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IDENTIFICATION AND CHANGES IN THE SEASONAL CONCENTRATIONS OF PROTEINS REGULATING THE BIOLOGICAL FUNCTIONS OF SPERMATOZOA AND PARTICIPATING IN THEIR CYTOSKELETON ORGANIZATION IN ROE DEER (*CAPREOLUS CAPREOLUS*) EPIDIDYMIDES*

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Abstract

In the case of animals characterized by a strongly marked seasonality of reproduction, extremely significant seem to be periodical fluctuations in the concentration of proteinaceous substances identified in tissues and fluids of epididymides, which take part in the maturation process of sperm cells. The aim of the present study was to compare the seasonal expression of identified proteins present in various regions of the mature roe deer (*Capreolus capreolus*) epididymides, which are regulating the biological functions of spermatozoa and participating in their cytoskeleton organization during the sperm maturation process. Epididymal tissue homogenates and epididymal fluids were analyzed by two-dimensional electrophoresis (2D-PAGE) and tandem mass spectrometry (MS/MS) to reveal 31 polypeptides with different biochemical functions. Moreover, among the identified polypeptides, twelve of them were similar to: alpha-enolase isoform 3; endoplasmic reticulum resident protein 29; calreticulin, calponin-1 isoform X1; transgelin; vimentin; tubulin; desmin; tropomyosin; actin; alpha-1 antitrypsin isoform X1 and 14-3-3 protein epsilon. Concentrations of the analyzed polypeptides, expressed in optical density units (ODU), differed significantly ($P \leq 0.05$) across the examined periods of the reproductive season. The highest ODU values of almost all analyzed proteins were observed during the rutting period. This variation in the identified proteins in the epididymal tissues and fluids of roe deer throughout the reproductive season could be indicative of their important roles in sperm maturation within the epididymis.

Key words: roe deer, reproductive season, epididymis, proteins, proteomics

Roe deer (*Capreolus capreolus*) are seasonal breeders characterized by a short rutting season (Goeritz et al., 2003). Mature males show synchronized cycles of con-

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siderable testicular growth and involution with completely arrested spermatogenesis in the winter. The reproductive season is defined by three periods: the pre-rutting period (May–June), the rutting period (July–August), and the post-rutting period (first half of August; Klonisch et al., 2005). In species with a seasonal breeding pattern, the well-timed regulation of testicular and epididymal functions is crucial to reproductive fitness and successful breeding (Goeritz et al., 2003). It is well known that the testicular spermatozoa are not fully mature cells. Their fertilizing ability is developed as a result of post-gonadal maturation in the epididymis (Dacheux and Dacheux, 2013). Sperm cells become motile; they undergo capacitation and acrosome reactions, and become able to recognize and bind to the oocyte as a result of their sequential interactions with numerous components present in the tubular fluid, particularly with the proteins secreted by the epididymal epithelium (Belleannee et al., 2011). Through the duct, the milieu composition is changing continuously. The regional variations observed in the luminal proteins are due to various modifications, such as proteolysis, reabsorption or sperm binding (Bassols et al., 2007).

The roe deer is a unique model to investigate epididymal functions throughout the entire reproductive cycle, among others because of the occurrence of a strongly marked cyclic change in the male genital tract during the transition between breeding and non-breeding seasons (Klonisch et al., 2005).

In our previous proteomic studies we showed for the first time ever changes in the expression of identified antioxidative and heat shock proteins in different segments of roe deer epididymis (Leciewicz et al., 2017; Majewska et al., 2017).

Therefore, the present study aimed to compare the seasonal expression of identified proteins within various regions of the mature roe buck (*Capreolus capreolus*) epididymis, which are regulating the biological functions of spermatozoa and participating in their cytoskeleton organization during the sperm maturation.

Material and methods

All reagents used in the experiment were supplied by Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise stated.

Materials and sample preparation

Epididymides were obtained from 21 mature roe bucks aged 3–7 years (defined based on dentition) which were hunted upon the consent of the Regional Directorate of the State Forests in Krosno, Poland (49°17'N; 22°7'E). Samples were collected during 3 periods of the rutting season: the pre-rutting period (May–June, n=7), the rutting period (July–August, n=7), and the post-rutting period (first half of August, n=7). Epididymides were dissected and sub-divided into the three well defined anatomical regions (caput, corpus and cauda). Samples with the volume from 0.5 to 3 cm³ were collected from the epididymal tissues and immediately frozen in liquid nitrogen. Tissue proteins were extracted with the TRI-Reagent Kit according

to a previously described method (Chomczyński, 1993). Caput, corpus and cauda epididymal fluids were collected by micro-perfusion using phosphate-buffered saline (PBS) at 38°C, followed by centrifugation at 3,000×g for 10 min (at 4°C) to separate fluid from spermatozoa. The supernatant was further centrifuged at 10,000×g for 10 min at 4°C, divided into aliquots and stored at -80°C until analysis. Protein content in the collected fluids was determined according to Bradford (1976). Additionally, protein content in the diluted samples was confirmed by Western blotting using monoclonal anti- β -tubulin antibody as a loading control (Majewska et al., 2017).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed according to the method of O'Farrell (1975) and Weiss and Görg (2009) with certain modifications, within 3-10 pH gradients. Protein samples (50 μ g) were applied onto strips and fixed in a cassette (Invitrogen Waltham, MA, USA). After isoelectrofocusing (200V for 20 min, 450V for 15 min, 750V for 15 min, and 2000 V for 30 min), the strips were equilibrated in 6M urea, 2% sodium dodecyl sulphate (SDS), 0.375M Tris, 20% glycerol, 2% dithiothreitol (DTT), and 0.2% bromophenol blue. Protein samples were placed on 12% SDS-PAGE gel (Laemmli, 1970), covered with molten agarose and electrophoresed at 150 V in the Mini Protean II Cell (BioRad Rockville, MD, USA). After electrophoresis, gels were stained in 0.025% Coomassie brilliant blue R-250 in 40% acetic acid and 7% methanol then destained in a solution of 5% methanol and 7% acetic acid in deionized water. The destained gels were analysed in the PDQuest Analysis Software (BioRad, Rockville, MD, USA).

Tandem mass spectrometry MALDI-TOF/TOF

Tandem mass spectrometry MALDI-TOF/TOF was performed according to Suckau et al. (2003). Briefly, spots of interest were excised from the gels, digested with trypsin using the trypsin profile IGD kit, desalted and concentrated with Zip-Tip® C₁₈ (Merck Millipore, Billerica, MA, USA). Samples were analyzed with the Autoflex III Smartbeam MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Selected peaks were fragmented using the TOF/TOF Lift technology and identified in BioTools software (Bruker Daltonics, Bremen, Germany). Identification was performed by searching the National Center for Biotechnology Information (NCBI) database using the MASCOT search engine (Matrix Science, Boston, MA, USA). In all searches, protein scores higher than 77 were considered statistically significant ($P < 0.05$).

Statistical analysis

Differences in the optical density of protein spots across seasons were analysed by the Kruskal-Wallis (1952) test in statistical software (StatSoft, Tulsa, OK, USA). Differences between mean values were considered significant at $P \leq 0.05$.

Results

The evaluated extracts and fluids were analyzed by tandem mass spectrometry (MS/MS) to reveal 31 polypeptides with different biochemical functions, including polypeptides similar to proteins, which are regulating the biological functions of spermatozoa and participating in their cytoskeleton organization (Table 1). Exemplary electrophoregrams of the corpus epididymal tissue and cauda epididymal fluids revealed 12 identified proteins (Figures 1 A, 1 B).

▭ Identified conservative proteins

○ Identified proteins showing variable expression

- 1 Alpha-enolase isoform 3
- 2 CTR
- 3 ERp29
- 4 Calponin-1 isoform X1
- 6 Transgelin
- 7 Vimentin
- 8 Tubulin
- 9 Desmin
- 10 Tropomyosin
- 11 Actin

Figure 1 A. Two dimensional electrophoresis (2D-PAGE) of identified proteins in the corpus epididymal tissue during rutting period (Lecewicz et al., 2017)

○ Identified conservative proteins

Identified proteins showing variable expression

1 Alpha-enolase isoform 3

2 CTR

3 ERp29

5 Alpha-1 antitrypsin isoform X1

8 Tubulin

11 Actin

12 14-3-3 protein epsilon

Figure 1 B. Two dimensional electrophoresis (2D-PAGE) of identified proteins in the cauda epididymal fluids during rutting period

Table 1. Proteins regulating the biological functions of spermatozoa and participating in their cytoskeleton organization identified in epididymal tissues and fluids

The identified protein	NCBI database index number	Calculated MW (kDa)	Protein sequence coverage %	Mascot score
Alpha-enolase isoform 3	426239778	43.820	25	250
CTR	545920	46.524	11	98
ERp29	674076726	29001	12	110
Calponin-1 isoform X1	126322811	33.690	14	100
Alpha-1 antitrypsin isoform X1	545842993	49.035	8	126
Transgelin	594030751	21.362	40	170
Vimentin	14389299	53.526	32	134
Tubulin	4115724	62.725	18	125
Desmin	2959452	52.587	38	210
Tropomyosin	512924259	39.313	35	256
Actin	924442847	42.053	40	200
14-3-3 protein epsilon	109119486	12.023	39	100

Proteins regulating the biological functions of spermatozoa and participating in their cytoskeleton organization identified in the epididymal tissues

Two conservative polypeptides similar to alpha-enolase isoform 3 and endoplasmic reticulum resident protein 29 (ERp29) were identified in the three (caput, corpus, cauda) segments of epididymis in all analysed periods of the rutting season. Furthermore, a protein similar to calreticulin (CTR) was also detected in the corpus epididymis regardless of the period of the reproductive season. This protein was also noted in the caput epididymis before and after the reproductive season, while in the cauda epididymis it was observed before and during the rutting season. Furthermore, six identified proteins similar to calponin-1 isoform X1, transgelin, vimentin, tubulin, desmin, tropomyosin, and actin, involved in the cytoskeletal organization of spermatozoa were detected in the three segments of epididymis regardless of the rutting season.

Proteins regulating the biological functions of spermatozoa and participating in their cytoskeleton organization identified in the epididymal fluids

Three conservative polypeptides similar to CTR, ERp29 and alpha-1 antitrypsin isoform X1 (A1AT) were identified in all epididymal fluids throughout all periods of the breeding season. A protein similar to alpha-enolase isoform 3 was observed in the caput and cauda epididymis only in the rutting season. The presence of proteins resembling tubulin, actin and 14-3-3 protein epsilon, as in the case of molecules involved in spermatozoa cytoskeletal organization observed in the epididymal tissues, were also constitutive. All analysed protein spots showed high protein sequence coverage and scores with predicted proteins from the NCBI database (Table 1).

The concentrations of the analysed polypeptides, expressed in optical density units (ODU), differed significantly ($P \leq 0.05$) across all examined periods of the rutting season. In all segments of the epididymis, the highest ODU values of nearly all analysed proteins were observed during the rutting period. The results of analyses were validated statistically (Tables 2 A and 2 B).

Table 2 A. Changes in optical density (mean $\pm$ SD) of the analyzed proteins in the epididymal tissues depending on the period of the reproductive season

Epididymal segment \ Reproductive season	Pre-rutting period		Rutting period		Post-rutting period	
	mean	SD	mean	SD	mean	SD
1	2	3	4	5	6	7
Caput epididymis						
Alpha-enolase isoform 3	0.208 a	0.021	0.284 b	0.008	0.249 ab	0.014
Calreticulin	0.340 a	0.012	-	-	0.254 b	0.016
Endoplasmic reticulum resident protein 29	0.187 a	0.031	0.369 b	0.020	0.260 ab	0.015
Calponin-1 isoform X1	0.212	0.017	0.223	0.026	0.217	0.013
Transgelin	0.144	0.024	0.153	0.032	0.146	0.011
Vimentin	0.240 a	0.022	0.317 b	0.018	0.266 a	0.019
Tubulin	0.267 a	0.007	0.314 b	0.012	0.299 b	0.011
Desmin	0.210 a	0.013	0.270 b	0.016	0.213 a	0.013

Table 2 A – contd.

1	2	3	4	5	6	7
Tropomyosin	0.329 a	0.031	0.381 b	0.039	0.349 ab	0.009
Actin	0.489 a	0.016	0.532 b	0.004	0.519 b	0.041
Corpus epididymis						
Alpha-enolase isoform 3	0.213 a	0.023	0.277 b	0.013	0.272 b	0.010
CTR	0.255 a	0.015	0.268 b	0.032	0.266 b	0.019
Endoplasmic reticulum resident protein 29	0.298 a	0.020	0.362 b	0.018	0.308 ab	0.022
Calponin-1 isoform X1	0.232 a	0.009	0.253 b	0.016	0.265 ab	0.011
Transgelin	0.169 a	0.017	0.171 a	0.006	0.132 b	0.013
Vimentin	0.253 a	0.021	0.272 b	0.010	0.270 b	0.024
Tubulin	0.262 a	0.019	0.304 b	0.015	0.301 b	0.031
Desmin	0.249 a	0.012	0.280 b	0.023	0.253 a	0.008
Tropomyosin	0.340 a	0.016	0.389 b	0.020	0.373 ab	0.018
Actin	0.536	0.012	0.541	0.012	0.538	0.014
Cauda epididymis						
Alpha-enolase isoform 3	0.282 a	0.005	0.354 b	0.013	0.310 ab	0.021
CTR	0.272	0.016	0.269	0.011	-	-
ERp29	0.242 a	0.009	0.312 b	0.021	0.248 a	0.025
Calponin-1 isoform X1	0.227	0.012	0.229	0.016	0.231	0.035
Transgelin	0.189 a	0.023	0.219 b	0.021	0.220 b	0.004
Vimentin	0.257 a	0.041	0.289 b	0.012	0.214 ab	0.028
Tubulin	0.254	0.030	0.260	0.013	0.257	0.010
Desmin	0.235 a	0.011	0.220 b	0.036	0.213 b	0.018
Tropomyosin	0.378 a	0.017	0.406 b	0.006	0.340 ab	0.009
Actin	0.524	0.010	0.529	0.015	0.521	0.012

a, b – values with different letters in row are significant at $P \leq 0.05$ .Table 2 B. Changes in optical density (mean $\pm$ SD) of the analyzed proteins in the epididymal fluids depending on the period of the reproductive season

Reproductive season Epididymal segment	Pre-rutting period		Rutting period		Post-rutting period	
	mean	SD	mean	SD	mean	SD
1	2	3	4	5	6	7
Caput epididymis						
Alpha-enolase isoform 3	-	-	0.223	0.013	-	-
CTR	0.235 a	0.005	0.285 b	0.011	0.280 b	0.015
ERp29	0.132 a	0.036	0.186 b	0.006	0.140 a	0.023
Alpha-1 antitrypsin isoform X1	0.138 a	0.012	0.076 b	0.017	0.062 b	0.009
Tubulin	0.174 a	0.021	0.225 b	0.012	0.182 b	0.025
Actin	0.384	0.015	0.390	0.024	0.392	0.010
14-3-3 protein epsilon	0.225	0.014	0.230	0.004	0.224	0.017
Corpus epididymis						
Alpha-enolase isoform 3	-	-	-	-	-	-
CTR	0.340 a	0.011	0.346 a	0.012	0.293 b	0.010
ERp29	0.116 a	0.042	0.172 b	0.016	0.110 a	0.021

Table 2 B – contd.

1	2	3	4	5	6	7
Alpha-1 antitrypsin isoform X1	0.092 a	0.015	0.153 b	0.007	0.106 a	0.017
Tubulin	0.082 a	0.019	0.119 b	0.015	0.093 a	0.028
Actin	0.323 a	0.005	0.386 b	0.027	0.330 a	0.009
14-3-3 protein epsilon	0.176	0.025	0.181	0.010	0.191	0.032
Cauda epididymis						
Alpha-enolase isoform 3	-	-	0.320	-	-	-
CTR	0.274	0.014	0.269	0.022	0.285	0.012
ERp29	0.196 a	0.023	0.254 b	0.016	0.231 b	0.014
Alpha-1 antitrypsin isoform X1	0.065 a	0.012	0.229 b	0.014	0.242 b	0.033
Tubulin	0.169	0.036	0.178	0.012	0.181	0.024
Actin	0.335 a	0.010	0.371 b	0.028	0.342 a	0.019
14-3-3 protein epsilon	0.215 a	0.007	0.251 b	0.024	0.228 a	0.019

a, b – values with different letters in row are significant at $P \leq 0.05$.

Discussion

For the first time ever, 2D-PAGE revealed the highest number of polypeptides in both tissue homogenates and epididymal fluids in the analysed periods of the breeding season in the roe deer (Lecewicz et al., 2017; Majewska et al., 2017). In the case of animals characterized by a strongly marked seasonality of reproduction, extremely significant seem to be periodical fluctuations in the concentration of proteinaceous substances identified in tissues and fluids of epididymides, particularly proteins regulating the biological functions of spermatozoa and participating in their cytoskeleton organization.

Among the identified proteins regulating the biological functions of spermatozoa was α -enolase isoform 3. It is characterized by a significantly conservative nature amongst isoenzymes occurring in organisms of mammals where the homology of an amino acid sequence accounts for nearly 82% (Pancholi, 2001). When interacting with cholesterol, this protein was also demonstrated to affect the extent of cholesterol esters accumulation in cells (Bottalico et al., 1993). Cholesterol as a constituent of cell membranes plays a significant role in preserving both the functional and structural integrity of all mammalian cells (Seweryn et al., 2007). The presence of α -enolase, being an enzyme of the glycolysis pathway, was detected in tails and cytoplasmic droplets of human sperm (Cooper, 2011). Its low activity was also demonstrated in primary spermatocytes. In addition, a relationship was found between enolase and microtubules (Gitlits et al., 2001). Alpha-enolase was detected in cauda epididymal fluids of bull and ram, where it most likely binds to sperm surface (Souza et al., 2012).

Noteworthy, in the current study the presence of α -enolase was detected in tissue homogenates of roe buck epididymides constitutively, whereas it was identified in the caput and the cauda epididymal fluids only in the breeding season. This result may indicate that in the case of animals characterized by a strongly marked season-

ality of reproduction, α -enolase is expressed only in epididymal tissues. Moreover, the highest expression of this protein is observed in caput and cauda epididymides in the breeding season, which may explain the presence of this molecule in epididymal fluids of indicated anatomical regions. Another identified protein regulating the biological functions of spermatozoa turned out to be CTR, a substance with molecular weight of 46 kDa commonly expressed in all tissues of the mammals. The Ca^{2+} -dependent CTR was identified in acrosomes of both round spermatids and mature spermatozoa of rat (Nakamura et al., 1992). A previous study conducted by Nakamura et al. (1993) demonstrated that in the cascade of spermatogenesis in rat, CTR may be incorporated into the acrosomal vesicle through a Golgi apparatus, with glycosylation process being omitted. These authors suggest also a significant role of CTR in the regulation of sperm motility and in the acrosome reaction. Moreover, CRT together with calnexin and with co-participation of protein disulfide-isomerase A3 (PDIA3) assists in glycoprotein folding in germ cells (Tokuhiro et al., 2012).

Another protein identified in the present study, was ERp29 which has a function similar to that of CTR and has a structure with traits specific to protein disulphide isomerases. The ERp29 was demonstrated to assist in protein folding together with other chaperon proteins present in the endoplasmic reticulum (Park et al., 2001). Additionally, colocalization of ERp29 and CTR was reported in the endoplasmic reticulum (Ferrari et al., 1998). In turn, the investigations carried out by Guo et al. (2007) revealed the presence of ERp29 precursor in rat spermatozoa. A significant increase was noted in the protein expression within both spermatozoa and plasma membranes along with progressive maturation of the reproductive cells during their residence in the epididymis. The results of immunofluorescence techniques revealed an intensive reaction in the mid-piece and tail regions of spermatozoa accumulated in the cauda epididymis, while only low fluorescence was observed in the mid-piece region in the caput epididymis. The spermatozoa accumulated in the cauda epididymis are characterized by intact outer mitochondrial membrane and the cytoplasmic membrane of which requires ERp29 involvement to reach maturity (Guo et al., 2007). In turn, the activation of epididymal sperm motility is determined by a short-term increase in the Ca^{2+} ions concentration in the cytoplasm, with subsequent increase in cAMP concentration. As ERp29 is incapable to bind with Ca^{2+} , it most likely enters into reactions with other proteins including canalexin or CTR (Guo et al., 2007).

Further studies in the near future are required to emphasize that ERp29 protein is a major component in both epididymal tissues and fluids of roe bucks.

A protein identified only in tissue fluids turned out to be alpha-1-antitrypsin, encoded by SERPINA1 gene and belonging to the family of serine protease inhibitors. Presumably, A1AT has anti-proteolytic effect to protect sperm cells of the male reproductive system against microorganisms. Furthermore, the presence of serpin 2 was detected in seminal vesicles and their secretions as well as in epididymis and deferent ducts of mouse (Lu et al., 2011).

The cytoskeleton is an intracellular system of protein filaments and microtubules which are responsible for the shape, internal organization and motility of spermatozoa (Virtanen et al., 1984). Among the commonly occurring structural proteins, six conserved polypeptides were identified in this study, namely: tubulin, actin, desmin,

vimentin, tropomyosin, and transgelin. Proteins regulating functions of spermatozoa cytoskeleton were detected as well, including calponin-1 isoform X1 which is characterized by its capability to bind actin, troponin, and calmodulin (Gong et al., 1993). In the presence of Ca^{2+} ions, this protein induces actin polymerisation and, simultaneously, inhibits depolymerisation of actin filaments (Kake et al., 1995). In addition, it is phosphorylated in vitro by kinase C, calmodulin and Ca^{2+} -dependent kinase II and kinase Rho (Kaneko et al., 2010). Calponin was identified in cytoskeleton of Sertoli cells in rat (Zhu et al., 2004).

In mammals, several studies have shown the expression of epsilon proteins belonging to the 14-3-3 family (Wong et al., 2009; Graf et al., 2011). They are characterized by the capability to form homo- and heterodimers and to bind with various proteins, like phosphatases, kinases or intra-membrane receptors (Dougherty and Morrison, 2004). Also, they can modify the conformation of proteins they enter into reaction with, thereby modulating their activity (Yaffe, 2002). This phenomenon is feasible owing to the presence of phosphoserine/threonine-containing specific motifs in proteins. These proteins are involved in multiple cellular processes, like for instance intercellular communication, signal transduction or formation of cellular structures (Aitken, 2006). Wong et al. (2009) demonstrated that 14-3-3 protein epsilon isoform theta plays a significant role in cell adhesion at the blood-testis barrier. In turn, investigations conducted by Sun et al. (2009) pointed to a key role of 14-3-3 proteins in maintaining the appropriate course of spermatogenesis process through the regulation of cell polarity and adhesion. Furthermore, these proteins were directly interacting with vimentin and tubulin (Wong et al., 2009). Finally, these proteins were found to have stabilizing and signaling roles in elongated phosphovimentin (Graf et al., 2011).

In conclusion, using proteomic techniques we succeeded to identify proteins that regulate the biological functions of spermatozoa and participate in their cytoskeleton organization within the different segments of the epididymal tissues and fluids in roe deer. The presence of these molecules in different periods of the reproductive season could indicate that they are involved in sperm maturation in the epididymis, in particular at the peak of the breeding season.

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