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Original article

Isolation and identification of zinc ions and heparin binding proteins in yellow fraction of the red deer *Cervus Elaphus L.* semen

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Abstract

During the rutting season, stag semen is accompanied by a sticky, dense secretion called yellow fraction (YF). There is little information about the role, biology, physiology, and most importantly, the composition of this fluid. The aim of this study was to isolate and identify zinc ions (ZnBPs) and heparin binding proteins (HBPs) from YF of the red deer (*Cervus elaphus L.*).

Using liquid chromatography, the presence of 6 fractions of ZnBPs (71, 65, 55, 16, 14 and 12 kDa) and 22 fractions of HBPs (163, 140, 96, 78, 71, 65, 55, 49, 33, 31, 26, 25, 24, 22, 18, 16, 13, 12, 11, 10, 9 and 8 kDa) in YF proteome was demonstrated. By means of two-dimensional electrophoreses and MALDI-TOF/TOF mass spectrometry some of them were then identified. Amongst ZnBPs the following were identified: glutaminyl-peptide cyclotransferase, inhibitor of carbonic anhydrase-like, potassium voltage-gated channel subfamily E member 2, WD repeat-containing protein 38 isoform X4. Amongst the HBPs metalloproteinase inhibitor 2 (TIMP2), seminal plasma glycoprotein PSP-I and adseverin (scinderin) were identified.

Identifying all ZnBPs and HBPs present in YF may broaden up-to-date knowledge concerning the biology, physiology and preservation of red deer semen.

Key words: red deer, yellow fraction, ZnBPs, HBPs, proteomics

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Introduction

Asher et al. (1996) studied the quality and quantity of deer semen collected in autumn, winter and spring by electroejaculation. They demonstrated that semen concentration and volume reached its highest values in autumn during the rutting season (the rut). The period of greatest libido (from the end of September until the end of October) coincides with the highest semen quality (Giżejewski et al. 2003). The presence of sperm-free yellow fraction (YF) is typical for the time of the rut. The volume of yellow fraction is significantly affected by the season, individuals and the number of ejaculate collection (Giżejewski 2004). The yellow fraction (YF), which is secreted mainly by the stag vesicle glands, is known to store some energetic substances (such as fructose and phospholipids) and proteins which probably protect the sperm during its transport in the hind's reproductive tract (Strzeżek et al. 1998).

Some authors noticed that spermatozoa which came into contact with YF reduced their motility, so others proposed that this fraction might be involved in post--copulatory sexual selection (Pintus and Ros-Santaella 2014). However, by providing the components necessary for spermatozoa during their transit through the female reproductive tract this fraction may also enhance the sperm quality. Yellow fraction is characterized by high total protein content, which crests in October (average: 345±96.3 mg/ml) (Giżejewski 2004). Such an amount of total protein content during the rut suggests that some protein fractions are essential in the process of fertilization. Up-to-date knowledge concerning the proteins in stag yellow fraction includes only expression of albumins and globulins (data not published) and, to some extent, the enzymatic activity of chosen proteins (Koziorowska-Gilun et al. 2016). Koziorowska-Gilun et al. (2016) evaluated the antioxidant status of YF and demonstrated that the highest activities of superoxide dismutase, glutathione peroxidase and glutathione content are observed during the rutting season.

Many previous studies among different species demonstrated that ZnBPs and HBPs affect sperm quality and thus indirectly affect successful fertilization. The roles of zinc ions in sperm physiology are various: they may increase the stability of membrane lipid bilayers, stabilize the structure of the chromatin in the sperm nuclei, or affect sperm motility and acrosome reaction (Mogielnicka-Brzozowska et al. 2014). ZnBPs in boar seminal plasma improve sperm motility and acrosome integrity of preserved spermatozoa, presumably by coating the sperm surface (Mogielnicka-Brzozowska et al. 2011). Moreover, ZnBPs of canine seminal plasma (especially between 14.6 and 11.6 kDa) might be implicated in key events associated with sperm-egg fertilization processes (Mogielnicka-Brzozowska et al. 2014). Heparin binding proteins (HBPs) and their homologs derive from seminal fluid, which is secreted by seminal glands. Binding of HBPs to the sperm membrane is necessary not only to obtain fertilizing ability by spermatozoon but also a successful cryopreservation process. HBPs from seminal plasma have been shown to participate in capacitation and acrosome reaction in species such as the human (Kumar et al. 2008) and buffalo (Singh et al. 2016).

Artificial insemination in deer is still underutilized due to the lack of understanding of species-specific reproductive traits (Pintus and Ros-Santaella 2014). Thus, research is required not only for knowledge enhancement but also for the development of reliable preservation methods of the red deer semen. There is no data concerning profiles of ZnBPs and HBPs present in the yellow fraction of this species. Thus, the aim of this study was to isolate, identify and analyze this group of proteins.

Materials and Methods

The experimental materials were 64 samples of yellow fractions (YF) derived from 5 red deer stags (A-E) from 1999 to 2004 (all yellow fractions were gathered in October). The animals were kept in Research Station for Ecological Agriculture and Conservative Animal Breeding of the Polish Academy of Sciences in Popielno. The ejaculates were collected with the use of a modified artificial vagina (AV) as described by Giżejewski (2000). Methods with the use of a typical AV (Giżejewski 1991) or a modified AV (Giżejewski 2000) have been considered as sufficient to separate sperm fractions. The modifications were made as follows: shortening the AV body by changing the semen collector into a proportionally prolonged rubber funnel, introducing a scaled final glass collector and inserting a pressure-release opening. Following the development of the modified artificial vagina adapted for use in red deer, the white and yellow fractions could be clearly distinguished. All applicable national and institutional guidelines for the care and the use of animals were followed.

Total protein content

In order to measure total protein content, all samples were first diluted a thousand fold with 0.85% NaCl first. After dilution, total protein content was measured according to the Bradford method (Bradford 1976).

Isolation of ZnBPs and HBPs from YF

Before conducting chromatography, all YFs were diluted with 0.85% NaCl to obtain 5 mg/mL of protein content in every sample. All separations were performed using a Biologic Duoflow (BioRad, USA) chromatographic system.

The isolation of ZnBPs was conducted on a Chelating Sepharose Fast Flow bed (GE Healthcare, USA). In order to stabilize and prepare the bed, the column was rinsed with 10 ml of starting buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 7.5). Secondly, 2 ml of 100 mM $ZnCl_2$ was added to the column and it was rinsed once again with 10 ml of starting buffer. Thirdly, 1 ml of sample was added to the column and it was re-washed with 10 ml of starting buffer. ZnBPs were then eluted using 15 ml of elution buffer (50 mM Tris-HCl, 100 mM imidazole, pH 7.5) and gathered to 1.5 ml fractions. The last stage of chromatography involved removing zinc ions from the bed by washing the column with 2 ml of EDTA buffer.

The isolation of HBPs was carried out with a Heparin Sepharose bed (GE Healthcare, USA). The first stage of the isolation process was washing the gel bed with 10 ml of starting buffer (50 mM Tris-HCl, pH 7.5) in order to stabilize the parameters and prepare the column. 1 ml of sample was then added to the column. The column was then immediately rinsed with 10 ml of starting buffer. The HBPs were eluted with 10 ml of elution buffer (50 mM Tris-HCl, 2.5 M NaCl, pH 7.5). The volume of gathered fractions was 1 ml.

Based on the obtained chromatograms, 4 fractions with the highest total protein contents from every chromatographic session were separated by electrophoreses.

SDS-PAGE

Prior to electrophoresis proteins were precipitated from the probes using 2% sodium deoxycholate (DOC) and 100% trichloroacetic acid (TCA). At first 100 µl of every sample was mixed with 1 µl of 2% DOC. Subsequently, all samples were vortexed and kept at 4°C for 30 min in a refrigerator. After incubation, 10 µl of 100% TCA was added to every probe. The samples were vortexed once again, kept at 4°C for 15 min and then centrifuged at 15 000 x g for 15 min. The pellets obtained were washed with acetone, centrifuged at maximum speed twice and finally dried in the open air. The precipitations were mixed with 100 µl of diluted loading sample buffer.

Electrophoresis of the proteins was conducted according to the method described by Laemmli (1970) with the use of 15% gels and a constant voltage of 150 V. Precision Plus Protein Standards (Bio-Rad, USA) were used as molecular weight standards in all separations. Subsequently, the gels were stained with colloidal Coomassie buffer (0.02% Coomassie Brilliant Blue G-250, 2% orthophosphoric acid, 5% aluminum sulfate, 10% methanol) for 24 h and destained with deionized water. Destained gels were analysed using Multi-Analyst™ software (version 1.1, BioRad, USA).

Two dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was conducted according to the method described by O'Farrell (1975) with some modifications, within the 3-10 pH gradient. Samples, containing 50 µg of total protein, were applied onto strips and inserted into a cassette (Invitrogen Waltham, USA). After isoelectrofocusing (200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 1000 V for 120 min), strips were equilibrated in 6 M urea, 2% SDS, 0.375 M Tris, 20% glycerol, 2% DTT and 0.2% bromophenol blue. They were then placed on 12% SDS-PAGE gel, covered with heated agarose and separated at 150 V in a Mini Protean II Cell (BioRad, USA). Precision Plus Protein Standards (Bio-Rad, USA) were used as molecular weight standards in all separations. After electrophoreses, the gels were stained with Coomassie buffer as described above. Destained gels were analysed using PDQuest analysis software (BioRad, USA).

MALDI –TOF/TOF mass spectrometry

Identification of chosen proteins was done by matrix-assisted laser desorption-ionization-time-of-flight-mass spectrometry (MALDI-TOF/TOF MS). Gel sections containing particular proteins were cut out with a scalpel and hydrolyzed with Trypsin (Trypsin Profile IGD Kit, Promega, USA) in overnight incubation at 37°C. They were then desalted and concentrated with ZipTip® C18 (Merck Millipore, USA). Polypeptides samples of 1.0 µl volume were applied onto MTP 384 polished steel TF targets and covered with 1.0 µl of matrix solution containing 5 mg/mL α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) in 50% acetonitrile and 0.1% trifluoroacetic acid. Samples were analyzed using the Autoflex III Smartbeam MALDI-TOF/TOF mass spectrometer. Certain parent peaks were fragmented using TOF/TOF Lift technology. Protein identification was conducted using BioTools 3.0 (Bruker Daltonics, Germany) by comparing spectra (combined MS and MS/MS) data using the MASCOT programme (Matrix Science, USA) against all entries of NCBI numbers.

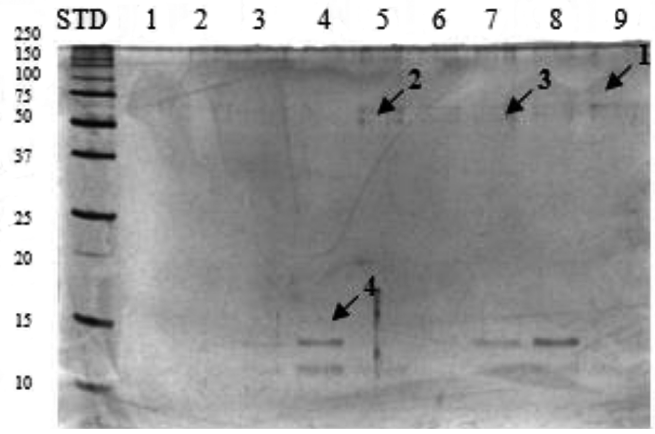

Fig. 1. ZnBP profiles of yellow fractions (stags A and B); Proteins marked with arrows: **1** – 71 kDa, **2** – 65 kDa, **3** – 55 kDa, **4** – 16 kDa

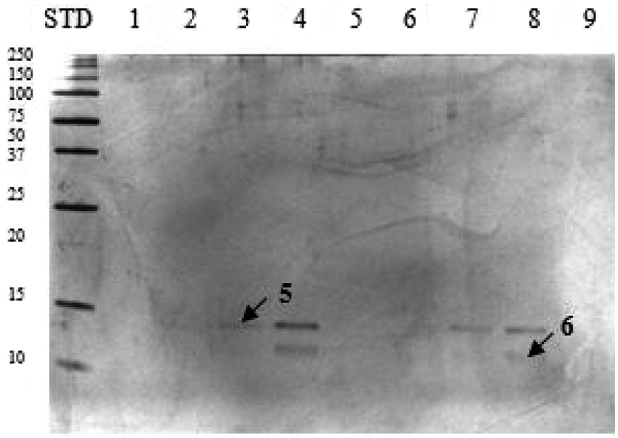

Fig. 2. ZnBP profiles of yellow fractions (stags C and D); Proteins marked with arrows: **5** – 14 kDa, **6** – 12 kDa

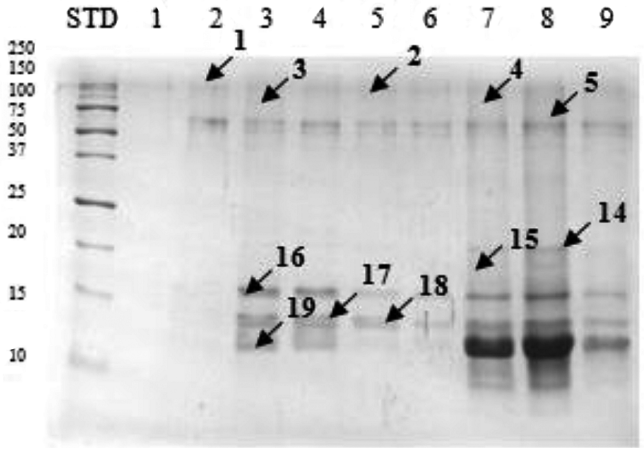

Fig. 3. HBP profiles of yellow fractions (stags A and C); Proteins marked with arrows: **1** – 163 kDa, **2** – 140 kDa, **3** – 96 kDa, **4** – 78 kDa, **5** – 71 kDa, **14** – 22 kDa, **15** – 18 kDa, **16** – 16 kDa, **17** – 13 kDa, **18** – 12 kDa, **19** – 11 kDa

Results

One-dimensional separations of isolated ZnBPs demonstrated the occurrence of approximately 6 bands with molecular weights of 71, 65, 55, 16, 14 and 12 kDa (Figs. 1 and 2), whereas separations of HBPs showed the presence of approximately 22 bands with molecular weights of 163, 140, 96, 78, 71, 65, 55, 49, 33, 31, 26, 25, 24, 22, 18, 16, 13, 12, 11, 10, 9 and 8 kDa (Figs. 3 and 4). Proteins with molecular weights of 71, 65, 55, 16, 14 and 12 kDa bound to both Zn and heparin.

The 2D separations showed the presence of approximately thirty-two and thirty-one spots of ZnBPs and HBPs respectively. Protein profiles of ZnBPs and HBPs were partially convergent (spots marked with triangles)

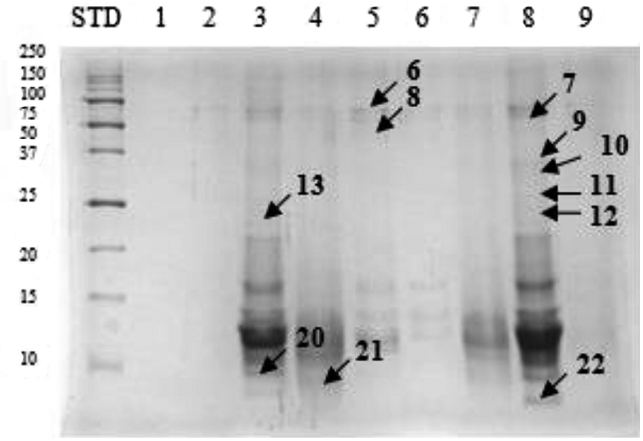

Fig. 4. HBP profiles of yellow fractions (stags B and E); Proteins marked with arrows: **6** – 65 kDa, **7** – 55 kDa, **8** – 49 kDa, **9** – 33 kDa, **10** – 31 kDa, **11** – 26 kDa, **12** – 25 kDa, **13** – 24 kDa, **20** – 10 kDa, **21** – 9 kDa, **22** – 8 kDa

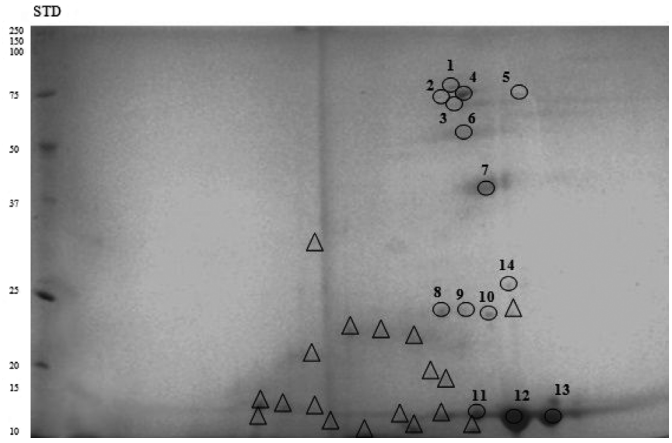

Fig. 5. ZnBPs profile of yellow fraction (stag A) obtained after 2D-PAGE separation; spots marked with numbers 1-14 were subjected to MALDI-TOF/TOF mass spectrometry

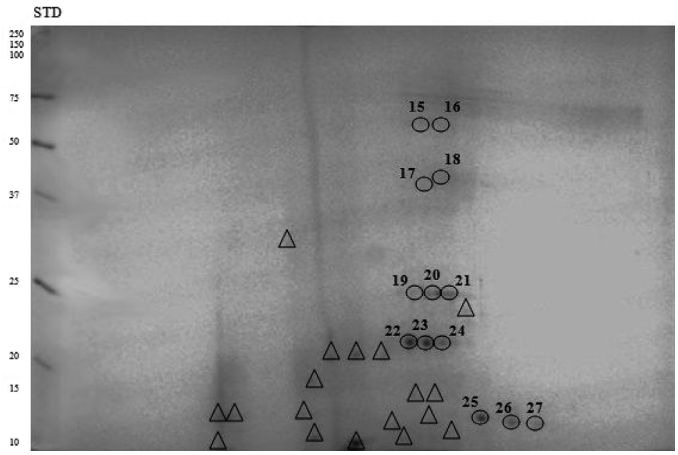

Fig. 6. HBPs profile of yellow fraction (stag E) obtained after 2D-PAGE separation; spots marked with numbers 15-27 were subjected to MALDI-TOF/TOF mass spectrometry

(Figs. 5 and 6). Certain ZnBPs and HBPs were subjected to an identification procedure using MALDI-TOF/ /TOF MS. Spots that gave the strongest signals in gels, and repeatedly appeared among 2-D separations of all YFs, were chosen for trypsin digestion and subsequent mass spectrometry.

Using mass spectrometry, some ZnBPs were identified i.e. glutaminyl-peptide cyclotransferase, inhibitor of carbonic anhydrase-like, potassium voltage-gated channel subfamily E member 2 and WD repeat-containing protein 38 isoform X4. Amongst the HBPs metalloproteinase inhibitor 2 (TIMP2), seminal plasma glyco-

Table 1. Identification of zinc binding proteins (ZnBPs) after 2D-PAGE and MALDI –TOF/TOF mass spectrometry.

IDENTIFIED ZINC BINDING PROTEINS (ZnBPs)							
Spot No.	Protein	Species	Score	Sequence coverage (%)	NCBI No.	MW (kDA)	pI
		with a score > 78					
7	Glutaminyl-peptide cyclotransferase	*Pentholops hadgsonii*	100	14	556765725	41324	6.30
	Glutaminyl cyclase	*Ovis aries*	98	16	3170172	27424	6.51
	Glutaminyl-peptide cyclotransferase, isoform X1	*Ovis aries*	94	11	803030626	41182	6.40
	Glutaminyl-peptide cyclotransferase	*Capra hircus*	94	11	548485318	41228	6.40
	Glutaminyl-peptide cyclotransferase precursor	*Ovis aries*	94	11	261244988	41253	6.40
5	Inhibitor of carbonic anhydrase-like	*Condylura cristata*	81	10	830030413	79498	6.32
11	Potassium voltage-gated channel subfamily E member 2	*Octodon degus*	78	59	507657581	14445	5.97
		with a score < 78					
3	Carbonic anhydrase 2	*Bison bison*	72	41	742151424	29112	4.87
14	WD repeat-containing protein 38 isoform X4	*Equus asinus*	70	24	958695042	34431	8.76
12	Sperm-associated antigen 11-like	*Leptonychotes weddellii*	57	52	585168032	10091	8.20
13	rCG 58926	*Rattus norvegicus*	57	82	149015569	7327	9.90
6	Immunoglobulin gamma 2 heavy chain constant region	*Capra hircus*	56	55	147744654	21648	6.33
9	Short chain dehydrogenase/reductase 3	*Cricetulus griseus*	49	42	625292753	24364	6.58

protein PSP-I and adseverin (scinderin) were identified (Tables 1 and 2). However, there is one limitation that should be mentioned - results for ungulates and other animals were presented as the MASCOT database does not yet possess numerous entries of proteins in the red deer species.

Discussion

Recently, in the reproductive biology of deer species emphasis has been laid on the possible recovery, evaluation and preservation of their sperm (Martinez--Pastor et al. 2006). Knowing the composition of stag semen we can establish the strategies and management practices for better reproductive use (Hernandez-Souza et al. 2014).

Different studies have shown the relationships between zinc concentrations, semen volume and pH, as well as spermatozoa concentration and morphology (Colagar et al. 2009). Knowledge of seminal plasma zinc binding protein-ligands is deficient and concerns only humans (Siciliano et al. 2000), boars (Hołody and Strzeżek 1999) and dogs (Mogielnicka-Brzozowska et al. 2014).

This was the first attempt to isolate and identify ZnBPs present in the yellow fraction of the red deer. In this study we demonstrated the presence of a few proteins which can specifically bond to Zn and may take part in subsequent stages of fertilization. The polypeptide with a molecular weight of 55 kDa was identified as glutaminyl-peptide cyclotransferase. This enzyme catalyses the conversion of L-γ-glutamylamines to free amines and 5-oxo-L-proline, and is suspected of participating in the posttranslational processing of hormonal precursors (Busby et al. 1987) such as neurototensin (NT) or thyrotropin-releasing hormone (TRH) and its analogues. NT receptors have been found in mice epididymal spermatozoa, whilst TRH, TRH--homologous peptide and TRH-binding substances have been found in human and rat semen. It was demonstrated that neurotensin indices both capacitation and acrosome reaction by intensifying protein phosphorylation (Hiradate et al. 2014), whereas the TRH-related peptide became known as the fertilization-promoting peptide (FPP) because of its ability to enhance the in vitro fertilizing potential of mouse epididymal spermatozoa (Cockle et al., 1994). The next two ZnBPs identified seem to take part in the maintenance of intracellular pH. The former, with a molecular weight of 79 kDa, was identified as an inhibitor of carbonic anhydrase (CA). CA catalyzes the reversible hydration of CO_2 to HCO_3^-, which is the main regulator of sperm motility.

Table 2. Identification of heparin binding proteins (HBPs) after 2D-PAGE and MALDI –TOF/TOF mass spectrometry.

IDENTIFIED HEPARIN BINDING PROTEINS (HBPs)							
Spot No.	Protein	Species	Score	Sequence coverage (%)	NCBI No.	MW (kDA)	pI
		with a score > 78					
	Metalloproteinase inhibitor 2	*Acinonyx jubatus*	121	44	961749875	37949	8.47
	Metalloproteinase inhibitor 2 isoform x2	*Nomascus leucogenys*	113	42	821013742	21669	6.79
	Metalloproteinase inhibitor 2 isoform x1	*Nomascus leucogenys*	109	36	332258792	25111	7.45
	Metalloproteinase inhibitor 2 isoform x2	*Ursus maritimus*	108	50	671002377	22138	7.51
	Metalloproteinase inhibitor 2	*Felis catus*	108	43	410981890	25179	8.40
	Metalloproteinase inhibitor 2	*Ailuropoda melanoleuca*	107	39	752399803	28048	7.88
	Metalloproteinase inhibitor 2,	*Sus scrofa*	105	41	927192733	21089	6.88
	Metalloproteinase inhibitor 2,	*Balaenoptera acutorostrata scammoni*	105	41	594676072	21094	7.49
	Metalloproteinase inhibitor 2, partial	*Pteropus alecto*	105	41	431908708	21011	7,61
24	Metalloproteinase inhibitor 2 isoform x3	*Sus scrofa*	105	41	545856428	21083	8.02
	Metalloproteinase inhibitor 2	*Cricetulus griseus*	105	32	344241777	22097	7.47
	Metalloproteinase inhibitor 2,	*Panther tigris altaica*	105	32	987419629	32796	9.01
	Metalloproteinase inhibitor 2 isoform x3	*Oryctolagus cuniculus*	104	39	655898023	22327	6.93
	Metalloproteinase inhibitor 2 isoform x2	*Oryctolagus cuniculus*	104	39	8478957	22519	7.59
	Tissue inhibitor of metalloproteinase-2, partial	*Bubalus bubalis*	104	37	808039704	22421	7.00
	Metalloproteinase inhibitor 2 isoform x2	*Colobus angolensis palliatus*	104	37	795093002	22294	7.45
	Metalloproteinase inhibitor 2	*Pteropus alecto*	103	35	989946491	25056	6.79
	Metalloproteinase inhibitor 2 isoform x2	*Ovis aries*	103	35	965956656	24563	8.64
	Metalloproteinase inhibitor 2 isoform x2	*Bubalus bubalis*	103	29	594081108	25168	7.93
	Spermadhesin PSP-I- pig	*Sus scrofa*	106	17	108346	12203	7.82
22	Major seminal plasma glycoprotein PSP-I precursor	*Sus scrofa*	105	14	47523176	14719	8.33
	Major seminal plasma glycoprotein PSP-I isoform x1	*Sus scrofa*	104	12	545830790	17610	9.14
23	Metalloproteinase inhibitor 2; short=TIMP-2	*Equus caballus*	78	16	6094482	10106	5.76
		with a score < 78					
	Spermadhesin PSP-I- pig	*Sus scrofa*	73	13	108346	12203	7.82
15	Major seminal plasma glycoprotein PSP-I precursor	*Sus scrofa*	73	11	47523176	14719	8.33
	Major seminal plasma glycoprotein PSP-I isoform x1	*Sus scrofa*	72	9	545830790	17610	9.14
16	Adseverin	*Fukomys damarensis*	60	28	676269833	77450	5.22
27	L-serine/L-threonine deaminase	*Pteropus alecto*	59	41	431914227	29065	6.17
	28S ribosomal protein S11, mitochondrial	*Pteropus alecto*	57	39	431920217	16998	11.53
20	Hyaluronan and proteoglycan link protein 3 isoform x2,	*Ovis aries musimon*	58	25	803257538	49312	8.15
25	AP-4 complex subunit beta-1	*Mustela putorius furo*	57	24	511900737	84125	5.57
	rCG58926	*Ratus norvegicus*	55	85	149015569	7327	9.90

CAII is presumed to be involved in the acidification process of the epididymal fluid. Strong acidification aids the maintenance of sperm immotility (Zhou et al. 2015). The latter, with a molecular weight of 14445 Da was identified as potassium voltage-gated channel subfamily E member 2 (KCNE2). Most likely, it is a part of the KCNQ1/KCNE2 K^+ channels that recirculate K^+ over the apical membrane. The voltage-gated ion channel allows potassium ions in sperm to flow outwardly during plasma membrane hyperpolarization and sperm–egg interaction triggered events (Chavez et al. 2014). On the other hand, protein with a 71 kDa molecular weight which bound to zinc manifested also an ability to bind to heparin. It was identified as WD repeat-containing protein 38 isoform X4. Although WD repeat proteins, which are important for regulation of flagellar motility, are mainly found on axoneme structures (Perrone et al. 1998), it was demonstrated that WD--repeat containing protein 1 can be found among the proteome of bull seminal vesicle proteome (Westfalewicz et al. 2017). The presence of a specific protein containing WD repeats, with a molecular weight of 71-kDa, in extracts of mouse sperm and testis (Zhang et al. 2002) was previously demonstrated.

Another group of proteins investigated in the present study were heparin binding proteins. Until now, profiles of HBPs derived from seminal plasma and sperm membranes have been associated with bull fertility (Fernandes et al. 2009). The majority of HBPs found in the YF of red deer were low-molecular weight proteins. We suppose that they may participate in stabilizing spermatozoa functionality. Patel et al. (2016) demonstrated that the treatment of bull semen with SP-HBP improves spermatozoa motility, viability, HOST and acrosome integrity. The protein with a molecular weight of approximately 24 kDa was identified as metalloproteinase inhibitor 2 (TIMP-2). The 24 kDa-HBP previously found in bull semen was also identified as a tissue-inhibitor of metalloproteinases-type 2 (McCauley et al. 2001). Generally, the matrix metalloproteinase (MMP) family is a group of calcium and zinc-dependent proteases which are changed into their active forms and regulated by specific tissue inhibitors called TIMPs. Metalloproteinase inhibitor 2 (TIMP-2) was localized in the testes, epididymis and ejaculated spermatozoa of the dog (Warinrak et al. 2015). Decreased levels of TIMP-2 and MMP-2 in seminal plasma may be associated with a higher level of sperm DNA fragmentation (Belardin et al. 2019). Furthermore, Alvarez-Gallardo et al. (2013) indicated that addition of rTIMP-2 (recombinant protein) to semen before cryopreservation helps to stabilize the acrosome membrane of the bovine sperm by decreasing post-thaw acrosome damage. Another HBP, found in deer YF, was identified as spermadhesin PSP-I (12.2 kDa). Spermadhesins with molecular weights ranging from 16 to 12 kDa are common in seminal plasma and spermatozoa of many domestic species. In our study, the heterogeneity of low molecular weight HBPs in stag YF was ascertained by many visible and strong electrophoretic bands. This indicates that the presence of low-molecular spermadhesins during the time of the rut is relatively diversified. PSP-I/PSP-II spermadhesin complex contributes to maintaining high viability, motility and mitochondrial activity of spermatozoa. Interestingly, it may serve as an additive to improve the viability of highly diluted porcine sperm. Isolated PSP-I protein addition exerts a beneficial effect on viability, motility and mitochondrial membrane potential of diluted porcine sperm (Garcia et al., 2006). HBP with a molecular mass of approximately 78 kDa was identified as scinderin (SCIN). Scinderin is engaged in actin polymerization which occurs during capacitation and acrosome reaction of sperm. This protein takes part in capping actin filaments which are free or newly synthesized. A study performed by Pelletier et al. (1999) demonstrated the presence of scinderin with a molecular mass of 80 kDa in, among others, bovine fetal and adult testes, epididymides (interstitial cells) and spermatozoa.

Summing up, many changes are regulated by the direct action of seminal plasma proteins. In contrast to the capacitating action of some polypeptides, others regulate sperm function, including suppression of capacitation and acrosome reaction (Perez-Pe et al. 2002). It is supposed that some YF proteins play a protective and supporting role in stag sperm during fertilization at the time of the rut. Our study focused on zinc ions and heparin binding proteins present in YF as they have a connotation with gamete interaction, sperm structure stabilization and protection of sperm in the female reproductive tract. We suspect that this group of proteins may participate in subsequent stages of fertilization in red deer species. There is a vital need to identify all the ZnBPs and HBPs present in stag yellow fraction in the future. This may broaden our knowledge of the composition of stag semen and develop the procedures concerning its preservation.

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