sperm in female reproductive tract (Manjunath *et al.*, 2007; Talevi & Gualtieri, 2010). Conversely, some authors reported a negative effect of SP on spermatozoa motility (Iwamoto *et al.*, 1993; Graham, 1994), viability (Garcia & Graham, 1987) and fertilising ability (Leahy *et al.*, 2010).

In rams, extensive analysis of seminal plasma by gel electrophoresis followed by liquid chromatography-tandem mass spectrometry has shown the presence of more than 700 proteins (Soleilhavoup *et al*., 2014). In accordance with Bernardini *et al.* (2011) the composition of some SPPs is highly conserved among seasonal breeds of rams. However, Asadpour (2012) and Carvajal-Serna *et al*. (2018) have reported variations in the SP protein composition of different ram breeds and suggested that they could be influenced by the season, age and rearing management. The data related to breed and age differences in the composition of SPP are very important for characterisation of the rams. The specificity of the individual SPP profiles may be an informative and safe marker characterising the semen quality at an early age. It allows finding an individual approach for selection of male breeders and ensures an economic efficiency of breeding and animal production.

The aim of the present study was to determine the effect of different breeds and age on the protein profile in ram seminal plasma and its relationship with the main semen characteristics.

MATERIALS AND METHODS

Animals

The study was carried out with three healthy rams at 3, 6 and 10 years of age from Merino, Pleven Blackhead and Ile de France breeds respectively. The animals were housed in individual pens for small ruminants located at N 42.25 and E 25.38 with the same feeding and water intake *at libitum*. All experimental procedures were in accordance with the standard requirements for human attitude and animal protection, which conform to the relevant provisions of Council Directive 86/609/ EEC. The study was conducted in the non-breeding season (February-March 2019).

Semen collection and evaluation

For the purposes of the present study, 6 ejaculates from each ram were collected by the artificial vagina method. After collection the semen was immediately placed in a water bath (37 ^{o}C) and submitted to primary assessment. The volume was determined by graduated pipette and mass motility was evaluated on the base of wave motion (scale 0–5, Evans & Maxwell, 1987). The sperm concentration ($\times 10^9$/mL) was determined by Photometer SpermaCue (Minitübe, Germany), calibrated for small ruminant semen. Only ejaculates with normal colour and transparency, volume >0.5 mL, concentration $>2\times10^9$ /mL, mass motility >3.5 and ab-

normal spermatozoa <20% were evaluated.

After that one part of semen was diluted with protein free semen extender until adjustment of the sperm concentration to 200×10^6 cells per mL, cooled to 4 °C and transported to the laboratory for computer assisted semen analysis (CASA) while the second part was used for SPP evaluation.

CASA was performed by means of System Sperm Class AnalyzerR (MicropticR, Spain) and analytical software module "motility and concentration" determining progressive, non-progressive and static spermatozoa (%); velocity curvilinear – VCL (μm/s); linearity – LIN (%) and head area (μm^2). The vitality (live and dead) and the percentage of abnormal sperm (injured tails – coiled and bent; cytoplasmic droplets) were recorded in semen smears stained with BrightVit kit (Microoptic C.L., Spain) by counting 200 cells in different fields under microscope Olympus (Japan).

Seminal plasma collection and total protein content analysis

The semen was centrifuged at 2000×*g* for 10 min at 4 °C (K24D centrifuge) to separate the seminal plasma and the sperm fraction. The supernatant was centrifuged again at 12000×*g* for 5 min and the SP was recovered and filtered through 0.22 mm Millipore membrane (Millipore®). The samples were kept at – 80 °C for future analyses. The total protein content was determined by Ultraspec 2000 UV/VIS Spectrophotometer (Pharmacia Biotech®) equilibrated for PBS. Each sample was diluted 1:100 with PBS and measured at λ=280.

Chromatographic separation of proteins from seminal plasma

The separation of SPP was performed by Binary HPLC Pump 1525 chromatographic system with UV/VIS detector 2489 (Waters Company®) using two wavelengths: λ = 220 nm and λ = 280 nm. An analytical column (TSK gel® Size Exclusion SW – Type HPLC, 30 mm × 7.5 mm) for 10 μm particle size and resolution of proteins from 10 up to 500 kDa was used. The sample injection volume was 50 μL and contained 1 mg of protein. The optimum system parameters were set to 20 min (time) and 1.2 mL/min (flow rate). Protein Standard Mix 15-600 kDA (Sigma-Aldrich®) was used as protein marker. The obtained chromatograms reflected protein profiles of analysed seminal plasma samples.

One dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for the determination of the molecular weight (MW) and relative content of the main SPP. The analysis was done in the electrophoretic mini cell TV 100 (Bio-Rad®) with glasses 10×10 cm. The SP aliquots were diluted 1:3 in 4× Laemmli Sample buffer (Bio-Rad®) with addition of 10% β-mercaptoethanol. To create reducing conditions (denaturation of the protein molecules) the samples were heated in a water bath for 1 min at 100 °C. A concentration of 5 μg SPP in volume of 40 μL was applied at the starting gel. The electrophoretic separation was run at a constant voltage of 80 V while the samples were in the concentrating gel. After the samples entered the separating gel, the voltage was increased to 120 V. SDS-PAGE was performed on 12% and 15% gels to ensure good separation of the proteins with high (HMW) and low (LMW) molecular

weight. After electrophoresis, the protein bands were visualised by staining with Coomassie blue (Sigma-Aldrich®). The proteins, contained in the bands, were determined vs a standard molecular weight (MW) curve using Sigma Marker™ Wide Range 6,500–200,000 Da (Sigma-Aldrich®).

Statistical analysis

The results were processed by Statistica version 10 software (Stat-Soft., 1984–2000 Inc., Tulsa, OK, USA) and expressed as mean ± standard deviation. Data were tested for normality of distribution and parametric and non-parametric methods were used for comparison of means and proportions. Statistical significance was considered at $P<0.05$.

RESULTS

The results of CASA evaluation of the ejaculates and the main semen characteristics are presented in Table 1. Although the data did not show statistically significant differences, a tendency to decrease in ejaculate volume and sperm concentration in semen collected from Ile de France was established. A wide variation of the basic sperm kinematic parameters in ejaculates of Merino and Ile de France compared to Pleven Blackhead ejaculates (coefficients of variation 19.0±4.5 and 14.2±2.6 vs. 8.7±2.3 respectively, $P<0.05$) was found out.

The data related to the head area of different sperm populations are shown on Fig. 1. All evaluated breeds presented an increase in this parameter from static to fast moving spermatozoa. The head area, calculated on the total sperm population, was significantly ($P<0.05$) higher in Pleven Blackhead breed compared to Merino and Ile de France breeds (Fig. 2).

The number of dead sperm tended to be higher in Pleven Blackhead compared to Ile de France ejaculates, but the difference was not significant. The Ile de France ejaculates had more injured sperm compared to Pleven Blackhead ($P<0.05$). Significant differences in abovementioned parameters between the ejaculates of other rams were not established due to high individual variations. The total percentage

Table 1. Semen characteristics of ejaculates collected from rams of different breeds. Data are presented as mean ± SD (n=6)

Semen characteristics	Merino	Pleven Blackhead	Ile de France
Volume of ejaculate (mL)	0.90±0.35	1.73±0.25	0.76±0.11
Sperm concentration ($\times 10^9$/mL)	5.25±1.9	5.6±2.5	3.8±0.87
Mass motility (0–5)	4.8±0.70	4.2±0.42	4.2±0.71
Sperms with progressive motility (%)	29.9±6.4	29.9±3.1	31.9±4.7
Sperms with non-progressive motility (%)	61.8±4.3	62.8±2.1	61.5±3.6
Static sperms (%)	8.2±2.5	7.3±1.1	6.6±1.1
VCL (μm/s)	27.94±6.2	27.4±2.6	28.84±4.5
LIN (%)	27.54±4.5	28.44±2.5	29.94±3.3
Live sperms (%)	75.3±12.1	67.1±7.4	77.1±5.3
Dead sperms (%)	24.7±11.2	32.9±7.2	22.9±5.2
Sperms with injured tails (%)	3.4±3.9	0.5±0.2	4.9±2.3*
Sperms with cytoplasmic droplets (%)	1.5±0.76	0.3±0.01	4.9±2.7*

VCL: velocity curvilinear; LIN: linearity; * $P<0.05$ compared to Pleven Blackhead samples.

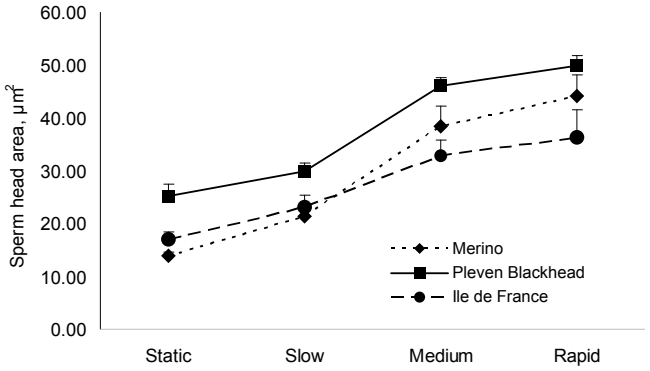


Fig. 1. Changes of the sperm head area according to the spermatozoa motility (static, slow, medium and rapid) in the ejaculates of rams from different breeds. Data are presented as mean ± SD (n=6).

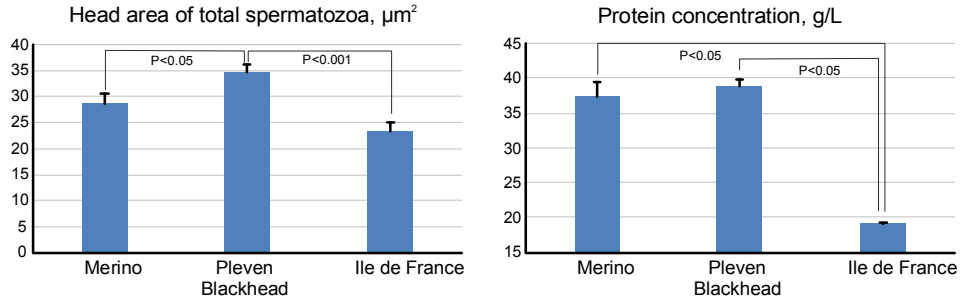


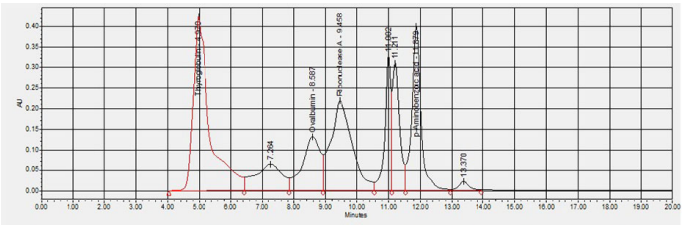
Fig. 2. Head area of all spermatozoa and protein concentration in seminal plasma of rams from different breeds. Data are expressed as mean±SD (n=6).

of spermatozoa with morphological abnormalities (coiled and bent tails and cytoplasmic droplets) in semen of all breeds was within the physiological norm and ranged between 1% and 10%.

The data from spectrophotometric measurement of total protein content in seminal plasma showed the highest value in Pleven Blackhead semen samples and the lowest in these from Ile de France (Fig. 2).

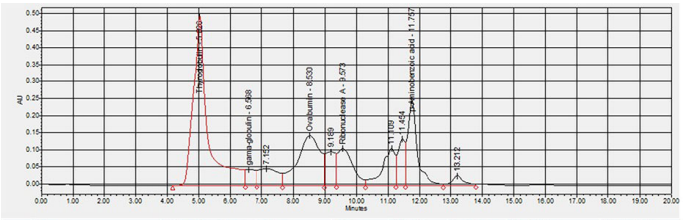
The chromatograms (Fig. 3) reflect the specificity of the protein content in seminal plasma of the individual animals. The samples from the three breeds produced different numbers of protein peaks, including proteins with molecular weights ranging from 670 kDa to 13.7 kDa (Table 2). All ejaculates of Merino, Pleven Blackhead and Ile-de-France breeds showed 9, 10 and 11 protein peaks, respectively. The amount of the same proteins detected in all breeds varied as well (Table 3). The highest quantity of these proteins was recorded in Merino ejaculates, while the lowest one was registered in the ejaculates of Ile de France breed.

The SDS-PAGE method allowed clarifying the molecular weights of proteins presented in the individual peaks that were separated by HPLC. Based on the standard curve for the molecular weight marker, more than 29 SPP bands were differentiated in the seminal plasma of the tested animals (Fig. 4). There were proteins with molecular weight from 12.6 up



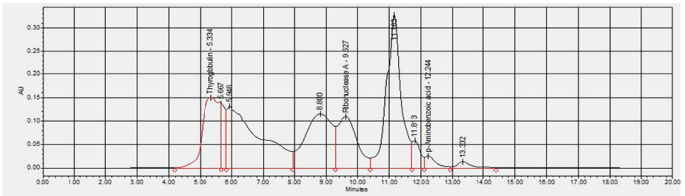
	Name	Retention Time	Area	% Area	Height	Int Type	Peak Type	Peak Codes
5	RIbonuclease A	9.458	10534028	20.00	218146	VV	Found	Q20
1	Thyroglobulin	4.970	15423392	29.28	423028	BV	Found	Q20
2	gama-globulin	6.859				Missing	Missing	
3		7.264	3819801	7.25	64148	VV	Unknown	
4	Ovalbumin	8.587	5139801	9.76	131372	VV	Found	Q20
6		11.002	4277655	8.12	328108	VV	Unknown	
7		11.211	4922742	9.35	309327	VV	Unknown	
8	p-Aminobenzoic acid	11.879	8045745	15.28	399695	VV	Found	Q20
9		13.370	508899	0.97	22162	VV	Unknown	

A



	Name	Retention Time	Area	% Area	Height	Int Type	Peak Type	Peak Codes
6	Ribonuclease A	9.573	3590932	8.35	107358	VV	Found	Q20
1	Thyroglobulin	5.026	15787068	36.70	497359	BV	Found	Q20
2	gama-globulin	6.568	999100	2.32	45868	VV	Found	Q20
3		7.152	2194442	5.10	48834	VV	Unknown	
4	Ovalbumin	8.530	7181681	16.69	145138	VV	Found	Q20
5		9.189	2197333	5.11	98140	VV	Unknown	
7		11.109	3010310	7.00	107941	VV	Unknown	
8		11.454	2104487	4.89	135204	VV	Unknown	
9	p-Aminobenzoic acid	11.757	5269932	12.25	248247	VV	Found	Q20
10		13.212	685667	1.59	28243	VV	Unknown	

B



	Name	Retention Time	Area	% Area	Height	Int Type	Peak Type	Peak Codes
3		5.946	9445675	23.22	132272	VV	Unknown	
1	Thyroglobulin	5.334	5735830	14.10	150038	VV	Found	Q20
2		5.667	1309001	3.22	138018	VV	Unknown	I06
4	Gama-globulin	6.859				Missing	Missing	
5	Ovalbumin	8.321				Missing	Missing	
6		8.800	6854833	16.85	115105	VV	Unknown	
7	Ribonuclease A	9.627	4790360	11.78	109379	VV	Found	Q20
8		11.163	10449313	25.69	327681	VV	Unknown	
9		11.813	1051321	2.58	58520	VV	Unknown	
10	p-Amirobenzoic acid	12.244	611623	1.50	26045	VV	Found	Q20
11		13.332	427900	1.05	13910	VV	Unknown	

C

Fig. 3. Chromatograms of the ejaculates from Merino breed (A), Pleven Blackhead breed (B) and Ile de France breed (C) showing different numbers of protein peaks (9, 10 and 11, respectively).

Table 2. Relative quantity of the main seminal proteins in ejaculates from rams of different breeds, calculated on the base of peak height.

Peaks	Retention time, s	Molecular weight (kDa)	Merino	Pleven Blackhead	Ile de France
Thyroglobulin	5.101	670	2.81	3.31	1
Gamma-globulin	6.859	150	0	1	0
Ovalbumin	8.321	44.3	1	1.1	0
Ribonuclease A	9.817	13.7	2	1	1.02
p-aminobenzoic acid	12.094	137.14	15.3	9.5	1

The lowest value was defined as 1 and missing as zero.

Table 3. Molecular weight of specific seminal plasma proteins in different ram breeds.

Ram breed	Molecular weight of specific proteins	Molecular weight of highly prevalent proteins
Merino	30.3 kDa, 27.4 kDa, 15.7 kDa and 15.2 kDa	< 12 kDa
Pleven Blackhead	39.7 kDa and 21.1 kDa	12.6–15 kDa
Ile de France	39.7 kDa, 21.1 kDa and 27.4 kDa	12.6–15 kDa

to 270.5 kDa as well as proteins with a lower MW (Table 3). The richest protein content was observed in seminal plasma of Merino breed. The data from SDS-PAGE analyses confirmed the findings from HPLC related to the quantitative and qualitative specific of proteins in seminal plasma of rams from different breeds. Most of specific proteins, detected for the Pleven Blackhead breed were similar to those in Ile-de-France breed (Fig. 4). In the seminal plasma of Merino breed three proteins with MW of 30.3 kDa, 15.7 kDa и 15.2 kDa were detected, missing in other two breeds. Also the prevailing proteins differed between breeds (for Pleven Blackhead and Ile-de-France they were from 12.6 kDa up to 15 kDa and for Merino were lower than 12 kDa.

DISCUSSION

The current study is the first report for specific protein profile in seminal plasma of Pleven Blackhead breed and differences compared to Merino and Ile de France breeds. It showed no significant differences between the ejaculates characteristics and the main kinematic parameters of the sperm in the breeds studied. The probable reason for this was the non-breeding season and the uniform rearing technology of the rams, which are considered to be major factors affecting semen parameters (David *et al*., 2007; Benia *et al*., 2018). However, significant ($P<0.05$) differences in the sperm head area in semen collected from the investigated breeds was determined by CASA. This parameter has been extensively investi-

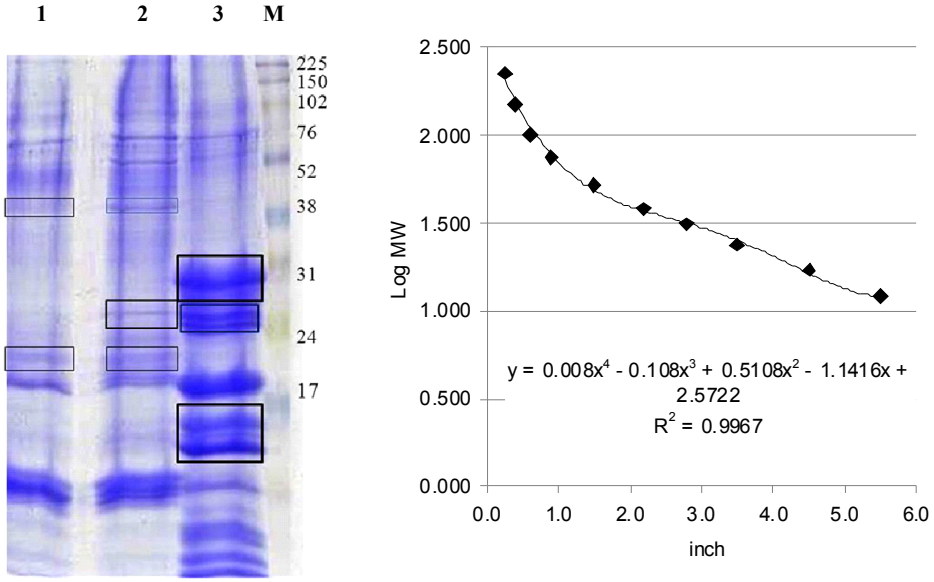


Fig. 4. Electrophoretic profiles (SDS-PAGE) of seminal plasma proteins of the investigated sheep breeds on 12% acrylamide gel stained with Coomassie blue. Breed-specific proteins are marked with rectangles. 1: Pleven Blackhead breed; 2: Ile de France breed; 3: Merino breed; M – protein marker. Right – SDS-PAGE standard curve for calculating MW of protein bands; MW – molecular weight.

gated in the recent years, because it correlated with sperm fertility in different males as horse, boar and dog (Phetudomsinsuk *et al.,* 2008). Regardless of the variations in this parameter between individual ejaculates, our results support the thesis for genetic origin of these differences in rams from different herds (Sancho *et al.,* 1998; Maroto-Morales *et al.,* 2010).

The seminal plasma of rams is extremely protein-rich with around 727 identified proteins with molecular weight ranging from 12 kDa up to 773.6 kDa (Manjunath *et al.,* 2005; Soleilhavoup *et al.,* 2014). Our data obtained by HPLC analysis confirmed the presence of a wide range of proteins with a molecular weight between 670 kDa and 13.7 kDa in seminal plasma of rams from Merino, Pleven Blackhead, and Ile-de-France breeds. Despite of the identity of the major seminal proteins, different numbers of protein peaks were observed with each breed. This study indicated no significant difference in the chromatographic profiles of three breeds, especially in the content of protein peaks separated between the 8th and 10th minutes (13.7 kDa – 44.3 kDa). A large group of these proteins are members of the spermadhesin (Bergeron *et al.,* 2005) and binder of sperm protein (BSP) families (Cardozo *et al.,* 2008). Some of them play important roles in the function and fertility of sperm and even have been suggested as fertility markers (Rickard *et al.* 2015). *In vitro* studies have shown that these proteins have a beneficial effect on sperm motility by stabilising the plasma membrane (Gwathmey *et al.,* 2006). Additionally, SDS-PAGE analyses demonstrated that proteins with a MW of about 12.6–15 kDa were predominant in the seminal plasma of Pleven Blackhead and

Ile-de-France breeds, which confirms the assumption that 45% of the seminal plasma proteins in rams belonged to the spermadhesin group (Bergeron *et al.,* 2005). The RSVP proteins as a part of BSP family are also included in these peaks and they have proven effects on the formation of the sperm reservoir in the female reproductive tract (Souza *et al.,* 2012). For rams, of particular interest in the aforementioned protein peaks is the newly analysed zinc alpha glycoprotein (ZAG) I with a molecular weight of 32.14 kDA. It can improve motility but, at the same time, its prolonged contact with the sperm membrane has a negative effect on the survival ability of spermatozoa (Rickard *et al.,* 2015).

Analyses of protein chromatograms with molecular weights corresponding to gamma-globulin and ovalbumin peaks (150 kDa and 44.3 kDa, respectively) revealed specific patterns of quantitative and qualitative distribution of proteins in examined breeds. Gamma-globulin peak was detected only in the Pleven Blackhead breed and was absent in the other two breeds, while an ovalbumin peak was observed in Merino and Pleven Blackhead semen but was not registered in Ile de France samples. The amounts of the similar proteins for the three breeds have shown a clear tendency to decrease in Ile de France ejaculates. It was in correspondence with the results regarding the total protein content in seminal plasma. The lowest value was observed in the ejaculates collected from Ile de France breed. A number of authors have pointed out that the total protein in seminal plasma differ not only depending on age but also on the breed (Ledesma *et al.,* 2014; Carvajal-Serna *et al.* 2018), environmental and seasonal factors (Marti *et al.,* 2007). However, the obtained results disagree with data related to the total protein content in seminal plasma of bulls showing the higher level in older than young bulls (Vince *et al.,* 2017).

Seminal plasma and its components affect semen quality and have crucial role in the regulation of sperm function (Rodríguez-Martínez *et al.,* 2011). The information about morphology of the spermatozoa in Ile de France indicated that a high percentage of morphologically damaged cells correspond with both low total protein and a reduced amount of individual protein fractions in seminal plasma.

In conclusion, the present investigations by HPLC and SDS-PAGE analysis have shown that the content and quantity of proteins in the seminal plasma of examined three sheep breeds were different and specific regardless of the identity in the content of the major proteins. This study is the first report for determination of specific protein profile in seminal plasma of Pleven Blackhead ejaculates and its difference compared to Merino and Ile de France breeds. The specific proteins detection can be used as a biological marker for the animal breed identification.

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