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AGE RELATED CHANGES IN PROTEOME AND QUALITY PARAMETERS OF SONICATED DOG EPIDIDYMAL SPERMATOZOA

Doctoral thesis conducted
at the Department of Animal Biochemistry and Biotechnology
under the supervision of
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WPŁYW WIEKU PSA NA ZMIANY W PROTEOMIE I PARAMETRACH JAKOŚCI PLEMNIKÓW NAJĄDRZOWYCH PODDANYCH SONIKACJI

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I dedicate this doctoral dissertation
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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper I: Zmudzinska, A.; Bromke, M.A.; Strzezek, R.; Zielinska, M.; Olejnik, B.; Mogielnicka-Brzozowska, M. **Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa and Sperm Structure Separation**. *Animals* 2022, *12*, 772. DOI: 10.3390/ani12060772 (**IF[1] = 3.231; pts[2] = 100**).

Paper II: Zmudzinska, A.; Wisniewski, J.; Mlynarz, P.; Olejnik, B.; Mogielnicka-Brzozowska, M. **Age-Dependent Variations in Functional Quality and Proteomic Characteristics of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa**. *International Journal of Molecular Sciences* 2022, *23*, 9143. DOI: 10.3390/ijms23169143 (**IF[1] = 6.208; pts[2] = 140**).

[1] *Impact Factor* indicates the scientometric index of the journal in the year of the paper publication

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List of Abbreviations

ACRBP	-	acrosin-binding protein
ACTB	-	actin, cytoplasmic 1
ALB	-	albumin
ASPM	-	abnormal spindle-like microcephaly-associated protein homolog
CASA	-	computer-assisted semen analysis
CE10	-	CE10 protein
CEMIP	-	hyaluronoglucosaminidase
CES5A	-	carboxylesterase 5A
CLU	-	clusterin
CRISP2	-	cysteine-rich secretory protein 2
CSNK1G1	-	peptidyl-prolyl cis-trans isomerase
DAPs	-	differentially abundant proteins
DPBS	-	Dulbecco's Phosphate-Buffered Saline
EF	-	epididymal fluid
ELSPBP1	-	epididymal sperm-binding protein 1
ENGASE	-	mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase
FAM135A	-	Family with sequence similarity 135 member A
G1, G2, G3	-	Group 1, Group 2, Group 3
GO	-	Gene Ontology
GPX5	-	glutathione peroxidase
HEXB	-	beta-N-acetylhexosaminidase
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
LCNL1	-	lipocalin-like protein
LOC607874	-	cystatin domain-containing protein
LRRN3	-	leucine-rich repeat neuronal protein 3
LTF	-	lactotransferrin
MW	-	molecular weight
NanoUPLC-Q-TOF/MS	-	nanoliquid chromatography coupled to quadrupole time-of-flight mass spectrometry
NPC2	-	intracellular cholesterol transporter/epididymal secretory protein E1
OD	-	optical density
OLFM4	-	olfactomedin 4
PB	-	protein bands
PFs	-	protein fractions
PLEKHH1	-	pleckstrin homolog, MyTH4, and FERM domain-containing H1
PTGDS	-	prostaglandin-H2 D-isomerase
PVDF	-	polyvinylidene fluoride
RIPA	-	Radioimmunoprecipitation Assay Buffer
RNASE9	-	inactive ribonuclease-like protein 9
SDS-PAGE	-	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SIPs	-	sperm intracellular proteins
SMAPs	-	sperm membrane-associated proteins
UBA52	-	ubiquitin-60S ribosomal protein L40
UPs	-	unique proteins
WAPdcp	-	WAP domain-containing protein

Abstract

This PhD thesis was performed in two studies. The first study was provided for proteomic analysis of intracellular and membrane-associated fractions of canine (*Canis lupus familiaris*) epididymal spermatozoa and additionally to find optimal sonication parameters for the epididymal sperm morphological structure separation and sperm protein isolation. Sperm samples were collected from 15 dogs. Protein fractions (PFs): intracellular (SIPs) and membrane-associated (SMAPs) were isolated. After sonication, sperm morphology was evaluated using Spermac Stain™. The sperm PFs were analyzed using gel electrophoresis (SDS-PAGE) and nanoliquid chromatography coupled to quadrupole time-of-flight mass spectrometry (NanoLC-Q-TOF/MS). UniProt database-supported identification resulted in 42 proteins identified in the SIPs and 153 proteins in the SMAPs. Differentially abundant proteins (DAPs) were found in SIPs and SMAPs. Based on a gene ontology analysis, the dominant molecular functions of SIPs were catalytic activity (50%) and binding (28%). Hydrolase activity (33%) and transferase activity (21%) functions were dominant for SMAPs. Bioinformatic analysis of SIPs and SMAPs showed their participation in important metabolic pathways in epididymal sperm, which may suggest their potential as sperm quality biomarkers.

The second study aimed to investigate the relationship between the functional quality and proteome of epididymal spermatozoa of dogs that were differing in age. The study was conducted on 30 male dogs that were divided into three age groups. G1 – 12 to 41 months old, G2 – 42 to 77 months old, and G3 – 78 to 132 months old. The sperm samples were assessed using a computer-assisted semen analysis (CASA). The epididymal sperm proteins were analyzed using SDS-PAGE, NanoUPLC-Q-TOF/MS and bioinformatic tools. The sperm quality parameters were significantly lower in older dogs. NanoUPLC-Q-TOF/MS identification resulted in 865 proteins that were found in the G1, 472 in G2, and 435 in G3.

There were seven proteins that were present in all three age groups, and four of them (ACTB, CE10, NPC2, CRISP2) showed significant changes among the studied groups.

The studies showed, for the first time, mass spectrometry and bioinformatic analysis of intracellular and membrane-associated proteins of canine epididymal spermatozoa. Additionally, sonication was used for the separation of dog epididymal sperm morphological elements (heads, tails and acrosomes). The results revealed the presence of DAPs in both sperm protein fractions significant for sperm function and fertilizing ability. Additionally, the age-dependent variations were detected in the sperm proteome composition and were related to important metabolic pathways, which might suggest that some proteins implicated in sperm maturation could be potential aging biomarkers.

Keywords: epididymal spermatozoa; semen quality; aging; proteins; sonication; canine

Abstract (Polish)

Przedłożona rozprawa doktorska została przygotowana w oparciu o wyniki uzyskane i zaprezentowane w cyklu dwóch publikacji naukowych. Pierwsze badanie miało na celu analizę proteomiczną wewnątrzkomórkowych i związanych z błoną frakcji plemników najądrzowych psa oraz dodatkowo określenie optymalnych parametrów sonikacji do separacji struktur morfologicznych plemników najądrzowych i izolacji białek plemników. Plemniki zostały pobrane od 15 psów, wyizolowano z nich następujące frakcje białkowe: wewnątrzkomórkowe (SIPs) oraz związane z błoną (SMAPs). Po sonikacji oceniano morfologię plemników przy użyciu Spermac Stain™. Frakcje białkowe plemników analizowano za pomocą elektroforezy żelowej (SDS-PAGE) i ultrasprawnej chromatografii cieczowej o nano przepływach połączonej z tandemową spektrometrią mas typu kwadrupol z analizatorem czasu przelotu (NanoUPLC-Q-TOF/MS). Identyfikacja oparta na bazie UniProt pozwoliła wyróżnić 42 białka zidentyfikowane jako SIPs i 153 białka zidentyfikowane jako SMAPs. W obu frakcjach wyodrębniono białka charakteryzujące się zróżnicowaną zawartością (DAPs). Na podstawie analizy ontologii genów określono dominujące funkcje molekularne. W przypadku SIPs były to: aktywność katalityczna (50%) i funkcja wiązania innych substancji (28%). Aktywność hydrolazowa (33%) i transferazowa (21%) były dominujące we frakcji SMAPs. Analiza bioinformatyczna SIPs i SMAPs wykazała ich udział w ważnych szlakach metabolicznych plemników najądrzowych, co może sugerować ich potencjał jako biomarkerów jakości nasienia.

Drugie badanie miało na celu określenie związku pomiędzy jakością funkcjonalną a proteomem plemników najądrzowych psów w różnym wieku. Badanie przeprowadzono na 30 samcach, które podzielono na trzy grupy wiekowe: G1 – 12 do 41 miesięcy, G2 – 42 do 77 miesięcy i G3 – 78 do 132 miesięcy. Próbki plemników oceniano za pomocą komputerowego systemu analizy nasienia (CASA). Białka plemników najądrzowych

analizowano za pomocą SDS-PAGE, NanoUPLC-Q-TOF/MS i narzędzi bioinformatycznych. Parametry jakości nasienia były istotnie niższe u starszych psów. Identyfikacja NanoUPLC-Q-TOF/MS pozwoliła wyróżnić 865 białek w G1, 472 w G2 i 435 w G3. Wszystkie trzy grupy wiekowe charakteryzowały się obecnością siedmiu białek, a cztery z nich (ACTB, CE10, NPC2, CRISP2) wykazywały istotne zmiany zawartości wśród badanych grup.

W badaniach po raz pierwszy przedstawiono wyniki spektrometrii mas i analizy bioinformatycznej białek wewnątrzkomórkowych i związanych z błoną plemników najądrzowych psa. Dodatkowo zastosowano sonikację do separacji elementów morfologicznych plemników najądrzowych psa (główki, witki i akrosomu). Wyniki wskazują na obecność DAPs istotnych dla funkcji plemników i ich zdolności do zapłodnienia w obu analizowanych frakcjach białkowych. Ponadto wykryto zależne od wieku zmiany w składzie proteomu plemników, które były powiązane z ważnymi szlakami metabolicznymi, co może sugerować, że niektóre białka zaangażowane w dojrzewanie plemników mogą być potencjalnymi biomarkerami procesów starzenia.

Słowa kluczowe: plemniki najądrzowe; jakość nasienia; proces starzeniowy; białka; sonikacja; pies

1. Introduction

The increasing popularity of dog breeding and the fact that this knowledge may be transferred to endangered *Canidae* species provides reasons for many scientists to place an increasing emphasis on understanding the specificity of canine reproduction (Luvoni and Morselli 2016). Epididymal spermatozoa have great potential in current dog reproductive technologies. In the case of azoospermia, or when the male dies, the recovery of the epididymal spermatozoa opens up a new possibility for generating offspring (Luvoni and Morselli 2016; Hassan et al. 2021). The epididymal spermatozoa may be obtained by various *ex vivo* or *in vivo* techniques and then frozen for later use in assisted reproduction technologies (Varesi et al. 2013; Chaveiro et al. 2015). Since the dog is also a good model for human reproduction, the acquired knowledge may be used to understand problems in human infertility and improve assisted reproduction methods (Kirchhoff 2002; Switonski 2014) also regarding aging (Ellerbrock et al. 1994; Kirchhoff 2002).

For dog breeders, it is important to know how long they may use semen from old high-quality reproducers without losing the semen quality. Thus, the possible effect of male age on fertility has become increasingly important in animal reproduction. While the influence of female age on fertility is well established, the impact of male age is poorly characterized (Silva et al. 2019). It has been observed that age is associated with diminished dog sperm quality (Rijsselaere et al. 2007; Rota et al. 2016; Bhanmeechao et al. 2018). Age-dependent changes in sperm quality have been reported in the rat (Wang et al. 1993; Wright et al. 1993), hamster (Calvo et al. 1999), ferret (Wolf et al. 2000), cat (Elcock and Schoning 1984), boar (Fraser et al. 2020), and in human (Collodel et al. 2021).

Many different techniques have been used to assess the reproductive potential of male dogs, but little is still known about the protein composition changes during the aging processes of the epididymis and epididymal spermatozoa. Proteomic studies contribute to

explaining the role of proteins at various stages of epididymal sperm maturation, capacitation, acrosomal reaction and sperm–egg fusion (Mann and Lutwak-Mann 1951; Mogielnicka-Brzozowska and Kordan 2011). The epididymis undergoes morphological changes with aging, which probably affects sperm maturation (Calvo et al. 1999; Elzanaty and Malm 2007). However, the particular molecular mechanisms of epididymal sperm aging based on changes in the protein structures and function have not yet been determined. Knowledge of the functions of proteins offers potential opportunities to use them as markers of biological value in reproductive processes or as future components of contraceptives (Gobello et al. 2002; Mogielnicka-Brzozowska and Kordan 2011). The search for marker proteins associated with the process of sperm preservation is of great importance as it allows the identification of males or semen samples with greater or lesser suitability in this regard (Jobim et al. 2011; Schafer-Somi and Palme 2016).

The sonication is a method used to break cells to isolate their components (Toyoshima et al. 2019). Sonication was also used for protein extraction from ejaculated and epididymal spermatozoa of human and stallion (Dias et al. 2014; Fanny et al. 2018; Guasti et al. 2020). Sonicated spermatozoa were found to be suitable for the preparation of membrane fractions used to identify proteins that mediate sperm–egg interactions (Baker et al. 2002). In this study we used sonication to improve protein extraction from epididymal spermatozoa and to separate epididymal sperm structures.

Epididymal sperm are different from those ejaculated because they are covered only by epididymal fluid proteins and do not come into contact with accessory sex glands-secreted proteins. To understand the epididymal sperm fertilizing potential and the role of particular proteins in it, it would be necessary to provide proteome analysis together with recognition of most important sperm metabolic pathways responsible for the quality of the sperm.

2. Aims

Main objective of this research was to investigate the relationship between the functional quality and proteome of epididymal spermatozoa of dogs that were differing in age. Additionally, studies provide the proteomic analysis of intracellular and membrane-associated fractions of canine epididymal spermatozoa and to find optimal sonication parameters for the epididymal sperm morphological structure separation and sperm protein isolation. The selection of possible protein markers derived from the dog's epididymal spermatozoa may constitute a diagnostic potential and support the treatment of diseases of the reproductive system in the future.

The specific aims were to:

- **I.** analyze the effect of various sonication parameters on the type and percentage of secondary morphological changes in the dog's epididymal spermatozoa (**Paper I**);
- **II.** analyze the influence of various sonication parameters on the total protein content in the intracellular and membrane-associated fractions of the sperm (**Paper I**);
- **III.** demonstrate the differences in the protein composition of intracellular and membrane-associated fractions of the sperm and to identify selected proteins by mass spectrometry (**Paper I**);
- **IV.** study the parameters of cauda epididymal sperm quality (concentration, motility) and to search for the relationship between the above-mentioned parameters and the age of the dogs (**Paper II**);
- **V.** analyze the effect of male age on morphological changes in the dog's epididymal spermatozoa (**Paper II**);
- **VI.** analyze total proteome of the dog epididymal spermatozoa according to the age group (**Paper II**);
- **VII.** identify proteins showing correlation with the age of dogs in order to distinguish epididymal sperm proteins as potential markers of aging (**Paper II**).

3. Material and Methods

3.1. Institutional Review Board Statement

The study was performed under the guidance of Directive 63/2010/EU and the Journal of Laws of the Republic of Poland (2017) regarding the protection of animals that are used for scientific or educational purposes. The exemption letter was obtained from the Local Ethics Committee for Animal Experimentation, Olsztyn, Poland (LKE/01/2022). The authors have permission to conduct animal experiments according to the Polish Laboratory Animal Science Association (Numbers: 1432/2015; 1508/2015).

3.2. Animals

The studies were performed on mixed-breed dogs from 1 to 11 years old with body weight from 9 to 33 kg. Depending on the research, the biological material came from 15 to 30 dogs. The dogs were fed and kept in the same environmental conditions in the Shelter for Homeless Animals in Tomaryny (Poland). All of the dogs were presented for a routine orchiectomy by a veterinary doctor as a part of the program to prevent animal homelessness and promote adoption.

3.3. Cauda epididymal semen collection

The materials, i.e., the testis with the epididymis, were placed in sterile plastic containers in 0.9% NaCl solution and then in a thermobox at a temperature of 4 ˚C and delivered within one hour to the laboratory of the Department of Animal Biochemistry and Biotechnology (University of Warmia and Mazury in Olsztyn, Poland). Immediately after that, the gonads were washed with DPBS. The cauda epididymal tissue was cut carefully with a sterile scalpel to avoid sectioning the blood vessels. The effluent of the epididymal semen was aspirated from the cauda epididymal tissue using an automatic pipette according to Ramos Angrimani et al. (2017) with modification.

3.4. Laboratory assays for spermatozoa evaluation

3.4.1. Spermatozoa quality assessment

The epididymal sperm concentration was determined using a Bürker chamber under a light microscope (Olympus BX41TF, Tokyo, Japan). The sperm samples were subsequently assessed using a CASA system (HTM-IVOS, 12.3, Hamilton-Thorne Biosciences). The procedure was described previously by Mogielnicka-Brzozowska et al. (2020).

3.4.2. Morphology assessment of epididymal spermatozoa

The dog epididymal spermatozoa were prepared as smears on glass slides using 10 µL of each sample (1×10^8 spermatozoa) and left to dry on a thermoblock (5 min, 37 ˚C). Spermac Stain™ (FertiPro) staining was then performed according to the manufacturer's recommendations with modifications.

3.5. Preliminary sample preparation for the sonication

The sperm samples were centrifuged at 800× g for 10 min, 4 ˚C to remove epididymal fluid. The supernatant was removed, and the sperm pellet was resuspended in 1 mL DPBS and again centrifuged at 800× g for 10 min, 4 ˚C, to remove loosely bound proteins (Cebi et al. 2016).

3.5.1. Sonication and sample preparation for analysis

Prepared epididymal spermatozoa samples were placed in an ice bath and subjected to sonication using the Omni Sonic Ruptor 250 Ultrasonic Homogenizer (Omni International). Following sonication, the sperm samples were centrifuged (8000× g for 10 min at 4 ˚C). The resulting supernatant containing SIPs was collected into another Eppendorf tube. To the remaining sperm pellet, an aliquot of 1 mL of RIPA was added, and the sample was dissolved, incubated for 5 min at 4 ˚C, and then vortexed and left overnight in buffer (Intasqui et al. 2018; Mogielnicka-Brzozowska et al. 2020) with modifications. The

samples were then centrifuged at 8000× g for 10 min, 4 ˚C to obtain a clear lysate of the SMAPs. The clear lysate was collected into another Eppendorf tube. The SIPs and SMAPs were frozen and kept at −80 ˚C until further analyses.

3.5.2. Total protein content measurement

The total protein content was measured using Bradford Reagent (Sigma-Aldrich/B6916).

3.6. Polyacrylamide gel electrophoresis (SDS-PAGE)

For proteomic analysis, prepared samples isolated from the spermatozoa of individual dogs were pooled. Each pool was run in triplicate (technical replicate). The SDS-PAGE procedure was described previously by Mogielnicka-Brzozowska et al. (2020).

3.7. Identification of proteins by mass spectrometry

3.7.1. In gel samples – trypsin digestion

The SDS-PAGE gel samples were processed according to the protocol described by Shevchenko et al. (2006).

3.7.2. Liquid samples

3.7.2.1. Sample preparation for LC-MS

The sperm samples were extracted, which allowed to separate the polar and non-polar metabolites. The upper non-polar phase for analysis of lipids was collected into a new tube. After a short centrifugation (5 min), the lower methanol-water phase was transferred into a new tube. Finally, the residual methanol-water phase was discarded, and the pellets were frozen at −80 ˚C. The collected liquid extracts were dried and stored at −80 ˚C prior to the LC-MS analysis.

3.7.2.2. In solution digestion

The protein pellets were dissolved in 10 µL 6M Guanidine-HCl in a 25 mM bicarbonate ammonium solution (pH 8.0). A total of 1 µL of 200 mM DTT in 25 mM bicarbonate ammonium solution (pH 8.0) was then added to each sample and incubated for 30 min at 37 °C. Then protein reduction and alkylation was made. Protein digestion was performed by adding a trypsin solution. The samples were desalted on Pierce™ C18 Spin Columns (Thermo Fisher Scientific), vacuum-dried, and resuspended in acetonitrile.

3.7.3. NanoUPLC-Q-TOF/MS analysis

Waters Acquity liquid chromatography M-Class system (Waters Corp.) equipped with a Peptide BEH C18 analytical column (Waters Corp.), and Symmetry C18 precolumn (Waters Corp.) was performed to separate the digested samples. A mass spectrometry (MS) analysis was performed using Synapt G2-Si (Waters Corp.) with a nano-electrospray ionization (nESI) source, operating under a positive ion mode. Raw chromatography files were analyzed with Byonic software (Protein Metrics). The detected peptides were compared to the SWISSPROT dog proteome (CANLF).

3.8. Western blotting analysis

LC-MS analyses allowed to identify proteins that were confirmed by the western blotting. Sperm extract protein samples were separated by 12% SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore) according to the previously described method (Towbin et al. 1979). The blots were incubated with one of the primary antibodies (Thermo Fisher Scientific) and then with Peroxidase AffiniPure Goat Anti-Rabbit secondary antibody (Jackson ImmunoResearch).

3.9. Gene ontology and functional annotation

In **Paper I** functional enrichment of the proteins of the canine epididymal sperm in GO categories: molecular function (GO:MF), biological process (GO:BP), protein class and

pathways were obtained from PANTHER Classification System v. 16.0, online tool. In **Paper II** for the functional enrichment of proteins that were present in the dog epididymal spermatozoa in Gene Ontology (GO) categories: molecular function (GO:MF), biological process (GO:BP), and cellular component (GO:CC), was performed with the g:Profiler online tool.

The significance of the dog epididymal spermatozoa proteins were analyzed in the KEGG pathway database and the KOBAS 3.0 protein functional annotation tool (Xie et al. 2011). A Venn diagram was constructed using a web tool. Heatmaps, GO plots, and morphological characteristic plots were performed using GraphPad Prism software (GraphPad Prism v.9.2.0).

3.10. Statistical analysis

The data analysis was carried out using Statistica version 13.1 (StatSoft). The results were presented as means with a standard error (mean ± SE). Results were analyzed with ANOVA for independent samples to determine statistically significant differences. In **Paper I**, the Student's t-test was also used for analyses.

The detailed methodology was described in Paper I and Paper II.

4. Results and Summary of Papers

4.1. Paper I

The results show that sonication for 10 min at 150 W can be considered for the separation of dog epididymal sperm structures. After the above-mentioned treatment 97.4 ± 0.8% (mean ± SE) of dog epididymal spermatozoa were found to be damaged. Total number of damaged spermatozoa was higher ($p \leq 0.05$) than in the rest of tested variants. Damaged structures as detached sperm tail, acrosome loss and damaged acrosomal membrane noted the highest percentage in the above-mentioned sonication variant and they were: 39.4 ± 9.6%, 62.4 ± 6.1% and 31.8 ± 2.2%, respectively.

It can be stated that sonication with 150 W, 10 min, can be used to isolate the head and tail of dog epididymal spermatozoa. The results are consistent with studies by other authors in which sonication was used to isolate sperm outer acrosomal membrane (Somanath and Gandhi 2004) and to separate sperm head from the tail (Amaral et al. 2013). In this study it was demonstrated, that sonication with appropriate time and power resulted in the effective separation of dog epididymal sperm heads from tails.

A higher sonication power (150 W) resulted in more protein being released from the epididymal spermatozoa. The use of sonication slightly increased the content of SIPs in all sonication variants (from 0.05 ± 0.01 mg/mL, up to 0.17 ± 0.03 mg/mL), while the content of SMAPs decreased, from 1.46 ± 0.01 mg/mL to 1.38 ± 0.02 mg/mL ($p \leq 0.05$). Total protein content was not significantly influenced by sonication variant ($p > 0.05$).

It was shown that the cytoplasm from the sperm cells broken by ultrasound may leak out from the spermatozoa (Qin et al. 2018).

To date, an SDS-PAGE analysis of the SIPs and SMAPs of dog epididymal spermatozoa has not been shown. The SDS-PAGE protein profiles of pooled SIPs and SMAPs of the dog cauda epididymal spermatozoa were analyzed. The protein profile of SIPs

presented 21 PFs and SMAPs showed 19 PFs. OD analysis in PFs showed higher protein content ($p \leq 0.001$) for five PFs in the SIPs compared with corresponding (showing the same MW) SMAPs and for five PFs in the SMAPs when compared to the corresponding PFs of the SIPs. These ten differentially expressed PFs were analyzed using NanoUPLC-Q-TOF/MS and UniProt identifiers.

UniProt database-supported identification resulted in 42 proteins found in the SIPs and 153 proteins in the SMAPs. Ten polypeptides with the highest scores were identified as DAPs in the SIPs fraction: LTF, CES5A, ALB, OLFM4, PTGDS, GPX5, LCNL1, CSNK1G1, NPC2 and LRRN3. Eight polypeptides were identified as DAPs in the SMAPs fraction: NPC2, LTF, CRISP2, WAPdcp, ACTB, UBA52, HEXB and ACRBP.

According to the authors' knowledge, this is the first study concerning a mass spectrometry analysis of the protein present in the dog epididymal spermatozoa. It is worth noting that the SMAPs were much more abundant in different proteins than SIPs.

Based on GO enrichment, the dominant molecular functions of SIPs were catalytic activity (50%), binding (28%) and molecular function regulator (10%). The main biological processes shown for SIPs were the cellular process (26%), the metabolic process (18%) and biological regulation (18%). Dominant molecular functions of SMAPs were hydrolase activity (33%), transferase activity (21%) and catalytic activity, acting on a protein (17%). The biological processes dominant in SMAPs were the cellular process (29%), biological regulation (14%) and the metabolic process (13%). The GO pathways showed mainly glycolysis (23%), the integrin signaling pathway (11%) and the Wnt signaling pathway (11%) for SIPs. For the SMAPs, the main pathways were inflammation mediated by chemokine and cytokine signaling pathways (7%), the Wnt signaling pathway (7%) and the Alzheimer disease–presenilin pathway (5%).

The detailed results have been described in Paper I.

4.2. Paper II

Results showed, that the sperm concentration, VSL, and VCL that were measured in the three age groups differed statistically significantly ($p < 0.05$) between G1 and G3. The results also showed significant differences ($p < 0.05$) in the percentage of TMOT, PMOT, and VAP values between G3 compared with G1 and G2. Also a statistically significant negative correlation with dog age group was shown for the sperm concentration, TMOT, PMOT, VAP, VCL, and VSL. These findings are in agreement with the results of Verón et al. (2018), who have shown that several sperm quality parameters for men's ejaculate were also negatively affected by age. Bhanmeechao et al. (2018) also have shown that dog age was negatively correlated with epididymal sperm motility, sperm vigor, and viability.

When comparing the percentage of morphological defects of the dog epididymal spermatozoa among the three age groups, it was shown that the highest percentage of normal spermatozoa was found in G1 (76.3 ± 2.1%), and the lowest value was in G3 (64.0 ± 4.0%). The differences were statistically significant ($p < 0.05$) between these two groups. Sperm morphological defects as a irregular and asymmetrical midpiece occurred in the highest number in G3 (6.6 ± 1.4%) and G2 (9.5 ± 0.4%), respectively, while the lowest percentage of above-mentioned defects was found in G1 (1.3 ± 0.4% and 3.8 ± 2.0%, respectively). Results were statistically significant ($p < 0.05$). Other sperm morphological defects did not have significant differences among the age groups. Some authors described a positive correlation between male dog age and the percentage of sperm defects (Bhanmeechao et al. 2018). Similar results were presented in the current study. The highest percentage of morphological damage was noted in the epididymal sperm midpiece. This may indicate disturbances in the ATP production in aging sperm.

Additionally, the SDS-PAGE protein profiles of dog epididymal spermatozoa extracts were analyzed to find the differences between age groups (G1, G2, and G3). The

protein profiles for all three age groups were similar and were characterized by the presence of 36 PFs. An OD analysis of PFs showed higher protein content ($p < 0.05$) for one PF when compared with the corresponding (showing the same MW) PFs among the age groups. To date, SDS-PAGE of the dog epididymal spermatozoa proteins according to age has not been shown. In this study, there were no differences that were found in the number of protein fractions or in the range of molecular weights in the gel image presenting epididymal sperm proteins depending on the age of the dog. Only the 68 kDa protein showed changes in their intensity depending on the age group. According to the authors' earlier results, this might be LTF or CES5A.

Mass spectrometry analysis identified a total of 1772 proteins in all age groups: G1 – 865 proteins, G2 – 472 proteins, and G3 – 435 proteins. UniProt database-supported identification resulted in two UPs that were identified in G1 (GPX5 and CEMIP), five UPs identified in G2 (ENGASE, RNASE9, CLU, PLEKHH1 and ELSPBP1), and three UPs that were identified in G3 (LOC607874, FAM135A and ASPM), which were present in a statistically significant manner. Protein identification also resulted in seven proteins that were present in all three age groups (G1, G2, G3) and two proteins that were found in G1 and G2. The seven common proteins in groups G1, G2, and G3 were: LTF, ACTB, PTGDS, CE10, NPC2, ALB and CRISP2. The two proteins that were detected in both G1 and G2 were WAPdcp and LCNL1.

The study also included a semi-quantity analysis of protein content that was based on intensity measurement. When comparing the intensity of seven common proteins (presented in all groups G1, G2, and G3) among the age groups, statistical differences were found in the intensity of four proteins: ACTB, CE10, NPC2 and CRISP2. Expression of above-mentioned four proteins was analyzed with western blotting analysis, in which ACTB showed significant differences ($p < 0.05$) in G3 compared to G1 and G2. Low levels of

ACTB, CE10, NPC2, and CRISP2 in the epididymal sperm may be associated with incomplete maturity or aging of the dogs. ACTB is responsible for cell volume regulation, it builds the sperm cytoskeleton (Pedersen et al. 2001) and its role in sperm motility and capacitation was known (Cabello-Agüeros et al. 2003; Breitbart et al. 2005; Naresh 2016). NPC2 was found on the acrosome and equatorial region of the sperm (Osterhoff et al. 1994) and it is implicated in cell cholesterol metabolism (Naureckiene et al. 2000). A decrease in CRISP2 amount in sperm is associated with male infertility (Jamsai et al. 2008; Zhou et al. 2015; Gottschalk et al. 2016). CE10 was found for the first time in dogs and it may act as extracellular proteinase inhibitor (Kirchhoff et al. 1991).

Gene ontology analysis showed similar molecular functions, biological processes and cellular component for all the age groups.

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG), the pathways for each age group were determined. For the G1 group has been shown mainly protein export, protein processing in the endoplasmic reticulum and Ras signaling pathway. For the G2 group, the main pathways were: Salmonella infection, transcriptional misregulation in cancer and adherens junction. For the G3 group, the main metabolic pathways were established as mucin-type O-glycan biosynthesis, porphyrin chlorophyll metabolism and cell cycle. It seems that in different dog ages, different metabolic pathways in epididymal spermatozoa are intensified, which might be connected with the aging processes.

The detailed results have been described in Paper II.

5. Conclusions

1. Sonication for 10 min at 150 W can be used to separate of individual dog epididymal sperm structures and improve protein extraction.
2. Use of mass spectrometry and bioinformatics tools to analyze the proteome of canine epididymal spermatozoa allowed for obtaining protein profiles of the intracellular and membrane-associated fractions of the above-mentioned cells.
3. The results indicate the presence of proteins showing different expression in particular fractions of sperm proteins, they are crucial for the proper function of sperm and their fertilization ability. These proteins have been confirmed to be involved in important sperm metabolic pathways.
4. The aging of dogs is associated with a decrease in the functional quality of epididymal spermatozoa, which may result in reduced fertility or even sterility in the tested individuals.
5. Differences in the protein profiles of sperm in young, middle-aged and older dogs related to changes in the sperm metabolic pathways were found. It may affect the fertilization ability of the above-mentioned cells.
6. The results of the above studies concerning the marker proteins of the maturation and aging process of epididymal sperm can be used in clinical andrology and in the improvement of the technology of controlled reproduction of dogs.

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Papers I-II

Paper I

Zmudzinska, A.; Bromke, M.A.; Strzezek, R.; Zielinska, M.; Olejnik, B.; Mogielnicka-Brzozowska, M. **Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa and Sperm Structure Separation**. *Animals* **2022**, *12*, 77

animals

MDPI

Article

Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa and Sperm Structure Separation

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Simple Summary: Epididymal spermatozoa have great potential in current dog reproductive technologies. In the case of azoospermia or when the male dies, the recovery of epididymal spermatozoa opens new possibilities for reproduction. It is of great importance to analyze the quality of the sperm in such cases. Proteomic studies contribute to explaining the role of proteins at various stages of epididymal sperm maturation and offer potential opportunities to use them as markers of sperm quality. The present study showed, for the first time, mass spectrometry and bioinformatic analysis of intracellular and membrane-associated proteins of canine epididymal spermatozoa. Additionally, sonication was used for the separation of dog epididymal sperm morphological elements (heads, tails and acrosomes). The results revealed the presence of differentially abundant proteins in both sperm protein fractions significant for sperm function and fertilizing ability. It was also shown that these proteins participate in important sperm metabolic pathways, which may suggest their potential as sperm quality biomarkers.

Abstract: This study was provided for proteomic analysis of intracellular and membrane-associated fractions of canine (*Canis lupus familiaris*) epididymal spermatozoa and additionally to find optimal sonication parameters for the epididymal sperm morphological structure separation and sperm protein isolation. Sperm samples were collected from 15 dogs. Sperm protein fractions: intracellular (SIPs) and membrane-associated (SMAPs) were isolated. After sonication, sperm morphology was evaluated using Spermac Stain™. The sperm protein fractions were analyzed using gel electrophoresis (SDS-PAGE) and nanoliquid chromatography coupled to quadrupole time-of-flight mass spectrometry (NanoLC-Q-TOF/MS). UniProt database-supported identification resulted in 42 proteins identified in the SIPs and 153 proteins in the SMAPs. Differentially abundant proteins (DAPs) were found in SIPs and SMAPs. Based on a gene ontology analysis, the dominant molecular functions of SIPs were catalytic activity (50%) and binding (28%). Hydrolase activity (33%) and transferase activity (21%) functions were dominant for SMAPs. Bioinformatic analysis of SIPs and SMAPs showed their participation in important metabolic pathways in epididymal sperm, which may suggest their potential as sperm quality biomarkers. The use of sonication 150 W, 10 min, may be recommended for the separation of dog epididymal sperm heads, tails, acrosomes and the protein isolation.

Keywords: epididymal spermatozoa; semen quality; proteomic; sonication; canine

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1. Introduction

The increasing popularity of dog breeding and the fact that this knowledge may be transferred to endangered Canidae species provides reasons for many scientists to place an increasing emphasis on understanding the specificity of canine reproduction [1]. Epididymal spermatozoa have great potential in current dog reproductive technologies. In the case of azoospermia, or when the male dies, the recovery of the epididymal spermatozoa opens up a new possibility for generating offspring [1,2]. The epididymal spermatozoa may be obtained by various ex vivo or in vivo techniques and then frozen for later use in assisted reproduction technologies [3,4]. Since the dog is also a good model for human reproduction, the acquired knowledge may be used to understand problems in human infertility and improve assisted reproduction methods [5,6].

The sonication is a method used to break cells to isolate their components [7]. It is a physical method based on the phenomenon of cavitation, when the ultrasounds may also affect cell membranes [8]. This method was successfully used to isolate sperm structures, such as the sperm head [9,10], acrosome [11] and tail [12]. Sonication was also used for protein extraction from ejaculated and epididymal spermatozoa of human and stallion subjects [13–15]. Sonicated spermatozoa were found to be suitable for the preparation of membrane fractions used to identify proteins that mediate sperm–egg interactions [16].

Proteomic studies contribute to explaining the role of proteins at various stages of epididymal sperm maturation, capacitation, acrosomal reaction and sperm–egg fusion [17,18]. Bioinformatic analyses based on gene ontology provide insight into the protein localization, distribution and participation in exact metabolic pathways [19]. Knowledge of the functions of proteins offers potential opportunities to use them as markers of biological value in reproductive processes or as future components of contraceptives [18,20]. The search for marker proteins associated with the process of sperm preservation is of great importance as it allows the identification of males or semen samples with greater or lesser suitability in this regard [21,22]. Seminal plasma and ejaculated sperm proteome was proposed by Aquino-Cortez et al. [23] and Araujo et al. [24,25]. However, according to the authors' knowledge, the proteomic study of dog epididymal spermatozoa was not shown until now. Epididymal spermatozoa are different from those ejaculated because they are covered only by epididymal fluid proteins and do not come into contact with accessory glands-secreted proteins. To understand the epididymal sperm fertilizing potential and the role of particular proteins in it, it would be necessary to provide proteome analysis together with recognition of most important sperm metabolic pathways responsible for the quality of the sperm.

Since there are no published reports in this field, it was hypothesized that intracellular and membrane-associated fractions of canine epididymal spermatozoa were differentially composed and that the application of optimal sonication parameters could be established for the separation of the morphological structures of epididymal spermatozoa and protein isolation.

Therefore, the aim of this study was to (1) provide the proteomic analysis of intracellular and membrane-associated fractions of canine (*Canis lupus familiaris*) epididymal spermatozoa and (2) to find optimal sonication parameters for the epididymal sperm morphological structure separation and sperm protein isolation.

2. Materials and Methods

2.1. Chemicals and Media

All chemicals of the highest purity were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals

The study was performed on 15 mixed-breed dogs (1 to 6 years old; mean 3.5 years) with body weight from 12 to 33 kg (mean 21.5 kg) of unknown fertility. The dogs were fed and kept in the same environmental conditions in the Shelter for Homeless Animals

in Tomaryny (Poland). All of the dogs were presented for a routine orchiectomy by a qualified veterinary doctor as a part of the program of preventing animal homelessness and promoting adoption. The consent form was achieved from the director of the shelter.

2.3. Cauda Epididymal Semen Collection

The materials, i.e., the testis with epididymis, were placed in sterile plastic containers in 0.9% NaCl solution and then in a thermobox at a temperature of 4 °C and delivered within one hour to the laboratory of the Department of Animal Biochemistry and Biotechnology (University of Warmia and Mazury in Olsztyn, Poland). Immediately after that, the gonads were washed with DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco, Grand Island, NY, USA). The cauda epididymal tissue was cut carefully with a sterile scalpel to avoid sectioning the blood vessels. The effluent of the epididymal semen was aspirated from the cauda epididymal tissue using an automatic pipette [26] with modifications. Samples obtained from the cauda epididymis (right and left) of the same animal were pooled.

2.4. Spermatozoa Quality Assessment

The epididymal sperm concentration was determined using a Bürker chamber under a light microscope (Olympus BX41TF, Tokyo, Japan). The concentration of epididymal spermatozoa in the studied group of dogs ranged from 21.8 to 56.4 $\times$ 10^8 spermatozoa/mL, (36.9 $\pm$ 2.3 $\times$ 10^8 spermatozoa/mL, mean $\pm$ SE).

The sperm samples were subsequently assessed using a computer-assisted semen analysis (CASA-system, HTM-IVOS, 12.3, Hamilton-Thorne Biosciences, MA, USA). The procedure was described previously by Mogielnicka-Brzozowska et al. [27]. The following software settings recommended by the manufacturer for canine sperm analyses were used: frame acquired—30, frame acquisition rate—60 Hz, minimum cell contrast—75, minimum cell size—6 pixels, straightness threshold—75%, path velocity threshold—100 µm/s, low average path velocity (VAP) cut-off—9.9 µm/s, low straight-line velocity (VSL) cut-off—20 µm/s, static size gates—0.80–4.93, static intensity gates—0.49–1.68, static elongation gates—22–84. The percentages of spermatozoa with total motility (TMOT, %) and progressive motility (PMOT, %), were analyzed in each epididymal sperm sample.

2.5. Sample Preparation for the Sonication

For the sonication, the epididymal sperm concentration was established at 1 $\times$ 10^8 spermatozoa in 300 µL DPBS, and samples were stored at 4 °C no longer than two hours until further analyses. The sperm samples were centrifuged at 800$\times$ *g* for 10 min, 4 °C to remove epididymal fluid (EF). The remaining supernatant (EF) was removed, and the sperm pellet was resuspended in 1 mL DPBS (Gibco) and again centrifuged at 800$\times$ *g* for 10 min, 4 °C, to remove loosely bound proteins [28]. The remaining supernatant was removed and discarded.

The epididymal spermatozoa samples were placed in an ice bath and subjected to sonication using the Omni Sonic Ruptor 250 Ultrasonic Homogenizer (Omni International, Kennesaw, GA, USA). The maximum sonication power was 250 W. The following sonication parameters were used for the experiment variants: C—control (without sonication); S1—5 min, 50 W; S2—10 min, 50 W; S3—30 min, 50 W; S4—10 min, 150 W. The frequency of the ultrasound in all the sonication variants was 60 kHz. Following sonication, the sperm samples were centrifuged (8000$\times$ *g* for 10 min at 4 °C). The resulting supernatant containing sperm intracellular proteins (SIPs) was collected into another Eppendorf tube. To the remaining sperm pellet, an aliquot of 1 mL of Radioimmunoprecipitation Assay Buffer (RIPA), containing 50 mM Tris-HCl; 150 mM NaCl; 1% (*v*/*v*) Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; and ddH_2O, pH 7.4 was added, and the sample was dissolved, incubated for 5 min at 4 °C, and then vortexed and left overnight in buffer ([27,29]; with modifications). Protease Inhibitor Cocktail (Sigma-Aldrich/P8340, St. Louis, MO, USA) was added both to the SIPs and the remaining sperm pellet. The samples were then centrifuged at 8000$\times$ *g* for 10 min, 4 °C to obtain a clear lysate of the sperm membrane-associated proteins (SMAPs). The clear

lysate was collected into another Eppendorf tube. The SIPs and SMAPs were frozen and kept at −80 °C until further analyses.

2.6. Morphology Assessment of Epididymal Spermatozoa Using Spermac Stain

The dog epididymal spermatozoa from each variant of the sonication (S1 to S4) were prepared as smears on glass slides using 10 µL of each sample (1×10^8 spermatozoa) and left to dry on a thermoblock (5 min, 37 °C). The control samples were prepared in the same way. Spermac Stain™ (FertiPro, Beernem, Belgium) staining was then performed according to the manufacturer's recommendations with modifications. The morphological defects caused by sonication in dog epididymal spermatozoa were examined under bright light microscopy at the magnification of 1000× (Olympus BX41TF). Approximately 200 spermatozoa were counted in each sperm sample. The spermatozoa were classified into two categories: undamaged (without secondary defects) or damaged (at least one secondary defect), according to the World Health Organization guidelines [30]. During the determination of the sperm head defects, attention was paid to the shape of the acrosome and continuity of the membranes surrounding the sperm nucleus (Figure 1). The sperm acrosome was found to be normal after dark green staining, and it formed a thin, smooth border at the top of the sperm head (Figure 1A). According to the protocol of Spermac Stain™ (FertiPro), the epididymal sperm fragments were stained as follows: the nucleus (red), the equatorial region (pale green) and the midpiece and the tail (green). When assessing the sperm tail, a detached tail was identified as a defect (Figure 1B). Two types of sperm head defects were recognized: acrosome loss (Figure 1C) and a damaged acrosomal membrane (Figure 1D).

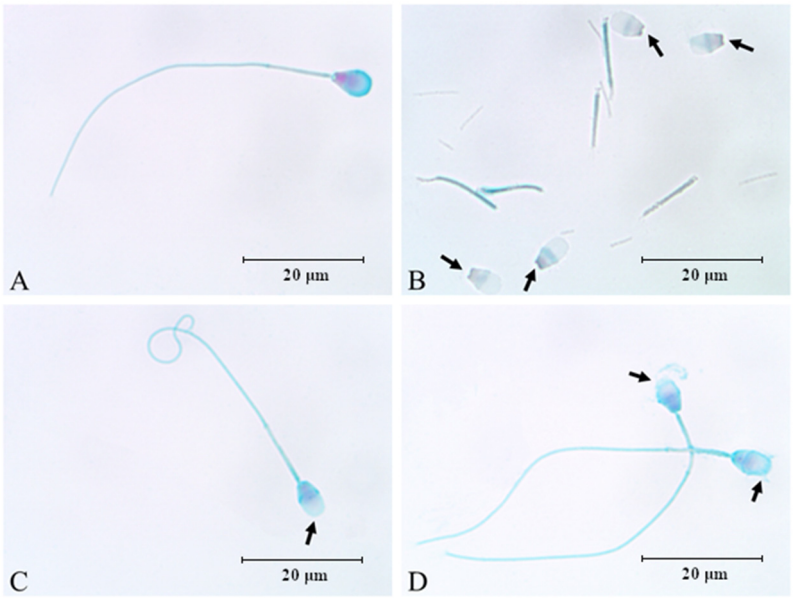

Figure 1. Morphological changes in dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15) after sonication stained with the Spermac stain (1000× magnification in light microscope). (**A**)—normal epididymal sperm; (**B**)—damaged epididymal sperm, with detached head and detached tail; (**C**)—damaged epididymal sperm showing acrosome loss; (**D**)—epididymal sperm showing damaged acrosomal membrane. Arrows show the exact sperm structures.

2.7. Total Protein Content Measurement

The total protein content was measured using Bradford Reagent (Sigma–Aldrich/B6916) in the control samples (without sonication) and in both the SIP and SMAP fractions subjected to the various sonication variants: S1, S2, S3, S4.

2.8. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For proteomic analysis, the SIPs and SMAPs isolated from the spermatozoa of individual dogs (n = 15) were pooled. Each pool was run in triplicate (technical replicate). The SDS-PAGE procedure was described previously by Mogielnicka-Brzozowska et al. [27]. The molecular weight (MW) and the optical density (OD) of the stained protein bands (PB) were determined using MultiAnalyst 1.1 software (BioRad, Laboratories, Hercules, CA, USA).

2.9. Identification of Proteins by Mass Spectrometry

2.9.1. In-Gel Trypsin Digestion

The SDS-PAGE gel samples were processed according to the protocol described by Shevchenko et al. [31]. Briefly, gel pieces with protein fractions (PFs) of SIPs and SMAPs were washed in acetonitrile and 25 mM ammonium bicarbonate to remove Coomassie stain, reduced in 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Next, the gel pieces were dehydrated with acetonitrile and dried in a vacuum centrifuge. Subsequently, the gel pieces were rehydrated for 10 min at 4 °C in 10 mM ammonium bicarbonate containing 13 ng/µL trypsin (Promega, Fitchburg, WI, USA). Gel samples were left overnight at 37 °C for complete digestion. After digestion, the peptides were extracted by adding 100 µL of extraction buffer (1:2 [*v*/*v*] 5% formic acid/acetonitrile) to each tube. All of the tubes were vortexed and incubated in a shaker for 15 min at 37 °C. Finally, the peptides were desalted using C18 zip tips (Millipore, Burlington, MA, USA), vacuum-dried and resuspended in water with 0.1% formic acid.

2.9.2. NanoUPLC-Q-TOF/MS Analysis

Waters Acquity liquid chromatography M-Class system (Waters Corp., Milford, MA, USA) equipped with a Peptide BEH C18 analytical column (150 mm × 75 µm; 1.7 µm, Waters Corp., Milford, MA, USA) and Symmetry C18 precolumn (180 µm × 20 mm; 1.7 µm, Waters Corp., Milford, MA, USA) was utilized to separate digested samples.

Each sample was injected on the precolumn and then washed with 99% solvent A (0.1% formic acid in water) at a flow rate of 5 µL/min for 5 min. After washing, the peptides were transferred to an analytical column and separated. The flow rate of the mobile phase was 300 nL/min. The total run time of the analytical gradient, including the column equilibration step, was set at 75 min. The elution gradient steps were as follows: From 0 to 2 min, the concentration of buffer B (0.1% formic acid in acetonitrile) was 5%. Then at 15 min, its concentration was increased to 30%. Next at 45 min, buffer B concentration was increased to 60% and then to 85% at 48 min. During the next 10 min, buffer B concentration was at 85%, before being reduced to 5%, within a time interval of 58 min to 58.5 min.

A mass spectrometry (MS) analysis was performed using Synapt G2-Si (Waters Corp., Milford, MA, USA) with a nanoelectrospray ionization (nESI) source, operating under a positive ion mode. The capillary voltage was set at 3.0 kV, and the cone voltage was set at 40 V. The cone gas flow was set at 40 L/h, and the source temperature was set at 100 °C. The nanoflow gas flow was set at 0.2 Bar. Data were acquired for m/z 70 to m/z 1800 using data-independent mode (MSE). Leucine enkephalin (m/z 556.2771) was used as a Lockspray. The lock mass was acquired every 45 s, and mass correction was applied automatically during acquisition.

Raw chromatography files were analyzed with Byonic software (Protein Metrics, Cupertino, CA, USA). The following settings were used for peak picking and identification: trypsin digestion, max. two miss-cleavages, max. three charges, possible modifications: carbamidomethylated Cys; oxidation of Met and Trp; dioxidation of Trp; pyro-Glu; de-carbamidomethylated Cys; oxidation of Pro; phosphorylation of Ser, Tyr, Thr; (di)methylation of Lys and Arg; acetylation of Lys; trimethylation at Lys; sulfation of Cys, Ser, Thr, Tyr. The detected peptides were compared to SWISSPROT dog proteome (CANLF)-downloaded April 2021. False identifications were limited by comparison with common

contaminants and decoys obtained by reverse amino acid sequencing of in silico-cleavage peptide models.

2.10. Gene Ontology and Functional Annotation

Functional enrichment of the proteins of the sperm intracellular proteins (SIPs) and sperm membrane-associated proteins (SMAPs) of the canine (*Canis lupus familiaris*) epididymal sperm in Gene Ontology (GO) categories: molecular function, biological process, protein class and pathways were obtained from PANTHER Classification System v. 16.0 (online tools, http://pantherdb.org, accessed on 23 November 2021). A Venn diagram was constructed using web tool (http://bioinformatics.psb.ugent.be/webtools/Venn, accessed on 23 November 2021). GO plots were performed using GraphPad Prism software (GraphPad Prism v. 9.2.0. for Windows, GraphPad Prism software, San Diego, CA, USA).

2.11. Statistical Analysis

The data analysis was carried out using Statistica version 13.1 (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA). The results of sonication variants are presented as means with a standard error (mean ± SE). The percentages of sperm morphological defects and protein separation were analyzed with ANOVA (Duncan's multiple range test). Different sonication variants were compared with their respective control and each by each. A comparison of the OD values of the DAPs was also performed using Student's *t*-test for independent samples to determine statistically significant differences.

3. Results

3.1. Sperm Motility Assessment

The sperm cells showed a total motility (TMOT, %) range from 84.0 to 95.0% (90.9 ± 0.9%, mean ± SE), while the progressive motility (PMOT, %) ranged from 41.0 to 72.0% (54.7 ± 9.9%, mean ± SE).

3.2. Influence of Sonication on Epididymal Spermatozoa Morphology

The influence of the sonication on the morphological changes in dog epididymal spermatozoa is shown in Figure 1.

Significant differences in the percentage of the total number of damaged spermatozoa were found between the control sample (C) and samples subjected to different sonication variants (S1, S2, S3, S4). After S4 treatment, 97.4 ± 0.8% (mean ± SE) of dog epididymal spermatozoa were found to be damaged. The effect of a sonication power of 150 W applied in variant S4 on the percentage of the total number of damaged spermatozoa was more significant ($p \leq 0.05$) than sonication power of 50 W in variants S1, S2 and S3 (Figure 2).

When comparing the occurrence of a detached tail in the spermatozoa samples, it was noted that the highest percentage of the abovementioned defect (39.4 ± 9.6%) ($p \leq 0.05$) was found after the application of the last sonication variant (S4). The percentage of the detached tail in the S4 variant (with the parameters 10 min, 150 W) was over 25% higher than the other tested variants (C, S1, S2, S3). There were no significant differences between the control and the sonicated samples (S1, S2, S3) ($p > 0.05$) in the percentage of the spermatozoa with a detached tail (Figure 2).

The use of a higher sonication power (150 W) resulted in more spermatozoa losing their acrosomes. The control sample contained 27.3 ± 3.4% spermatozoa without acrosome, but the use of sonication increased the occurrence of this defect to 62.4 ± 6.1% in the S4 variant ($p \leq 0.05$) (Figure 2).

When comparing the percentage of damaged acrosomal membranes in the spermatozoa samples, it was noted that the highest value of the defect occurred when the last sonication variant (S4) was applied (31.8 ± 2.2%) ($p \leq 0.05$). The percentages of damaged sperm acrosome membrane in S1, S2, S3 were 14.3 ± 3.3%, 18.5 ± 2.2% and 24.6 ± 2.2% ($p > 0.05$), respectively (Figure 2).

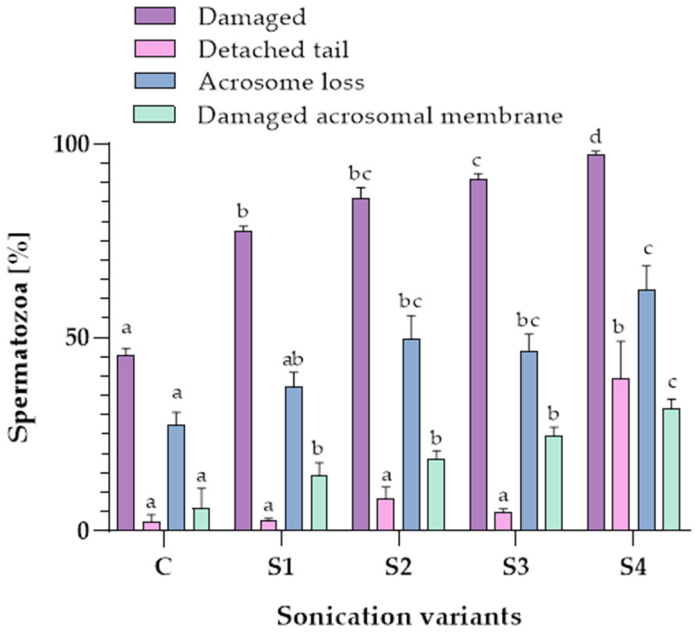

Figure 2. The evaluation of the morphological changes in dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15) after the treatment with different sonication variants. C—the control sample (without sonication), S1—5 min, 50 W; S2—10 min, 50 W; S3—30 min, 50 W; S4—10 min, 150 W. Values are presented as the mean ± SE. Different sonication variants were compared with their respective control each by each. Different letters indicate significant difference ($p \leq 0.05$).

3.3. Protein Content in Samples after the Sonication

After sonication, the cauda epididymal spermatozoa proteins were divided into two fractions. The first fraction contained the sperm intracellular proteins (SIPs) that flowed into the solution from the sperm cells that disintegrated during the sonication. The second fraction contained the sperm membrane-associated proteins (SMAPs) obtained after the protein extraction from the epididymal sperm residues remaining after the centrifugation of sonicated samples.

The protein contents in the SIP and SMAP fractions were interdependent (Figure 3). A higher sonication power (150 W) resulted in more proteins being released from the sperm cells. The control sample contained 0.05 ± 0.01 mg/mL SIPs. The use of sonication slightly increased the content of SIPs in all sonication variants up to 0.17 ± 0.03 in the S4 variant. The content of SMAPs in the control samples was 1.46 ± 0.01 mg/mL. The use of sonication slightly decreased the content of SMAPs in all sonication variants up to 1.38 ± 0.02 mg/mL in the S4 variant (Figure 3).

A comparison of the total protein content (as a sum of the SIPs and the SMAPs) of samples subjected to variants S1, S2, S3, S4 and the control sample indicate that the total protein content was not significantly influenced by sonication power or time ($p > 0.05$) (Figure 3).

3.4. SDS-PAGE Analysis

The SDS-PAGE protein profiles of pooled SIPs and SMAPs were analyzed. The protein profile of the SIPs of the dog cauda epididymal spermatozoa was characterized by the presence of 21 PFs with molecular weights (MW) ranging from 10.6 to 163.2 kDa (Figure 4, Line A).

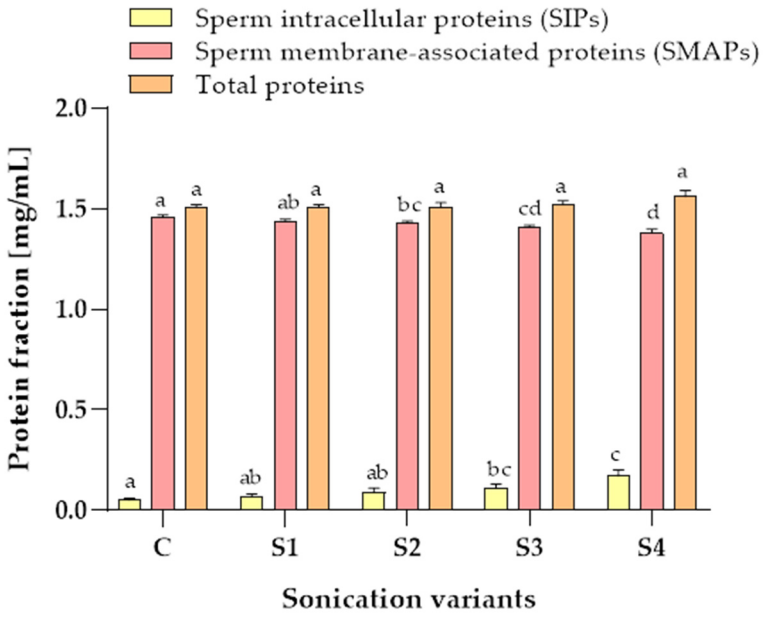

Figure 3. Average amounts of intracellular proteins (SIPs) and membrane-associated proteins (SMAPs) (mg/mL) of dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15) obtained using different sonication variants. C—control sample (without sonication), the S1—5 min, 50 W; S2—10 min, 50 W; S3—30 min, 50 W; S4—10 min, 150 W. Values are presented as the mean ± SE. Different sonication variants were compared with their respective control each by each. Different letters indicate significant difference ($p \leq 0.05$).

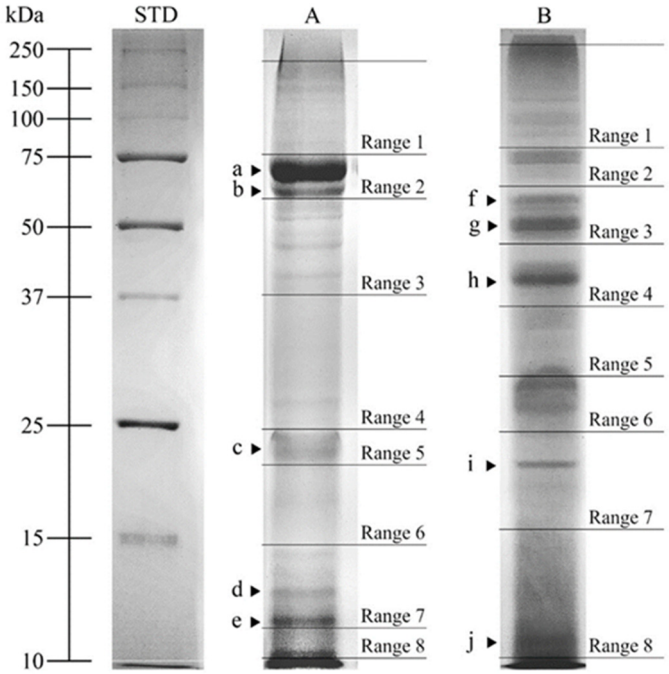

Figure 4. One-dimensional SDS-PAGE (12%). Line A: sperm intracellular proteins (SIPs) of dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15); Line B: sperm membrane-associated proteins (SMAPs) of the dog cauda epididymal spermatozoa. Proteins were identified using mass spectrometry in ranges 1–8 (Range 1 to 8) for SIPs and SMAPs. Differentially abundant protein fractions were marked with letters from a to j. STD—molecular weight markers.

The values of MW of PFs on SDS-PAGE gels were averaged for all sonication variants (C, S1, S2, S3, S4). Optical density (OD) analysis in PFs showed higher protein content ($p \leq 0.001$) for five PFs in the SIPs compared with corresponding PFs of the SMAPs. These

were marked with letters from a to e, with the following MW and average optical density (OD) values: (a) 69.0 kDa, 0.36 ± 0.012 OD; (b) 63.6 kDa, 0.32 ± 0.013 OD; (c) 20.2 kDa, 0.29 ± 0.008 OD; (d) 12.8 kDa, 0.28 ± 0.006 OD; (e) 11.9 kDa, 0.30 ± 0.007 OD (Table 1).

Table 1. Average optical density (OD) values (mean ± SE) of differentially abundant proteins (DAPs) of the sperm intracellular proteins (SIPs) and the sperm membrane-associated proteins (SMAPs) of dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15). Different superscripts within rows indicate statistically significant differences ($p \leq 0.001$) between fractions. DAPs were marked with letters from a to j. MW—average molecular weight.

DAPs	MW (kDa)	Optical Density	
		SIPs	SMAPs
a	71.8	0.36 ± 0.012 [a]	0.21 ± 0.006 [b]
b	65.0	0.32 ± 0.013 [a]	0.19 ± 0.005 [b]
c	20.5	0.29 ± 0.008 [a]	0.17 ± 0.007 [b]
d	12.8	0.28 ± 0.006 [a]	0.17 ± 0.005 [b]
e	11.9	0.30 ± 0.007 [a]	0.18 ± 0.005 [b]
f	57.5	0.22 ± 0.009 [a]	0.28 ± 0.006 [b]
g	50.0	0.18 ± 0.006 [a]	0.32 ± 0.008 [b]
h	38.6	0.17 ± 0.006 [a]	0.29 ± 0.014 [b]
i	18.0	0.18 ± 0.007 [a]	0.29 ± 0.006 [b]
j	10.9	0.20 ± 0.004 [a]	0.31 ± 0.013 [b]

The SDS-PAGE profile of the SMAPs of the dog epididymal spermatozoa was characterized by the presence of 19 PFs with MW range from 11.3 to >250.0 kDa (Figure 4, Line B).

The PF molecular weights were averaged for all sonication variants (C, S1, S2, S3, S4). OD analysis in these PFs showed higher protein content ($p \leq 0.001$) for five PFs in the SMAPs when compared with the corresponding (showing the same MW) PFs of the SIPs. They were marked with letters from f to j, with the following MW and OD values: (f) 61.1 kDa, 0.28 ± 0.006 OD; (g) 50.4 kDa, 0.32 ± 0.008 OD; (h) 40.0 kDa, 0.29 ± 0.014 OD; (i) 18.7 kDa, 0.29 ± 0.006 OD; (j) 11.3 kDa, 0.31 ± 0.013 OD (Table 1).

3.5. Mass Spectrometry Analysis

SDS-PAGE protein fractions were analyzed in Ranges 1–8, using NanoUPLC-Q-TOF/MS and UniProt identifiers (Figure 4). Differentially abundant protein fractions are shown in Tables 1 and 2.

Ten polypeptides with the highest scores were identified as DAPs in the SIPs fraction: lactotransferrin (*LTF*), carboxylesterase 5A (*CES5A*), albumin (*ALB*), olfactomedin 4 (*OLFM4*), prostaglandin-H2 D-isomerase (*PTGDS*), glutathione peroxidase (*GPX5*), putative lipocalin-like protein (*LCNL1*), putative peptidyl-prolyl cis-trans isomerase (*CSNK1G1*), intracellular cholesterol transporter (*NPC2*) and leucine-rich repeat neuronal protein 3 (*LRRN3*). Eight polypeptides were identified as DAPs in the SMAPs fraction: epididymal secretory protein E1 (*NPC2*), lactotransferrin (*LTF*), cysteine-rich secretory protein 2 (*CRISP2*), WAP domain-containing protein (N/A/WAPdcp), actin, cytoplasmic 1 (*ACTB*), ubiquitin-60S ribosomal protein L40 (*UBA52*), beta-N-acetylhexosaminidase (*HEXB*) and acrosin-binding protein (*ACRBP*). UniProt database-supported identification resulted in 42 proteins identified in the SIPs and 153 proteins in the SMAPs. The proteins (13) present in both SIPs and SMAPs were: *ALB*, *GPI*, *UBA52*, *CRISP2*, *PTGDS*, *LTF*, *GPX5*, *ACRBP*, *LCNL1*, *CES5A*, *OLFM4*, *NPC2*, *HEXB* (Figure 5).

Table 2. Differentially abundant proteins (DAPs) of the SMAPs and SIPs present in dog (*Canis lupus familiaris*) cauda epididymal spermatozoa (n = 15). Proteins separated in SDS-PAGE were analyzed with mass spectrometry (NanoUPLC-Q-TOF/MS) and identified by UniProt identifiers. The protein score represents the quality of identification.

DAPs	Protein Name	Swiss-Prot Accession Number	Gene Symbol	Sequence Coverage (%)	Molecular Weight (kDa)	Peptide Counts (Unique)	Peptide Counts (All)	Protein Score
	SIPs							
a	Lactotransferrin	F1PR54	*LTF*	34.6	77.3	21	72	440
	Carboxylesterase 5A	Q6AW47	*CES5A*	15.1	63.6	6	44	226
b	Albumin	P49822	*ALB*	9.5	68.6	4	25	337
	Olfactomedin 4	F1PB68	*OLFM4*	4.6	54.4	1	6	236
c	Prostaglandin-H2 D-isomerase	Q9XS65	*PTGDS*	28.3	21.1	5	25	212
	Glutathione peroxidase	F1PJ71	*GPX5*	16.7	25.3	3	20	282
d	Lipocalin_cytosolic_FA-bd domain-containing protein	E2R6E0	*LCNL1*	3.7	32.6	1	7	229
	Peptidyl-prolyl cis-trans isomerase	F1PLV2	*CSNK1G1*	5.4	26.6	1	1	147
e	NPC intracellular cholesterol transporter 2	Q28895	*NPC2*	12.1	16.1	1	33	247
	Leucine rich repeat neuronal protein 3	F1PYL2	*LRRN3*	1.6	79.6	1	2	135
	SMAPs							
f	Epididymal secretory protein E1	F1PAR9	*NPC2*	33.3	20.2	4	29	470
	Lactotransferrin	F1PR54	*LTF*	17.4	77.3	12	22	335
g	Cysteine-rich secretory protein 2	A0A5F4CCD	*CRISP2*	13.5	35.4	2	5	302
	WAP domain-containing protein	E2RCT1	N/A * WAPdcp **	14.7	13.0	1	3	303
h	Actin, cytoplasmic 1	O18840	*ACTB*	5.3	41.7	4	41	378
	Ubiquitin-60S ribosomal protein L40	P63050	*UBA52*	7.0	14.7	1	4	262
i	Epididymal secretory protein E1	F1PAR9	*NPC2*	25.4	20.2	3	29	591
	Beta-N-acetylhexosaminidase	F1Q1M8	*HEXB*	6.7	38.1	1	4	276
j	Epididymal secretory protein E1	F1PAR9	*NPC2*	32.3	20.2	3	21	540
	Acrosin-binding protein	E2RNS8	*ACRBP*	1.9	61.3	1	3	231

* not available, ** protein name abbreviation in this publication.

The proteins of the SIPs and SMAPs with significant MS scores are listed in Supplementary Tables S1 and S2, respectively.

3.6. Gene Ontology and Functional Annotation

Based on gene ontology (GO) enrichment, the dominant molecular functions of SIPs were catalytic activity (50%), binding (28%) and molecular function regulator (10%). The main biological processes shown for SIPs were the cellular process (26%), the metabolic process (18%) and biological regulation (18%) (Figure 6A). Dominant molecular functions of SMAPs were hydrolase activity (33%), transferase activity (21%) and catalytic activity,

acting on a protein (17%). The biological processes dominant in SMAPs were the cellular process (29%), biological regulation (14%) and the metabolic process (13%) (Figure 6B).

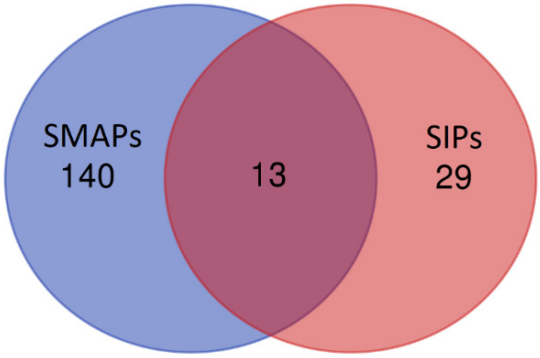

Figure 5. Venn diagram showing the number of proteins identified in the sperm intracellular proteins (SIPs) and the sperm membrane-associated proteins (SMAPs) of the dog (*Canis lupus familiaris*) cauda epididymal spermatozoa.

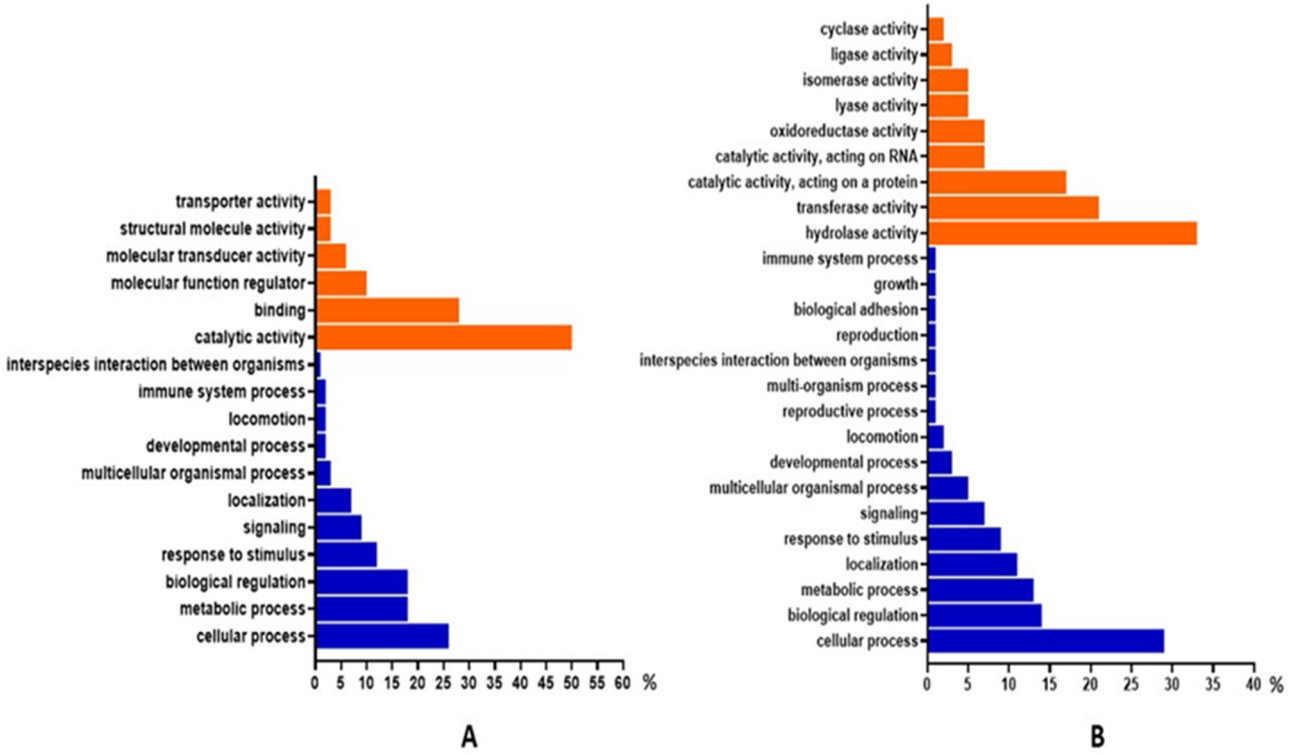

Figure 6. Gene ontology (GO) enrichment of (**A**) sperm intracellular proteins (SIPs) and (**B**) sperm membrane-associated proteins (SMAPs) of the dog (*Canis lupus familiaris*) cauda epididymal spermatozoa. Significant GO terms for molecular function (orange) and biological process (blue) are presented. The analyses were made by the PANTHER Classification System (v. 16).

The protein classes recognized in SIPs after analysis of GO are mainly metabolite interconversion enzyme (34%), transfer/carrier protein (15%) and protein modifying enzyme (9%) (Figure 7A). For SMAPs, a protein class analysis showed mainly metabolite interconversion enzyme (25%), transporter (14%) and transmembrane signal receptor (11%) (Figure 7B).

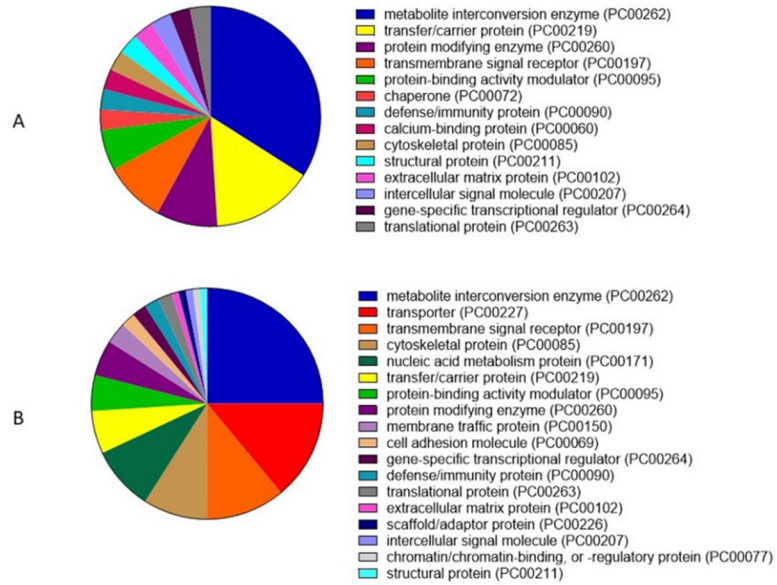

Figure 7. Protein classes in (**A**) sperm intracellular proteins (SIPs) and (**B**) sperm membrane-associated proteins (SMAPs) of the dog (*Canis lupus familiaris*) cauda epididymal spermatozoa. The protein class was analyzed by the PANTHER Classification System (v. 16). Each color represents percentage of protein participation in each protein class.

The GO pathways showed mainly glycolysis (23%), the integrin signaling pathway (11%) and the Wnt signaling pathway (11%) for SIPs (Figure 8A). For the SMAPs, the main pathways were inflammation mediated by chemokine and cytokine signaling pathways (7%), the Wnt signaling pathway (7%) and the Alzheimer disease–presenilin pathway (5%) (Figure 8B).

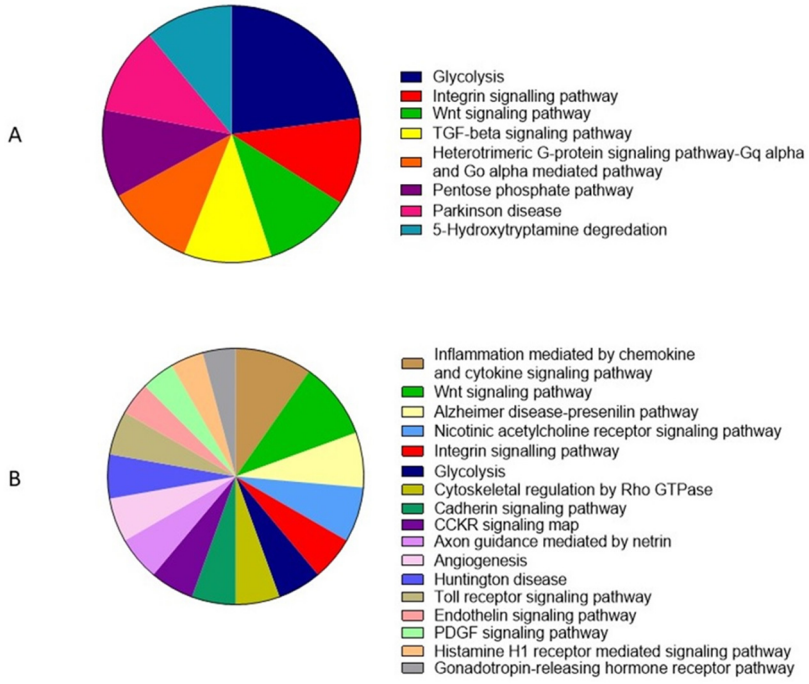

Figure 8. Pathways according to PANTHER Classification System (v. 16) for (**A**) sperm intracellular proteins (SIPs) and (**B**) sperm membrane-associated proteins (SMAPs) of dog (*Canis lupus familiaris*) cauda epididymal spermatozoa. Each color represents percentage of protein participation in each protein class.

4. Discussion

This is the first study presenting the isolation of intracellular and membrane-associated proteins from canine epididymal sperm and showing the comparative analysis of proteomes of the abovementioned fractions. Moreover, the present study showed the effect of sonication time and power on the percentage of damage in dog cauda epididymal spermatozoa and the sperm protein isolation.

Based on the sonication results, it is difficult to compare the results obtained in this study with the results of other scientists since different types of sonicators were used, as well as sonication parameters such as maximum power or time, which depends on the type and manufacturer of the equipment. However, it can be stated that sonication with 150 W, 10 min, can be used to isolate the head and tail of dog epididymal spermatozoa. The results are consistent with studies by other authors in which sonication was used to isolate the outer acrosomal membrane of goat ejaculated spermatozoa [11]. The acrosomal membranes were separated without damaging their structure, which was confirmed by electron microscopy. It has been shown that acrosomal membranes isolated in this way may provide good material for an analysis of the activity of specific enzymes, such as Ca^{2+} ATPase [11]. Some authors have also used sonication to separate the heads of human sperm from the tails [12]. Similar effects were achieved by Yamamoto et al. [10], who used sonication on ejaculated rat spermatozoa to remove sperm tails. In this study, a similar effect of sonication on dog epididymal spermatozoa was demonstrated, in which treatment with appropriate timing and power of sonication resulted in the effective separation of sperm heads from sperm tails. Given that sonication does not lead to damage of the sperm chromosomes, the separated sperm heads may be further used as material for in vitro fertilization [9].

It was shown that the cytoplasm from the sperm cells broken by ultrasound may leak out from the sperm cell [8]. In the current study, separate analysis was shown of the sperm intracellular proteins (SIPs) and the sperm membrane-associated proteins (SMAPs) obtained by protein extraction from the epididymal sperm residue. To date, an SDS-PAGE analysis of the SIPs and SMAPs of dog epididymal spermatozoa has not been shown. According to the authors' knowledge, this is also the first study concerning a mass spectrometry analysis of the protein present in dog epididymal spermatozoa. Until now, mass spectrometry has allowed the identification of epididymal spermatozoa proteins of animal species, among others, for stallions [13], bulls [32], boars [33] and mice [34]. In this study, UniProt database-supported identification resulted in 42 proteins identified in the SIPs and 153 proteins in the SMAPs. Thirteen proteins were present in both fractions: *ALB* (albumin), *GPI* (Glucose-6-phosphatase isomerase), *UBA52* (Ubiquitin-60S ribosomal protein L40), *CRISP2* (Cysteine-rich secretory protein 2), *PTGDS* (Prostaglandin-H2 D-isomerase), *LTF* (Lactotransferrin), *GPX5* (Epididymal secretory glutathione peroxidase), *ACRBP* (Acrosin-binding protein), *LCNL1* (Lipocln cytosolic FA-bd domain-containing protein), *CES5A* (Carboxylesterase 5A), *OLFM4* (Olfactomedin 4), *NPC2* (Epididymal secretory protein E1) and *HEXB* (Beta-N-acetylhexosaminidase). It is worth noting that the sperm membrane-associated fraction was much more abundant in different proteins than sperm intracellular fraction. According to proteins present in both SIPs and SMAPs, it is impossible to state if they were just contamination formed during the sperm protein isolation procedure (unavoidable) or if these proteins are really components of abovementioned sperm compartments.

The current study showed the presence of DAPs on SDS PAGE gels in both the SIPs and the SMAPs of dog epididymal spermatozoa (Figure 4, Table 2) and analyzed the potential function of these proteins in reproduction. Some of them were found in spermatozoa structures of different animal species: *LTF*, *ALB*, *OLFM4*, *CSNK1G*, *LRRN3*, *CRISP2*, *ACTB*, *UBA52*, *HEXB*, *ACRBP*, *GPX5*, *PTGDS* and *NPC2*. Three of the DAPs, however, were found only in different animal species' reproductive tissues, such as a testis or epididymis. These included *CES5A*, WAPdcp and *LCNL1*. However, it is known that the proteins present in the epididymis may exert an impact on sperm functions [35].

The functions of DAPs, which were found among others in sperm structures of different animal species and humans, are discussed below.

Lactotransferrin (*LTF*) was found in both SIPs and SMAPs of canine epididymal sperm. A high content of *LTF* was found in the canine seminal plasma [23], and its function in reproduction is well known [27]. As it was demonstrated, *LTF* is present in high concentrations and binds to sperm in the epididymis of boars and stallions and was immunolocalized in rodent epididymis [36]. According to its binding ability to the sperm membrane [36], it was found in high amounts in our study, and it may be postulated that *LTF* also coats canine epididymal sperm plasma membrane during the sperm maturation in epididymis and might exert protective function.

In the seminal plasma of different animal species, ALBs are quite abundant proteins, and they play an important role in reproductive processes [22]. The ALBs can absorb lipid peroxides, a feature that contributes to their protective effect on membrane stability and sperm motility [37]. Albumins were found in mice sperm acrosome. They are synthesized in the epididymis and aggregate in a high molecular mass glycoprotein complex involved in sperm–egg fertilization [38].

Olfactomedin 4 (*OLFM4*) is an olfactomedin domain-containing glycoprotein [39]. OLFM-1, -2, -3, -4 are known to regulate cellular growth, differentiation and pathological processes [40]. The absence of *OLFM4* gene expression is associated with the progression of human prostate cancer, but its role and the molecular mechanisms involved in this process have not been completely understood [41]. *OLFM4* was found in human spermatozoa [42].

Peptidyl-prolyl cis-trans isomerases (*CSN*) catalyze the isomerization between the cis and trans forms of peptide bonds, which are associated with new polypeptide conformation [43,44]. In bulls, the *CSN* may exert a negative impact on spermatogenesis and sperm maturation [45]. It was found in human and rat spermatozoa [46,47].

Leucine-rich repeat protein family (*LRTP*) have been found in the testis of humans and mice [48]. A downregulation of the *LRRN3* gene in sperm of high-fertility boars was found [49], with *LRRN3* being involved in Ras-MAPK signaling [50].

CRISP2 is a member of the cysteine-rich secretory protein (*CRISP*) family. Its expression is high in testis, and it is localized in sperm acrosome, sperm tail and the junction between germ and Sertoli cells within the seminiferous epithelium [51–54]. *CRISP2* is implicated in cell-cell adhesion and is capable of steroid binding [55,56]. It can also specifically regulate calcium flow through ryanodine receptors [57,58]. It is known that a decrease in *CRISP2* content in sperm is associated with male infertility in humans [59,60] and horses [61].

ACTB is the major cytoskeleton protein which is responsible for cell volume regulation [62]. The localization of the *ACT* in the flagellar and acrosomal membrane of spermatozoa suggests its role in sperm motility and capacitation [63–65].

UBA52 is a low molecular weight peptide that tags other proteins for proteasomal degradation and is also involved in the regulation of other protein functions. Its role in the elimination of defective spermatozoa during transit through the epididymis has been described in humans and cattle [66,67]. Vernocchi et al. [68] indicated the presence of ubiquitinated proteins in feline epididymal sperm.

HEXB is a lysosomal enzyme that hydrolyses acetylglucosamine and acetylgalactosamine residues in glycoconjugates [69]. The b-hexosaminidase enzymatic ability to remove N-acetylglucosamine residues from ZP glycoproteins maintains spermatozoa properties to penetrate human oocytes [70]. It is interesting that *HEXB* has the ability to bind zinc-ions, which may influence sperm motility [71].

ACRBP is an acrosomal protein (also known as Sp32), and it is a binding protein specific for the precursor (proACR) and intermediate forms of *ACR* [72,73]. *ACRBP* is normally expressed exclusively in the human testis but is also expressed in a wide range of different tumor types [74,75]. *ACRBP* influences acrosin activity and acrosome formation, which is connected with sperm fertilizing ability [76].

The glutathione peroxidase (*GPX5*) is highly expressed in the epididymis of mammals, where it is secreted into the lumen, and its role is to protect sperm from lipid peroxidation. The glutathione peroxidase was found, among others, in bull, boar and dog seminal plasma [77–79]. Functional network analysis showed the participation of this protein in important metabolic pathways in feline SP for *GPX 5* and 6 isoforms [27]. *GPX5* was found in boar epididymal sperm [79].

PTGDS was found in the epididymal fluid and seminal plasma of rats [80] as well as in the seminal plasma of dogs [27]. Prostaglandin (H2) D-isomerase, expressed in different reproductive organs, binds to small nonsubstrate lipophilic molecules and may act as a scavenger for harmful hydrophobic molecules as it is potentially involved in vesicle-mediated transport and defense response [81]. Araujo et al. [24,25] described this protein as a purebred dog sperm component.

NPC intercellular cholesterol transporter 2, also called epididymal secretory protein E1, was found in SIPs and SMAPs. In general, epididymal secretory protein E1 (*CE1*/*NPC2*) is expressed based on canine genes similar to those known in humans [5]. The *CE1* protein is a highly abundant, conserved secretory protein [82]. The mRNA was found in large amounts in the epididymal duct epithelium, while the protein was found in the duct lumen [83]. Recently, *CE1* has been identified as the second gene of Niemann-Pick type C disease (*NPC2*) involved in cholesterol efflux from lysosomes [84]. It is interesting that NPC2-based proteins were found in both the SIPs and SMAPs. This may suggest its epididymal sperm membrane coating ability. Similar results were shown for the human ejaculated spermatozoa in which an *HE2* human homologue of *CE1* was present on the acrosome and equatorial region of the cells [85]. Araujo et al. [24,25] found *NPC2* as a purebred sperm component.

It may be concluded that proteins which showed high fraction-dependent (SIPs or SMAPs) content play a very important role in canine epididymal sperm metabolism, mostly by sperm protection.

This paper also discusses DAPs, which were not isolated earlier and established as sperm components. In the current study, they were shown for the first time as such. These proteins were mostly described as being produced in the epididymis.

An interesting protein with sperm quality diagnostic potential is carboxylesterase (*CES5A*). It is known to be produced in the epididymis and is required for sperm capacitation and male fertility in the rat [86]. Gene expression of *CES* was found in the rat and rabbit epididymis [87] and rat testis [88]. It is hypothesized that *CES* plays a role in the maturation of the sperm lipid membrane. The function of *CES5A* in sperm capacitation is not fully understood as it does not seem to have direct interaction with spermatozoa in the epididymal lumen but is instead thought to alter the lipid content of the luminal fluid and then, indirectly, the lipid content of the sperm membrane [86,87].

Lipocalins (*LCNL1*) typically transport or store small molecules, including vitamins, steroid hormones, odorants and various secondary metabolites [89]. Gene expression of *LCN2* was found in the mouse, ram and human epididymis [90–92]. Since *LCN2* could facilitate cell proliferation of castration-resistant prostate cancer via androgen receptor, *LCN2* could be a novel target in cancer therapy [93].

WAP (whey acidic protein) domain-containing protein possesses serine-type endopeptidase inhibitor activity and plays a role in biological processes such as antibacterial humoral response and innate immune response in the dog [94]. mRNA expression for this protein was found in most regions of the canine epididymis [83]. However, its function in reproduction and in canine spermatozoa was not established.

The proteins recognized as DAPs in this study and mostly described as epididymis-produced probably might be coated on sperm plasma membrane or even inserted into membrane structure during canine epididymal sperm maturation.

Some the DAPs recognized in our study such as *ALB*, *PTGDS*, *LTF* and *NPC2* were identified by mass spectrometry as components of purebred dog spermatozoa by Araujo et al. [24,25]. There have been found 47 proteins in spermatozoa, 109 in seminal plasma and

6 in both samples. Serum albumin, tubulins and acrosin binding protein were statistically relevant. Gene ontology analysis has confirmed their role in numerous important cellular biological processes.

According to the GO analysis, the proteins present in the SIPs were mainly involved in glycolysis, the integrin signaling pathway and the Wnt signaling pathway. An obligatory role for glycolysis in spermatozoa is to support its motility and capacitation process [95]. Integrin signaling pathway is involved in a broad range of processes engaged in sperm activation and fertilization [96]. For SMAPs, the main metabolic pathways were inflammation mediated by the chemokine and cytokine signaling pathway, the Wnt signaling pathway and the Alzheimer disease–presenilin pathway. The chemokine and cytokine signaling pathways contribute to testicular function and the maintenance of male reproductive health [97]. Presenilins stabilize β-catenin in the Wnt signaling pathway and regulate calcium homeostasis [98]. It was found that the presence of amyloid precursor protein (APP) is an important element of the Alzheimer disease–presenilin pathway in human sperm and testis [99,100]. The Wnt signaling pathway (showed as important for both SIPs and SMAPs) is an ancient and evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development [101]. The Wnt signaling pathway has been successfully linked with the regulation of sperm maturation in the epididymis [102]. Post-transcriptional Wnt signaling influences sperm, maintaining protein homeostasis, initiating sperm motility and establishing a membrane diffusion barrier in the sperm tail [103].

As it was shown above, the protein present both in the canine epididymal sperm intracellular and membrane-associated fraction participates in important metabolic pathways regulating sperm functions and fertility. Taking into consideration the above results, some proteins may potentially be used as sperm quality markers and disease diagnostic markers of the male reproductive system.

5. Conclusions

The results of this study show that sonication for 10 min at 150 W can be considered for the separation of dog epididymal sperm structures and for improved protein extraction. Mass spectrometry identification and bioinformatic analysis of SIPs and SMAPs have been shown for the first time in the present study. The results show the presence of DAPs in both the sperm protein fractions, which are crucial for the functions of spermatozoa and their fertilizing ability. Finally, it has been confirmed that these proteins are implicated in important sperm metabolic pathways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12060772/s1, Table S1: Proteins present in epididymal sperm intracellular (SIPs) fraction of dog (*Canis lupus familiaris*), evaluated by SDS PAGE and mass spectrometry (NanoUPLC-Q-TOF/MS); Table S2: Proteins present in epididymal sperm membrane-associated fraction (SMAPs) of dog (*Canis lupus familiaris*), evaluated by SDS PAGE and mass spectrometry (NanoUPLC-Q-TOF/MS).

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Paper II

Zmudzinska, A.; Wisniewski, J.; Mlynarz, P.; Olejnik, B.; Mogielnicka-Brzozowska, M. **Age-Dependent Variations in Functional Quality and Proteomic Characteristics of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa**. *International Journal of Molecular Sciences* **2022**, *23*, 9143

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Age-Dependent Variations in Functional Quality and Proteomic Characteristics of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa

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Abstract: Increased male age is associated with a significant reduction in semen quality. Little is known about the sperm proteome changes resulting from the aging process. This study aimed to investigate the relationship between the functional quality and proteome of epididymal spermatozoa of dogs that were differing in age. The study was conducted on 30 male dogs that were divided into three age groups. G1—12 to 41 months old, G2—42 to 77 months old, and G3—78 to 132 months old. The sperm samples were assessed using a computer-assisted semen analysis (CASA). The epididymal sperm proteins were analyzed using gel electrophoresis (SDS-PAGE), nano-liquid chromatography coupled to quadrupole time of flight mass spectrometry (NanoUPLC-Q-TOF/MS) and bioinformatic tools. The sperm quality parameters were significantly lower in older dogs. NanoUPLC-Q-TOF/MS identification resulted in 865 proteins that were found in the G1, 472 in G2, and 435 in G3. There were seven proteins that were present in all three age groups, and four of them (ACTB, CE10, NPC2, CRISP2) showed significant changes among the studied groups. Age-dependent variations were detected in the sperm proteome composition and were related to important metabolite pathways, which might suggest that several proteins are implicated in sperm maturation and could be potential aging biomarkers.

Keywords: epididymal spermatozoa; aging; proteins; canine

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1. Introduction

Canine epididymal semen provides an additional source of gametes to preserve the genetics of valuable breeding dogs [1]. Moreover, the development of a gene bank of epididymal semen would greatly contribute to increasing the genetic diversity in dogs and also in endangered canids [1]. Additionally, the dog already fulfils many of the criteria of a good model of the human epididymis on a molecular level also regarding aging [2,3].

For dog breeders, it is important to know how long they may use semen from old high-quality reproducers without losing the semen quality. Thus, the possible effect of male age on fertility has become increasingly important in animal reproduction. While the influence of female age on fertility is well established, the impact of male age is poorly characterized [4]. It has been observed that age is associated with diminished dog sperm quality [5–7]. Age-dependent changes in sperm quality have been reported in the rat [8,9], hamster [10], ferret [11], cat [12], boar [13], and in humans [14]. Many different techniques have been used to assess the reproductive potential of male dogs, but little is still known about the protein composition changes during the aging processes of the epididymis and

epididymal spermatozoa. Associations have been shown among age and epididymal and accessory sex gland function in humans according to sperm progressive motility and protein activity such as NAG (neutral α-glucosidase) and PSA (prostate-specific antigen) [15]. The declining sperm motility that is observed in older men might be due to age-dependent changes in epididymal and accessory sex gland function [15]. The epididymis and accessory sex gland function are highly dependent on androgens. The epididymis undergoes morphological changes with aging, which probably affects sperm maturation [10,15]. However, the particular molecular mechanisms of epididymal sperm aging based on changes in the protein structures and function have not yet been determined.

Motility is believed to be one of the most important parameters in evaluating the fertilizing ability of spermatozoa [16]. Sperm motility is the result of a multitude of factors, including post-testicular molecular processes. Following spermatogenesis, the spermatozoa undergo maturation in the epididymis, including gaining their ability to be motile [17]. Protein components that are derived from epididymal ducts play a very important role in these events [2]. Sperm structures and functions are affected by protein degradation and post-translational modifications [18]. Both protein interaction with the sperm plasma membrane and inner protein recompositing play a basic role in the epididymal sperm maturation [2,3]. Many internal and external factors may influence these protein qualities and functions, which are closely related to sperm fertilizing ability [17].

Only a few studies have been conducted to determine the protein-based molecular mechanisms that are responsible for the age-dependent decline in epididymal sperm quality. Most notably, Silva et al. [4] noted increased levels of apoptotic/stress markers: cellular tumor antigen p53 (TP53) and mitogen-activated protein kinases (MAPKs) activity.

This study aimed to investigate the relationship between the functional quality and proteome of epididymal spermatozoa of dogs that were differing in age.

2. Results

2.1. Epididymal Sperm Quality Assessment

The concentration of epididymal spermatozoa in the studied group of dogs ranged as follows: Group 1 (G1), from 21.4 to 54.9 $\times 10^8$ spermatozoa/mL (38.2 $\pm$ 3.2 $\times 10^8$ spermatozoa/mL, mean $\pm$ SE); Group 2 (G2), from 9.7 to 49.4 $\times 10^8$ spermatozoa/mL (27.7 $\pm$ 3.7 $\times 10^8$ spermatozoa/mL, mean $\pm$ SE); and Group 3 (G3), from 6.8 to 39.6 $\times 10^8$ spermatozoa/mL (24.8 $\pm$ 3.9 $\times 10^8$ spermatozoa/mL, mean $\pm$ SE) (Table 1).

Table 1. Motion parameters of the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1: 12–41 months old; G2: 42–77 months old; G3: 78–132 months old).

Sperm Parameters	G1 (n = 10)	G2 (n = 10)	G3 (n = 10)	*p*-Value
Concentration ($\times 10^8$/mL)	38.2 $\pm$ 3.2 ^{a}	27.7 $\pm$ 3.7 ^{ab}	24.8 $\pm$ 3.9 ^{b}	0.018
Total motility (TMOT, %)	92.8 $\pm$ 0.3 ^{a}	89.1 $\pm$ 0.9 ^{a}	81.3 $\pm$ 2.4 ^{b}	0.002
Progressive motility (PMOT, %)	57.7 $\pm$ 2.7 ^{a}	53.8 $\pm$ 2.3 ^{a}	38.5 $\pm$ 5.8 ^{b}	0.007
Average path velocity (VAP, μm/s)	137.7 $\pm$ 3.4 ^{a}	132.5 $\pm$ 3.0 ^{a}	113.5 $\pm$ 7.3 ^{b}	0.002
Straight line velocity (VSL, μm/s)	114.0 $\pm$ 3.5 ^{a}	110.1 $\pm$ 2.5 ^{ab}	93.0 $\pm$ 7.5 ^{b}	0.003
Curvilinear velocity (VCL, μm/s)	206.6 $\pm$ 5.9 ^{a}	198.0 $\pm$ 8.0 ^{ab}	174.2 $\pm$ 9.3 ^{b}	0.014
Amplitude of lateral head displacement (ALH, μm)	7.1 $\pm$ 0.3 ^{a}	6.8 $\pm$ 0.3 ^{a}	6.5 $\pm$ 0.4 ^{a}	0.430
Beat cross frequency (BCF, Hz)	16.2 $\pm$ 1.0 ^{a}	17.2 $\pm$ 1.4 ^{a}	19.8 $\pm$ 2.0 ^{a}	0.115
Straightness (STR, %)	81.9 $\pm$ 1.3 ^{a}	82.1 $\pm$ 1.1 ^{a}	80.5 $\pm$ 2.2 ^{a}	0.354
Linearity (LIN, %)	57.8 $\pm$ 2.3 ^{a}	58.2 $\pm$ 2.2 ^{a}	54.5 $\pm$ 3.4 ^{a}	0.234

Values are represented as the mean $\pm$ SE. Different superscripts (a, b) with the same row indicate significant differences at $p < 0.05$.

The sperm concentration, VSL, and VCL that were measured in the three age groups differed statistically significantly ($p < 0.05$) between G1 and G3. The results also showed significant differences ($p < 0.05$) in the percentage of TMOT, PMOT, and VAP values between G3 compared with G1 and G2 (Table 1).

A statistically significant negative correlation with dog age group was shown for the following parameters: sperm concentration (r = −0.43; $p < 0.05$), TMOT % (r = −0.55; $p < 0.005$), PMOT % (r = −0.49; $p < 0.01$), VAP (r = −0.54; $p < 0.005$), VCL (r = −0.44; $p < 0.05$), and VSL (r = −0.52; $p < 0.005$).

2.2. Epididymal Sperm Morphology Assessment

The percentage of morphological changes that were found in dog epididymal spermatozoa among age groups (G1, G2, and G3) are shown in Figure 1.

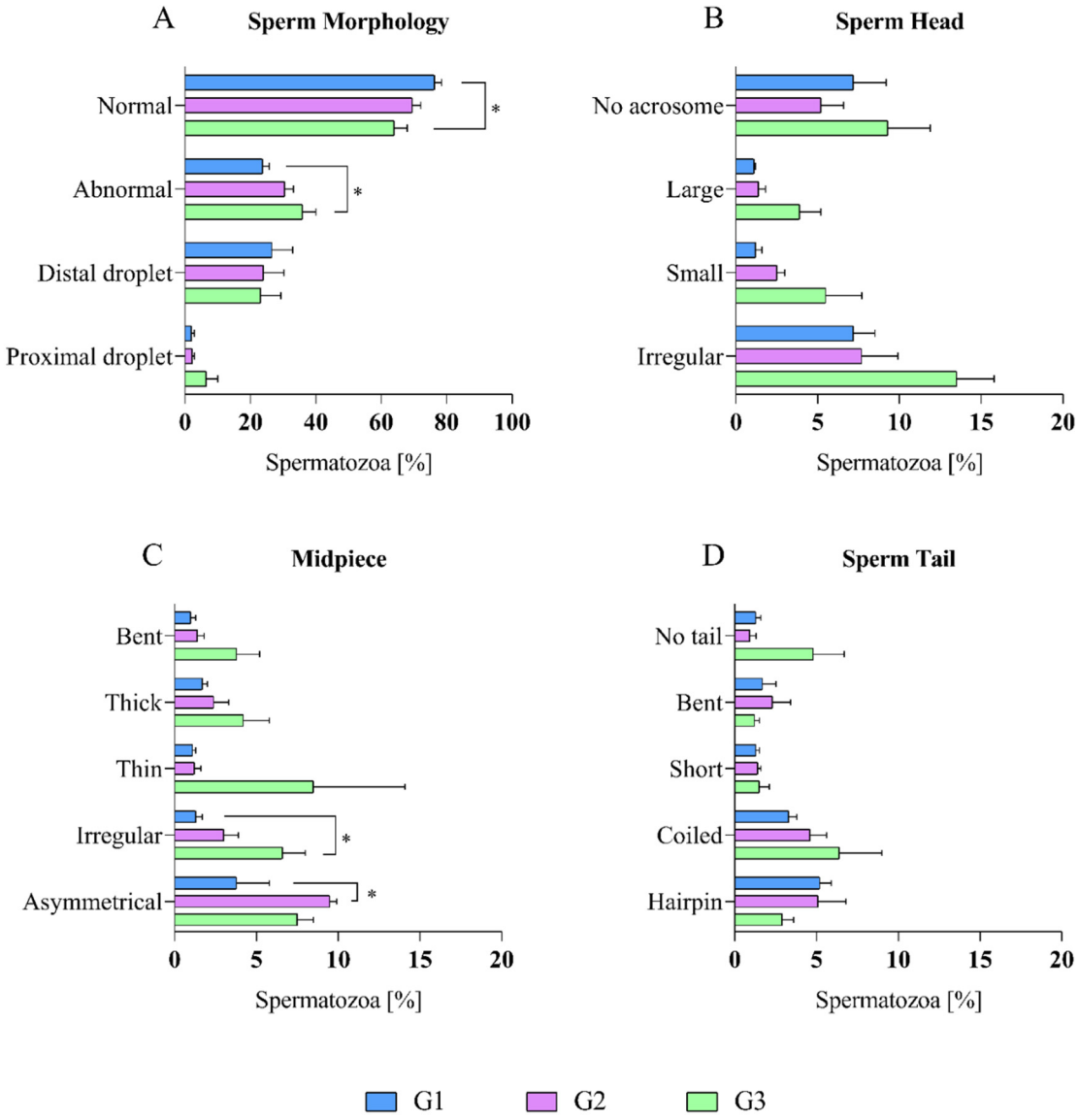

Figure 1. Morphological characteristics of the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1: 12–41 months old; G2: 42–77 months old; G3: 78–132 months old). The dog epididymal spermatozoa were stained with Spermac Stain™. (**A**) Sperm morphology was analysed according to the World Health Organization guidelines: normal (without primary defects), abnormal (at least one defect), sperm with distal droplet and sperm with proximal droplet. (**B**) Sperm head: no acrosome (sperm head without acrosome), large (sperm with large head), small (sperm with small head), irregular (irregular shape of sperm head). (**C**) Midpiece: bent, thick, thin, irregular (irregular shape of sperm midpiece), asymmetrical (asymmetrical position of sperm midpiece). (**D**) Sperm tail: no tail (sperm without tail), bent, short, coiled, harpin (harpin-like sperm tail). Values were given as the mean ± SE. * Significant at $p < 0.05$.

When comparing the percentage of morphological defects of the dog epididymal spermatozoa among the three age groups, it was shown that the highest percentage of normal spermatozoa was found in G1 (76.3 ± 2.1%), and the lowest value was in G3 (64.0 ± 4.0%). The differences were statistically significant ($p < 0.05$) between these two groups. G2 characterized the average percentage of normal spermatozoa (69.5 ± 2.6%) (Figure 1A).

There were no significant differences in the percentage of distal and proximal droplets among the age groups (Figure 1A). Also, no significant differences were found in the percentage of sperm head defects among the age groups (Figure 1B).

When comparing morphological defects of the midpiece among the age groups, there were statistical differences ($p < 0.05$) between two of them. The lowest percentage of irregular midpiece was found in G1 (1.3 ± 0.4%), and the highest was in G3 (6.6 ± 1.4%). The highest number of asymmetrical midpieces was found in G2 (9.5 ± 0.4%), and the lowest was in G1 (3.8 ± 2.0%). The average percentage of irregular and asymmetrical midpiece was 3.0 ± 0.9% (in G2) and 7.5 ± 1.0% (G3), respectively. Defects such as a bent, thick, and thin midpiece did not have significant differences among the age groups (Figure 1C).

When comparing morphological defects of the sperm tails among the age groups, there were no statistical differences that were found (Figure 1D).

2.3. SDS-PAGE Analysis

The SDS-PAGE protein profiles of dog epididymal spermatozoa extracts were analyzed according to the age group (G1, G2, and G3). The protein profiles for all three age groups were similar and were characterized by the presence of 36 protein fractions (PFs) with molecular weights (MW) ranging from 10.6 to >250.0 kDa (Figure 2).

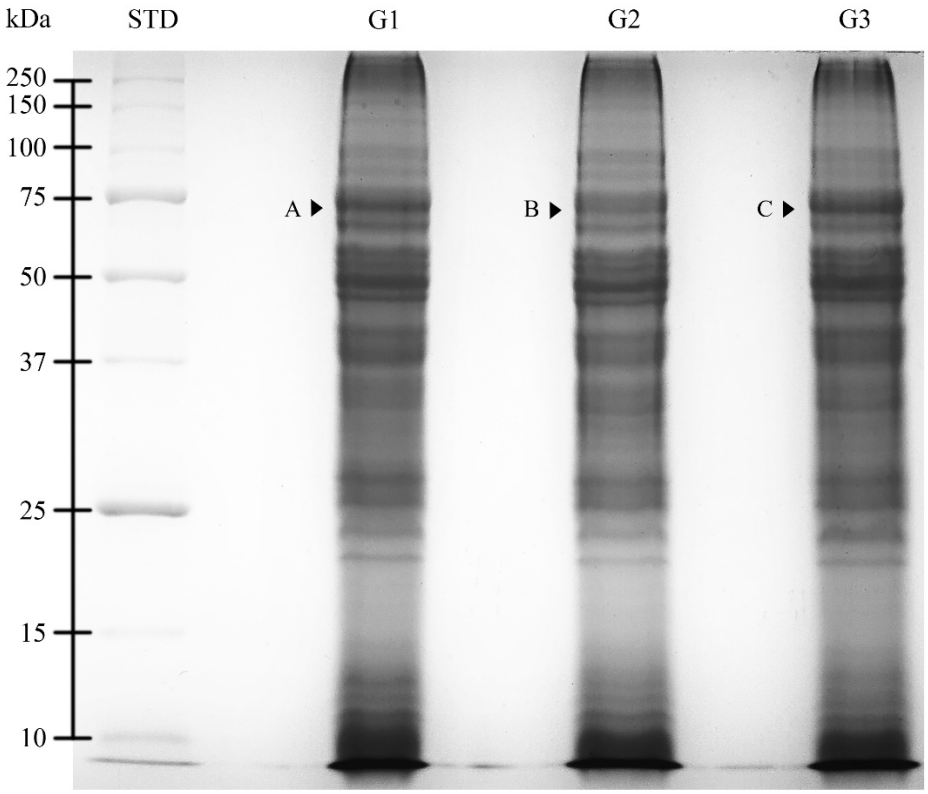

Figure 2. One-dimensional SDS-PAGE (12%) gel image of the cauda epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1: 12–41 months old; G2: 42–77 months old; G3: 78–132 months old). Differentially expressed proteins (DEPs) are marked with letters A, B, and C. STD—molecular weight markers.

An optical density (OD) analysis of PFs showed higher protein content ($p < 0.05$) for one PF (68 kDa) when compared with the corresponding (showing the same MW) PFs among the age groups. PFs in particular age groups were marked with letters from A to C (Table 2).

Table 2. Average optical density (OD) values (mean ± SE) of differentially expressed proteins (DEPs) of the cauda epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1: 12–41 months old; G2: 42–77 months old; G3: 78–132 months old). Different superscripts (a, b) within the same column indicate significant differences ($p \leq 0.05$) among the fractions. DEPs are marked with letters from A to C. MW—average molecular weight.

Age Groups	Protein Bands	MW (kDa)	OD ± SE
G1	A	68.48	0.32 ± 0.01 [a]
G2	B	68.37	0.25 ± 0.01 [b]
G3	C	68.07	0.33 ± 0.02 [a]

2.4. Qualitative and Quantitative Mass Spectrometry Analysis

2.4.1. Qualitative Analysis

Mass spectrometry analysis identified a total of 1772 proteins in all age groups: G1—865 proteins, G2—472 proteins, and G3—435 proteins.

UniProt database-supported identification resulted in two unique proteins (UPs) that were identified in G1, five UPs identified in G2, and three Ups that were identified in G3, which were present in a statistically significant manner (when present in 50% + 1 animals) (Figure 3). There were two polypeptides that were identified in G1 as UPs were: glutathione peroxidase (GPX5) and hyaluronoglucosaminidase (CEMIP). A total of five polypeptides that were identified in G2 as UPs were: mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase (ENGASE), inactive ribonuclease-like protein 9 (RNASE9), clusterin (CLU), pleckstrin homolog, MyTH4, and FERM domain-containing H1 (PLEKHH1) and epididymal sperm-binding protein 1 (ELSPBP1). There were three polypeptides that were identified in G3 as UPs were: cystatin domain-containing protein (LOC607874), Family with sequence similarity 135 member A (FAM135A), and abnormal spindle-like microcephaly-associated protein homolog (ASPM).

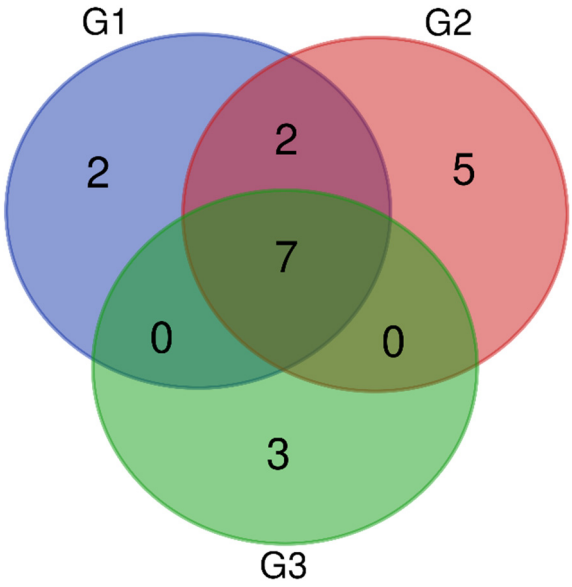

Figure 3. A Venn diagram showing the number of unique proteins (UPs) and common proteins that were identified in the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1, G2, G3).

Protein identification also resulted in seven proteins that were present in all three age groups (G1, G2, G3) and two proteins that were found in G1 and G2 (Figure 3). The seven common proteins in groups G1, G2, and G3 were lactotransferrin (LTF), actin, cytoplasmic 1 (ACTB), prostaglandin-H2 D-isomerase (PTGDS), CE10 protein (CE10), NPC intracellular cholesterol transporter 2 (NPC2), albumin (ALB), and cysteine-rich secretory protein 2 (CRISP2). The two proteins that were detected in both G1 and G2 were WAP domain-containing protein (N/A/WAPdcp) and lipocln_cytosolic_FA-bd_dom domain-containing protein (LCNL1).

2.4.2. Quantitative Analysis

The study also included a semi-quantity analysis of protein content that was based on intensity measurement. When comparing the intensity of seven common proteins (presented in all groups G1, G2, and G3) among the age groups, statistical differences were found in the intensity of four proteins.

ACTB intensity was low ($p < 0.05$) in G1 and G3 ($114.6 \pm 16.1 \times 10^6$ and $134.7 \pm 23.9 \times 10^6$, respectively). It was shown that it differed statistically significantly ($p < 0.05$) when compared with G2 ($240.2 \pm 36.2 \times 10^6$) (Figure 4).

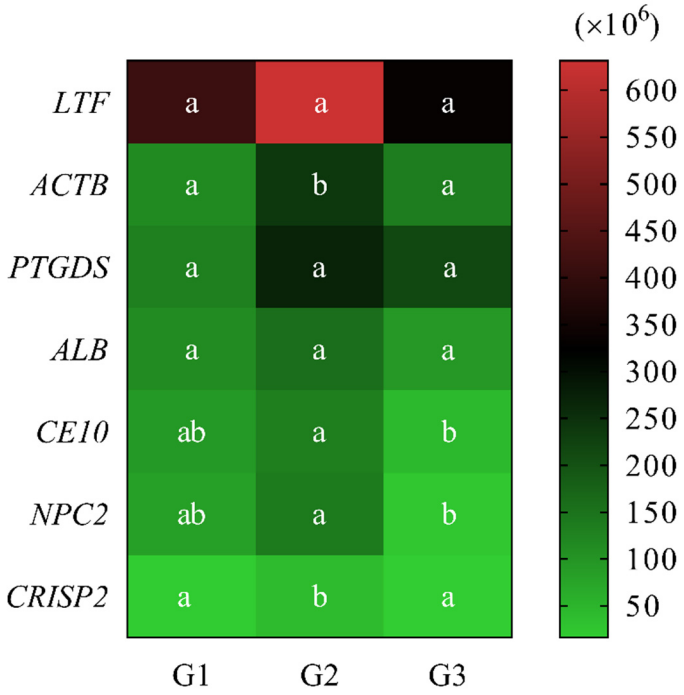

Figure 4. Heatmap showing the common proteins abundance (MS intensity) in epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1, G2, G3). Different superscripts within rows (a, b) means statistically significant differences ($p < 0.05$) among the age groups.

When comparing the intensity of CE10 and NPC2 among the age groups, it was noted that the lowest ($p < 0.05$) values of CE10 ($45.5 \pm 9.5 \times 10^6$) and NPC2 ($24.8 \pm 8.6 \times 10^6$) were found in G3. Values differed statistically significantly ($p < 0.05$) when they were compared with G2, $132.8 \pm 17.8 \times 10^6$ and $140.4 \pm 34.1 \times 10^6$, CE10 and NPC2, respectively. The intensity of the above-mentioned proteins in G1 showed no statistically significant differences when they were compared to other age groups and were $93.7 \pm 25.7 \times 10^6$ and $82.9 \pm 21.5 \times 10^6$, respectively (Figure 4).

The CRISP2 intensity was low in G1 and G3 ($17.1 \pm 6.3 \times 10^6$ and $16.0 \pm 5.0 \times 10^6$, respectively), and it differed statistically significantly ($p < 0.05$) when it was compared with G2 ($40.5 \pm 6.8 \times 10^6$) (Figure 4).

There were no significant differences in the values among the age groups in the intensity of LTF, PTGDS, and ALB (Figure 4). The intensity in LTF and ALB ranged from $331.6 \pm 77.4 \times 10^6$

and 94.3 ± 21.2 × 10^6 (G3) to 631.1 ± 109.6 × 10^6 and 157.2 ± 92.8 × 10^6 (G2), respectively, while the PTGDS intensity ranged from 130.2 ± 39.3 × 10^6 (G1) to 269.9 ± 85.4 × 10^6 (G2).

2.5. Western Blotting Analysis

Western blotting analysis showed differential protein expression in dog epididymal spermatozoa among the age groups (Figure 5A,B). Significant differences ($p < 0.05$) were found for ACTB in G3 when compared to G1 and G2 in the normalized mean band volume (Figure 5B).

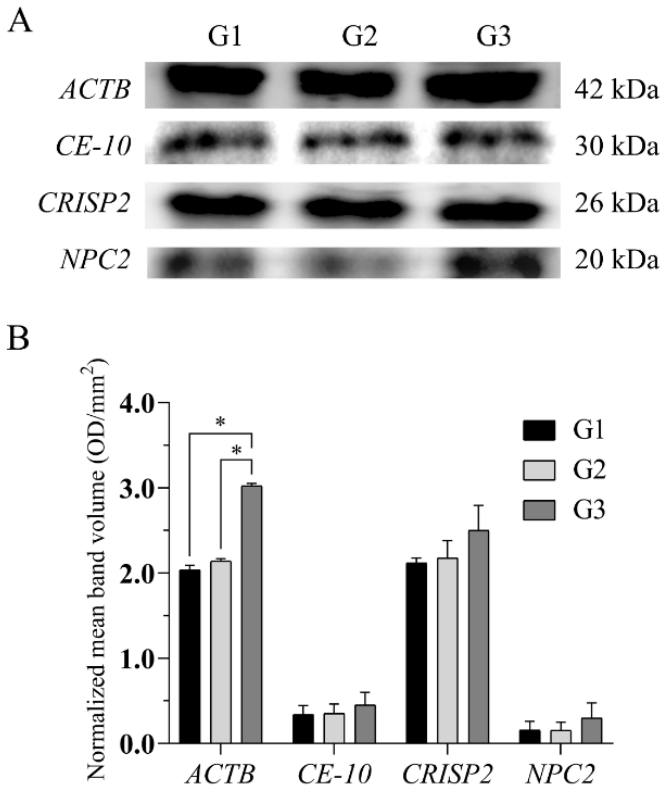

Figure 5. Protein expression levels in the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups: G1, G2, and G3 blotted with antibodies (**A**). Each column indicates the normalized value (mean ± SE) of protein expression from three replicates. Protein expression represents band optical density (OD) which is significantly different among the age groups. * Significant at $p < 0.05$ (**B**).

2.6. GO Analysis, KEGG Pathways, and Functional Annotation

The g.GOSt multi-query Manhattan plots of the GO analysis and KEGG pathways of G1, G2, and G3 dog epididymal spermatozoa proteins, performed with the KOBAS annotation tool (v.3.0), are shown in Figure 6A–C, respectively.

Gene ontology analysis showed similar main molecular functions for all the age groups. For the G1 proteins, the GO:MF terms were dominated by molecular function (LTF, CE10, PTGDS, AREL1), binding (LTF, PTGDS, ALB, SACS), and ion binding (LTF, ALB, SULF2, CYP2E1) (Figure 7A). Furthermore, for the G2 proteins, the GO:MF terms were represented by binding (LTF, PTGDS, ALB, ASPM), ion binding (LTF, ALB, MYO1D, CDC42BPA), and molecular function (LTF, PTGDS, CE10, ALB) (Figure 7B). Whereas for the analysis of the G3 proteins, GO:MF terms were dominated by molecular function (LTF, ASPM, TRPV1, PTGDS), binding (LTF, ASPM, TRPV1, PTGDS), and protein binding (ASPM, TRPV1, CBL, ALB) (Figure 7C).

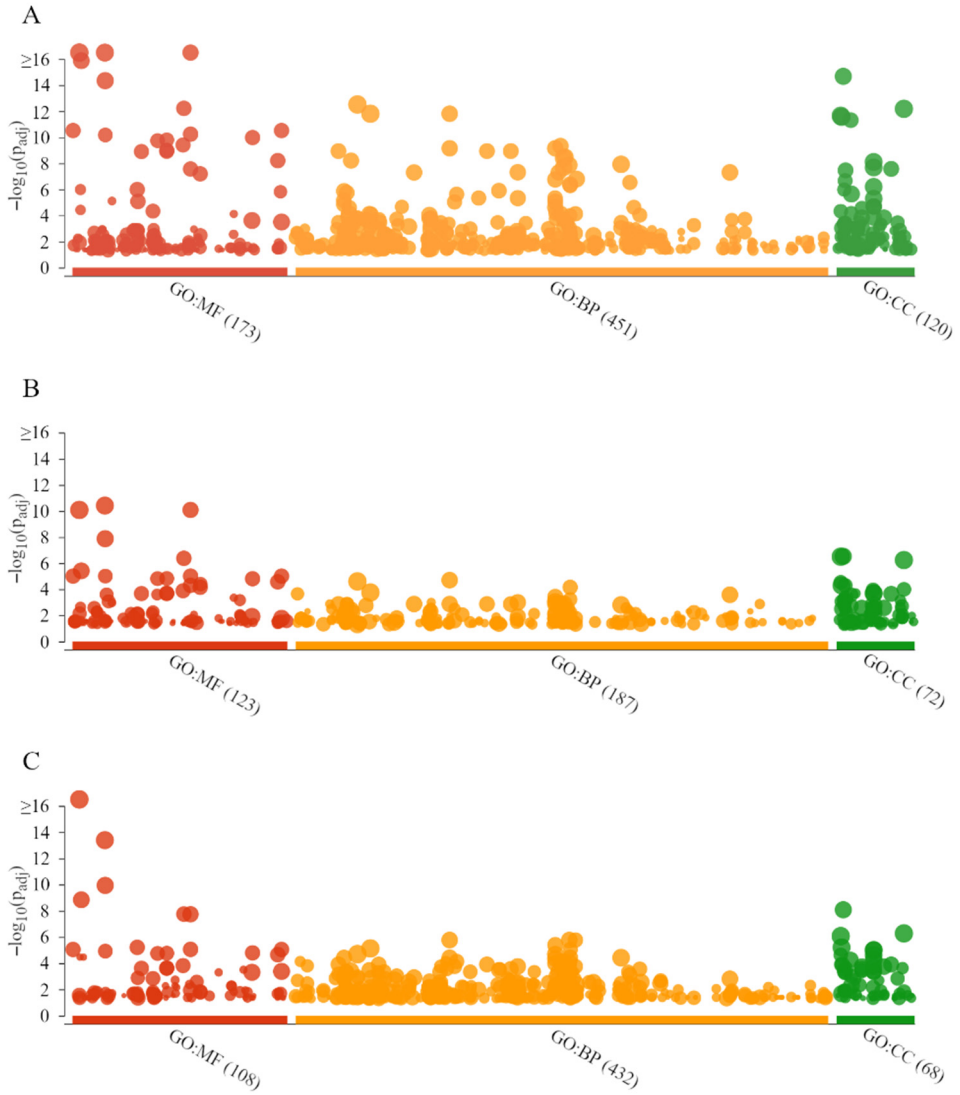

Figure 6. g.GOSt multi-query Manhattan plots showing the functional classification of proteins that were present in the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups: G1 (**A**), G2 (**B**), and G3 (**C**). The *x*-axis represents Gene Ontology (GO) functional terms, grouped and color-coded by data sources: molecular function (GO:MF, red), biological process (GO:BP, orange), and cellular component (GO:CC, green). The *y*-axis shows the adjusted enrichment *p*-values in negative log10 scale. Circles illustrates the enrichment of GO terms and shows the *p*-value. The circle sizes are in accordance with the corresponding GO term size. The number behind the source name in the *x*-axis labels shows how many significantly enriched GO terms there were from this source.

Gene ontology analysis showed similar biological processes for all the age groups. For the G1 proteins, the GO:BP terms were represented by biological processes (LTF, NPC2, PTGDS, AREL1), cellular processes (LTF, NPC2, PTGDS, AREL1), and multicellular organismal processes (LTF, PTGDS, PLCD3, AFP) (Figure 7A). Furthermore, for the G2 proteins, the GO:BP terms were dominated by multicellular organismal processes (LTF, PTGDS, ASPM, CEMIP), biological processes (LTF, PTGDS, ALB, NPC2), and regulation of multicellular organismal processes (LTF, PTGDS, ASPM, CLU) (Figure 7B). However, for the G3 protein analysis, GO:BP terms were represented by multicellular organismal processes (LTF, ASPM, TRPV1, PTGDS), localization (ASPM, TRPV1, CBL, ALB), and cellular localization (ASPM, TRPV1, ALB, CLU) (Figure 7C).

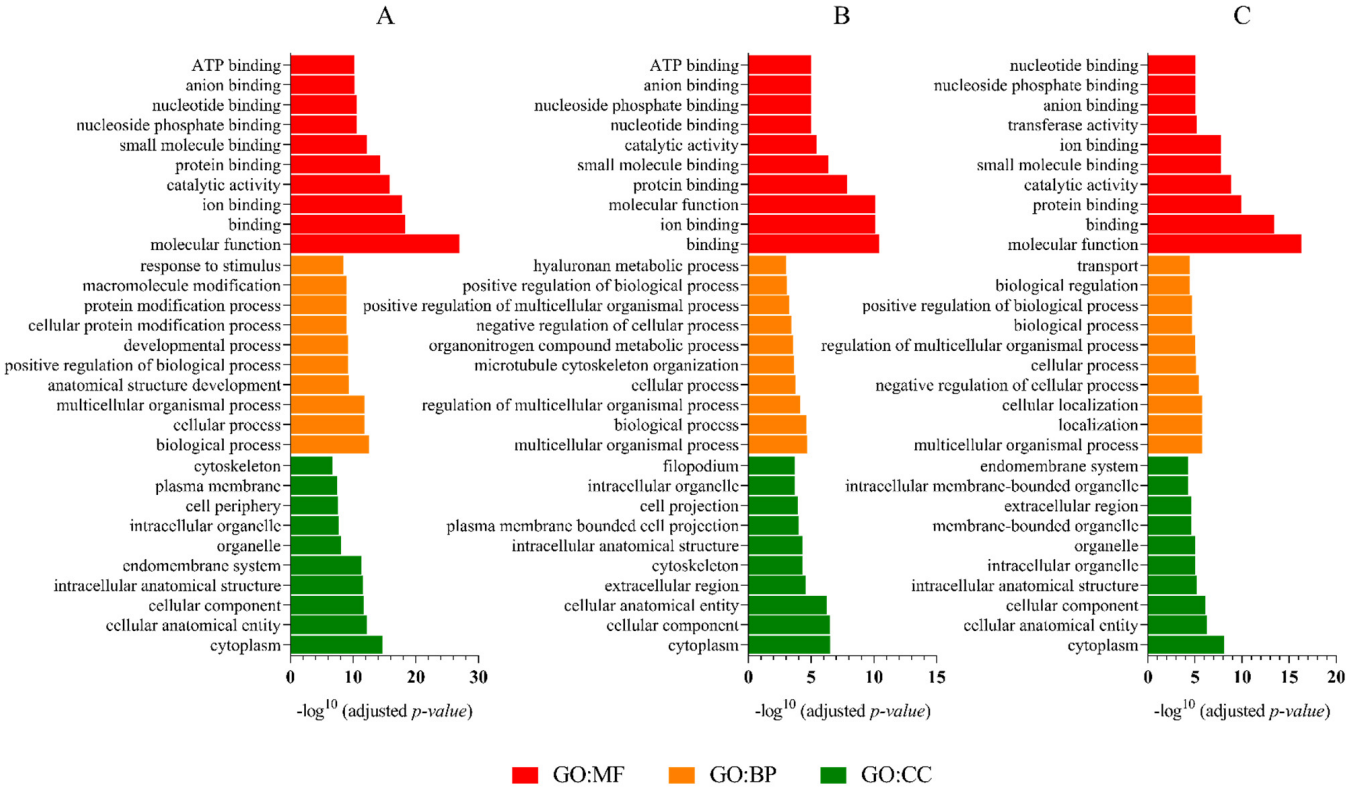

Figure 7. Gene Ontology (GO) of proteins that were present in the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups: G1 (**A**), G2 (**B**), and G3 (**C**). There are ten highly significant GO terms for molecular function (GO:MF, red), biological process (GO:BP, orange), and cellular component (GO:CC, green) that are presented.

Gene ontology analysis showed the same main cellular component for all the age groups. For the G1 proteins, the GO:CC terms were dominated by cytoplasm (LTF, AREL1, ALB, SACS), cellular anatomical entity (LTF, CE10, PTGDS, AREL1), and cellular component (LTF, CE10, PTGDS, AREL1) (Figure 7A). Furthermore, for the G2 proteins, the GO:CC terms were represented by cytoplasm (LTF, ALB, ASPM, CEMIP), cellular component (LTF, PTGDS, CE10, ALB), and cellular anatomical entity (LTF, PTGDS, CE10, ALB) (Figure 7B). However, for the G3 protein analysis, GO:CC terms were dominated by cytoplasm (LTF, ASPM, CBL, ALB), cellular anatomical entity (LTF, ASPM, TRPV1, PTGDS), and cellular component (LTF, ASPM, TRPV1, PTGDS) (Figure 7C).

Pathways analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed mainly protein export for the G1 group (SRP14, SEC61A1, SRPRA), protein processing in the endoplasmic reticulum (TUSC3, SEC24D, MAPK10, RRBP1, SEC61A1, ERN1), and Ras signaling pathway (RGL2, TIAM1, GRB2, MAPK10, ZAP70, ABL1). For the G2 group, the main pathways were: Salmonella infection (NFKB1, TJP1, DYNC2H1), transcriptional misregulation in cancer (MET, MLLT1, NFKB1), and adherens junction (MET, TJP1). For the G3 group, the main metabolic pathways were established as mucin-type O-glycan biosynthesis (GALNT6, GALNT18), porphyrin and chlorophyll metabolism (BLVRA, CP), and cell cycle (SMC1B, ANAPC11, MCM2) (Table 3).

Table 3. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of the epididymal spermatozoa of dogs (*Canis lupus familiaris*) in different age groups (G1: 12–41 months old; G2: 42–77 months old; G3: 78–132 months old). $p \leq 0.01$. Gene name abbreviations were explained in Tables S1–S3.

G1				
ID	**Pathway Name**	**Protein Counts**	**$-Log^{10}$ (Adjusted *p*-Value)**	**Protein Names**
cfa03060	Protein export	3	5.63×10^{-4}	SRP14 | SEC61A1 | SRPRA
cfa04141	Protein processing in endoplasmic reticulum	6	6.22×10^{-4}	TUSC3 | SEC24D | MAPK10 | RRBP1 | SEC61A1 | ERN1
cfa04014	Ras signaling pathway	6	3.58×10^{-3}	RGL2 | TIAM1 | GRB2 | MAPK10 | ZAP70 | ABL1
cfa04930	Type II diabetes mellitus	3	3.59×10^{-3}	ABCC8 | HK1 | MAPK10
cfa00330	Arginine and proline metabolism	3	3.80×10^{-3}	MAOB | PRODH2 | NOS3
cfa04979	Cholesterol metabolism	3	4.02×10^{-3}	LIPA | ABCG8 | ABCA1
cfa02010	ABC transporters	3	4.02×10^{-3}	ABCG8 | ABCC8 | ABCA1
cfa04530	Tight junction	5	5.10×10^{-3}	TIAM1 | SLC9A3R1 | MAPK10 | RAB13 | ROCK2
cfa04360	Axon guidance	5	6.14×10^{-3}	PLXNC1 | ABL1 | ENAH | ROCK2 | ABLIM2
cfa00360	Phenylalanine metabolism	2	6.20×10^{-3}	MAOB | IL4I1
cfa05135	Yersinia infection	4	7.34×10^{-3}	ROCK2 | MAPK10 | ZAP70 | NLRP3
G2				
ID	**Pathway Name**	**Protein Counts**	**$-Log^{10}$ (Adjusted *p*-Value)**	**Protein Names**
cfa05132	Salmonella infection	3	5.55×10^{-4}	NFKB1 | TJP1 | DYNC2H1
cfa05202	Transcriptional misregulation in cancer	3	4.54×10^{-3}	MET | MLLT1 | NFKB1
cfa04520	Adherens junction	2	8.47×10^{-3}	MET | TJP1
G3				
ID	**Pathway Name**	**Protein Counts**	**$-Log^{10}$ (Adjusted *p*-Value)**	**Protein Names**
cfa00512	Mucin-type O-glycan biosynthesis	2	3.37×10^{-3}	GALNT6 | GALNT18
cfa00860	Porphyrin and chlorophyll metabolism	2	3.79×10^{-3}	BLVRA | CP
cfa04110	Cell cycle	3	4.43×10^{-3}	SMC1B | ANAPC11 | MCM2
cfa04120	Ubiquitin mediated proteolysis	3	6.18×10^{-3}	ANAPC11 | MGRN1 | UBR5
cfa02010	ABC transporters	2	7.61×10^{-3}	ABCA12 | ABCC1
cfa05225	Hepatocellular carcinoma	3	9.79×10^{-3}	PLCG2 | TERT | WNT6

3. Discussion

Age-dependent changes in males (e.g., increase in systemic diseases, infections, abnormalities in testis structure, and lower sex hormones) may influence the quality of epididymal sperm [19,20].

This study aimed to verify the possible influence of the aging process on dog epididymal spermatozoa quality and on their proteome characteristics. According to the authors' knowledge, this is the first study to investigate the influence of dog age on the epididymal sperm proteome.

The aging process in semen has been mostly investigated in humans, and a significantly lower ejaculate volume [20–22], a decrease in sperm with progressive motility [21,22], and a higher percentage of sperm DNA fragmentation index [20] were found. In this study, it was shown that the motion parameters of the dog's epididymal sperm, such as TMOT, PMOT, VAP, VCL, and VSL, decreased with age. These findings are in agreement with the results of Verón et al. [23], who have shown that several sperm quality parameters for men's ejaculate, such as VSL, VCL, VAP, BCF, and ALH, were also negatively affected by age. Bhanmeechao et al. [7]

also have shown that dog age was negatively correlated with epididymal sperm motility, sperm vigor, and viability. Additionally, those authors described a positive correlation between male dog age and the percentage of sperm defects [7]. Similar results were presented in the current study. The highest percentage of morphological damage was noted in the epididymal sperm midpiece. This may indicate disturbances in the ATP production in aging sperm. Negative changes in dog ejaculated sperm morphology followed by male age were also shown by Rijsselaere et al. [5] and Rota et al. [6]. Lechner et al. [24] showed that the percentage of progressively motile, membrane-intact, and morphologically normal spermatozoa was the lowest in 10 and 11-year-old dogs.

The above studies clearly showed that senescence in dogs was associated with a decrease in the functional quality of the epididymal sperm, and consequently, this will be transferred on to the ejaculated sperm quality, which may result in reduced fertility or even infertility.

Much more research is needed on studying sperm proteomics, which would allow a better understanding of the molecular events affecting the biological functions of the reproductive cells [25]. The first step to proteome analysis is usually protein electrophoresis. To date, SDS-PAGE of the dog epididymal spermatozoa proteins according to age has not been shown. In this study, there were no differences that were found in the number of protein fractions or in the range of molecular weights in the gel image presenting epididymal sperm proteins depending on the age of the dog. Only the 68 kDa protein showed changes in their intensity depending on the age group. According to the authors' earlier results, this might be lactotransferrin or carboxylesterase 5A [26]. Lactotransferrin showed differences in the abundance in epididymal sperm from different age groups, but it was not statistically significant. The authors suggested that LTF that was produced and secreted in the dog epididymis may coat epididymal sperm for protection [26]. This phenomenon may also be age-dependent.

Baker et al. [27] used two-dimensional gel electrophoresis (2DE) to investigate changes in rat sperm proteomes during epididymal maturation, and Asano et al. [28] used iTRAQ mass spectrometry to characterize membrane fractions in murine sperm. The rat and mouse sperm proteomes were characterized by 2DE and LC-MS/MS identification [29,30]. The dog ejaculated spermatozoa proteome was characterized by Araujo et al. [31,32] using mass spectrometry.

Based on the authors' knowledge, this is also the first study concerning a mass spectrometry analysis of the proteins that are present in dog epididymal spermatozoa according to age, using liquid samples. The epididymal sperm proteome was characterized for different animal species, such as the horse [33], bull [34], swine [35], and mice [36]. In a recent study by the authors of fractionated canine epididymal sperm proteins [26], mass spectrometry identification resulted in a total of 195 proteins that were extracted from the gel. That may be a small number when compared with a liquid sample analysis involving 1772 proteins that were identified in all age groups. As can be noted, an analysis of liquid samples might provide much more information than an analysis of protein samples that were extracted from the protein bands from a gel, even for the same biological material.

Knowledge of the impact of aging processes on male dog fertility is limited. Brito et al. [37] indicated that aging processes have an adverse effect on spermatogenesis and sperm maturation in the epididymis. However, a proteomic study of the influence of aging processes on the sperm proteome in the dog has not yet been conducted. In this study, the whole proteome profiling of the dog's epididymal spermatozoa divided into age groups showed differences in the total number of peptides that were identified.

A total of four common proteins (ACTB, CE10, NPC2, CRISP2) showed statistically significant different expressions according to the dog's age. Their presence in the canine epididymal sperm was confirmed using Western blotting. Low levels of ACTB, CE10, NPC2, and CRISP2 in the epididymal sperm may be associated with incomplete maturity or aging of the dogs. However, a large-scale study is needed to confirm the potential of these proteins as epididymal sperm aging markers.

ACTB is responsible for cell volume regulation, and it builds the cytoskeleton of sperm cells [38]. It is localized in the flagellar and acrosomal membrane of the spermatozoa. Its role in sperm motility and capacitation was proposed [39–41].

NPC2 intercellular cholesterol transporter 2, also called epididymal secretory protein mRNA was found in high amounts in the epididymal duct epithelium, while the protein was found in the duct lumen [42]. NPC2 form was found on the acrosome and equatorial region of the sperm [43]. It is implicated in cell cholesterol metabolism [44].

CRISP2 is a cysteine-rich secretory protein (CRISP) family member. A decrease in CRISP2 amount in sperm is associated with male infertility [45–47]. It is known to be a part of the sperm acrosome and sperm tail [48–51].

All three proteins (ACTB, NPC2, CRISP2) were earlier found in dog epididymal spermatozoa and described in detail in a previous study by the authors [26].

The pattern of cysteine residues indicates that CE10 is similar to the epididymal CE4 protein [2]. Closely related gene products were abundant in the epididymis of stallions and bulls but not in rodents or men [52]. It was not found in dogs. The described protein may act as extracellular proteinase inhibitor [53].

A qualitative analysis of proteins showed the presence of two proteins that were unique for the group of the youngest dogs. The proteins were glutathione peroxidase (GPx) and hyaluronoglucosaminidase (CEMIP). GPx is a well-known, highly abundant protein in the canine epididymis [54] and was described in detail in a recent study by the authors [26]. CEMIP (also named KIAA1199) is expressed in the human testis [55]. It exerts significant changes in cell morphology and actin cytoskeletal dynamics and mediates its effects through the cooperative regulation of the canonical Wnt and P38/MAPK signaling [56]. The biological role of CEMIP has been studied in cancer biology in humans, but it has not been described in other species including the dog. The expression of CEMIP may be regulated depending on whether the cells are mortal or immortal rather than how old the cells are [55].

A total of five unique proteins were identified in the middle-aged dog group: endo-β-N-acetylglucosaminidase (ENGASE), epididymal sperm-binding protein 1 (ELSPBP1), inactive ribonuclease-like protein 9 (RNASE9), clusterin (CLU), and pleckstrin homolog, MyTH4 and FERM domain-containing H1 (PLEKHH1).

ENGASE is one of the key enzymes in the processing event of free oligosaccharides in the cytosol [57]. Recently reported evidence suggests that the enzyme can also directly act on misfolded N-glycoproteins [58]. The ENGASE gene exhibited a broad tissue distribution [57], but it was not characterized in the reproductive organs or canine spermatozoa.

ELSPBP1 was first described in humans and dogs as a sperm-binding protein of epididymal origin [59]. Since then, orthologs have been identified in horse [60], pig [61,62], and bovine [63] models. ELSPBP1 binds to the spermatozoa during their transit through the epididymis [63]. More recently, ELSPBP1 was shown to negatively correlate with bull fertility [64] and was, in fact, associated with the sperm population that was already dead before ejaculation [65].

RNASE 9 may be synthesized and secreted by principal cells of the epididymis and may bind to spermatozoa when they are passing by. Therefore, human RNASE 9 may be a sperm maturation-related protein [66]. Liu et al. [67] demonstrated that RNASE 9 protein inhibited sperm capacitation and acrosome reaction in humans. Epididymis-specific and androgen-dependent RNASE9 expression was shown in rats [68] and in mice [69]. This enzyme also exhibited antibacterial activity [66]. RNASE 9 presence in the canine epididymal spermatozoa was established for the first time in the current study. It might also act as an antibacterial factor and sperm capacitation inhibitor in canine sperm.

CLU is an extracellular chaperone that is known to be secreted by stallion testes [70] and epididymides [71] in a high amount. It is overexpressed in several human cancers such as prostate cancer [72]. CLU participates in sperm maturation by affecting lipid transport and membrane remodeling [72]. Morphologically defected sperm extensively bind CLU

to its plasma membrane [73]. CLU presence in the canine epididymal spermatozoa was established for the first time in the current study.

PLEKHH1 is a protein that is involved in intracellular signaling or as constituents of the cytoskeleton [74]. It can bind phosphatidylinositol lipids within biological membranes. Through these interactions, PLEKHH1 plays a role in recruiting proteins to different membranes. The protein expression was found in human testis, epididymis, seminal vesicles, and prostate [75]. However, although its presence was shown [26], its function in canine epididymal tissues or sperm has not yet been established.

In the oldest dog group (G3), three proteins were identified as unique: Cystatin domain-containing protein (LOC607874), Family with sequence similarity 135 member A (FAM 135A), and abnormal spindle-like microcephaly-associated protein homolog (ASPM). These proteins were not shown in dog tissues or spermatozoa until now.

Canis lupus familiaris cystatin-C-like (LOC607874) mRNA was found as recorded in the NCBI. A cystatin-related epididymal-specific (CRES) gene was found in the mouse epididymis, showing homology with those of well-established protein inhibitors (cystatins) [76]. The CRES gene is almost restricted to the epididymis and much less expressed in the testis, may be expressed in spermatids [77].

FAM 135A function is closely related to the regulation of cellular proliferation, differentiation, development, and cellular growth control [78]. The protein expression was found in human tissue: testis, epididymis, prostate, and seminal vesicle [79].

ASPM is expressed in a variety of embryonic and adult tissues and is upregulated in cancer [80]. A lack of a functional ASPM may affect the fidelity of chromosome segregation which leads to a reduced ability of fetal stem cells to produce neurons [80]. ASPM possesses a role in sperm flagellar function [81].

These three above-mentioned proteins may be potentially epididymal sperm aging markers, but a much broader study is needed to confirm these findings.

A gene ontology analysis showed the main function of the analyzed sperm proteins to be binding, independent of age groups. This might underline the importance of epididymal sperm protein affinity to other proteins or ions as one of the sperm function regulating mechanisms. This is a well-documented issue for both animal and human ejaculated sperm [82]. However, less is known about epididymal sperm in this regard. Epididymal sperm protein functions that were found in each of the three age groups were mainly involved in the regulation of biological processes, cellular processes, multicellular organismal processes, and cellular localization. Using a cellular components analysis for all three age groups, it was found that epididymal sperm proteins were derived mainly from the cytoplasm and cellular anatomical entities.

A pathway analysis using KEGG for the youngest dogs mainly showed protein export, protein processing in the endoplasmic reticulum, and the Ras signaling pathway.

Most secretory and membrane-bound proteins are co-translationally translocated. Proteins that reside in the endoplasmic reticulum (ER), Golgi, or endosomes also use the co-translational translocation pathway [83], even though most secretory proteins are co-translationally translocated. In addition, proteins that are targeted to other cellular destinations, such as mitochondria, chloroplasts, or peroxisomes, use specialized post-translational pathways [83]. These processes seem to be intensified in the epididymal spermatozoa of young dogs.

The Ras signaling pathway is one of the main pathways to transduce intracellular signals in response to mitogens controlling cell growth, survival, and anti-apoptotic programs. Ras, a low-molecular-weight GTP-binding protein, plays a key regulatory role in many biochemical processes. The presence of Ras was shown in hamster testicular, caput and cauda epididymal spermatozoa [84]. The interaction of Ras with both PI3-kinase and PKC suggests that Ras may regulate several signaling pathways in spermatozoa [84]. It has been recently reported that Rab 2A and 3A (members of the Ras family) are related to acrosomal exocytosis in spermatozoa, and Rab 2A can be used as a fertility-related biomarker in

males [85]. Rab proteins were located in the sperm head and tail. Ras is correlated with various sperm motility patterns and motion kinematics before capacitation [86].

For the middle-aged dogs, the main established pathways were: salmonella infection, transcriptional misregulation in cancer, and adherens junction.

In tumor cells, genes encoding transcription factors (TFs) are often amplified, deleted, rearranged via chromosomal translocation and inversion, or subjected to point mutations that result in a gain or loss of function. Similar processes might be connected with aging in a sperm cell.

Adherens junctional complexes, such as ectoplasmic specializations, facilitate cellular interactions that are critical for both adhesion and signaling between Sertoli cells and germ cells [87]. Previous studies indicated that Sertoli cell-germ cell adherens junctions undergo extensive restructuring to promote germ cell maturation and spermation [87,88].

For the oldest dogs, the main metabolic pathways were established as mucin-type O-glycan biosynthesis, porphyrin and chlorophyll metabolism, and cell cycle.

Mucin-type O-glycosylation is a protein modification that is present on membrane-bound and secreted proteins [89]. Mucins bind bacteria and viruses, and also function as receptors for carbohydrate-binding proteins. Mucin-type O-glycan biosynthesis was intensified in the spermatozoa of obese rats [90].

Metal complexes of porphyrins play important biological roles. A porphyrin- and chlorophyll-metabolism-enriched pathway was found in goat seminal plasma [91]. However, little is known about porphyrin metabolism in canine epididymal sperm.

The cell cycle includes the mitotic and meiotic cell cycle [92]. The meiotic proteins might be remnants of spermatogenesis, with no function in mature sperm [92].

In a study by Silva et al. [4], the activity of 12 proteins in human ejaculated spermatozoa was correlated with male age. Of those, half of them were the main components of the mammalian target of the rapamycin (mTORC1) signaling pathway.

It seems that in different dog ages, different metabolic pathways in epididymal spermatozoa are intensified, which might be connected with the aging processes.

In order to analyze the influence of male age on the epididymal semen, parameters such as sperm motility, membrane condition, antioxidant status, and chromatin status are usually examined. The current study additionally investigated the influence of age on the proteome of the dog's epididymal sperm and its metabolic pathways, which provides a broader point of view on the aging process.

4. Materials and Methods

The study was performed under the guidance of Directive 63/2010/EU and the Journal of Laws of the Republic of Poland (2017) regarding the protection of animals that are used for scientific or educational purposes. The exemption letter was obtained from the Local Ethics Committee for Animal Experimentation, Olsztyn, Poland (LKE/01/2022). The authors have permission to conduct animal experiments according to the Polish Laboratory Animal Science Association (Numbers: 1432/2015; 1508/2015).

4.1. Chemicals and Media

All chemicals of the highest purity were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

4.2. Animals

The study was performed on 30 mixed-breed dogs that were divided into three age groups according to the study by Ortega-Pacheco et al. [93], with modifications: G1 (young, 12 to 41 months old; n = 10); G2 (adult, 42 to 77 months old, n = 10); and G3 (old, 78 to 132 months old, n = 10) of unknown fertility. The weight of the dogs was from 9 to 28 kg (mean 16.5 kg) in G1, from 15 to 30 kg (mean 20.8 kg) in G2, and from 17 to 30 kg (mean 23.9 kg) in G3. Only dogs for whom the authors were able to prove animal age documented information were taken into consideration. The dogs were fed

and kept in the same environmental conditions in the Shelter for Homeless Animals in Tomaryny (Poland). All of the dogs were presented for a routine orchiectomy by a qualified veterinary doctor as a part of a program to prevent animal homelessness and promote adoption. The consent form was achieved from the director of the shelter.

4.3. Cauda Epididymal Semen Collection

The materials, i.e., the testis with the epididymis, were placed in sterile plastic containers in 0.9% NaCl solution and then in a thermobox at a temperature of 4 °C and delivered within one hour to the laboratory of the Department of Animal Biochemistry and Biotechnology (University of Warmia and Mazury in Olsztyn, Poland). Immediately after that, the gonads were washed with DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco, Grand Island, NY, USA). The cauda epididymal tissue was cut carefully with a sterile scalpel to avoid sectioning the blood vessels. The effluent of the epididymal semen was aspirated from the cauda epididymal tissue using an automatic pipette [94] with modification. The samples that were obtained from the cauda epididymis (right and left) of the same animal were pooled.

4.4. Spermatozoa Quality Assessment

The epididymal sperm concentration was determined using a Bürker chamber under a light microscope (Olympus BX41TF, Tokyo, Japan).

The sperm samples were subsequently assessed using a computer-assisted semen analysis (CASA-system, HTM-IVOS, 12.3, Hamilton-Thorne Biosciences, Beverly, MA, USA). The procedure was described previously by Mogielnicka-Brzozowska et al. [95]. The following software settings that were recommended by the manufacturer for canine sperm analyses were used: frame acquired—30, frame acquisition rate—60 Hz, minimum cell contrast—75, minimum cell size—6 pixels, straightness threshold—75%, path velocity threshold—100 μm/s, low average path velocity (VAP) cut-off—9.9 μm/s, low straight-line velocity (VSL) cut-off—20 μm/s, static size gates—0.80–4.93, static intensity gates—0.49–1.68, and static elongation gates—22–84. The total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), the amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity coefficient (LIN, %) were analyzed in each epididymal sperm sample.

4.5. Morphology Assessment of Epididymal Spermatozoa

The dog epididymal spermatozoa were prepared as smears on glass slides using 10 μL of each sample (1×10^8 spermatozoa) and left to dry on a thermoblock (5 min, 37 °C). Spermac Stain™ (FertiPro, Beernem, Belgium) staining was then performed according to the manufacturer's recommendations with modifications. The morphological structures in the dog epididymal spermatozoa were examined under bright light microscopy at the magnification of 1000× (Olympus BX41TF). Approximately 200 spermatozoa were counted in each sperm sample. The spermatozoa were classified into two categories: normal (without defects) or damaged (at least one defect), according to the World Health Organization guidelines [96]. During the determination of the sperm head defects, attention was paid to the shape of the acrosome and continuity of the membranes surrounding the sperm nucleus. A normal head was smooth and regularly contoured, the midpiece was slender and regular, and the tail was smooth along its length and thinner than the midpiece. According to the protocol of Spermac Stain™ (FertiPro), the epididymal sperm fragments were stained as follows: the acrosome (dark green), the nucleus (red), the equatorial region (pale green), and the midpiece and the tail (green).

4.6. Preliminary Sample Preparation

After the epididymal semen quality assessment, the sperm samples were centrifuged at 800× *g* for 10 min at 4 °C to remove epididymal fluid (EF). The remaining supernatant

(EF) was removed, and the sperm pellet was resuspended in 1 mL DPBS (Gibco) and again centrifuged at 800× *g* for 10 min at 4 °C to remove loosely bound proteins [97]. The remaining supernatant was removed and discarded. Each of the sperm samples was split in half. One half was used for the electrophoretic separation, and the other half was stored for 2 weeks at −80 °C for further LC-MS analysis.

4.6.1. Preliminary Sample Preparation for Protein Analysis

The epididymal spermatozoa samples were placed in an ice bath and subjected to sonication using the Omni Sonic Ruptor 250 Ultrasonic Homogenizer (Omni International, Kennesaw, GA, USA) with the following parameters: 150 W for 10 min with a frequency of 60 kHz. Following sonication, the sperm samples were centrifuged (8000× *g* for 10 min at 4 °C). The resulting supernatant containing sperm intracellular proteins (SIPs) was collected into another Eppendorf tube. An aliquot of 1 mL of Radioimmunoprecipitation Assay Buffer (RIPA) containing 50 mM Tris-HCl; 150 mM NaCl; 1% (*v*/*v*) Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; and ddH2O, pH 7.4 was added to the remaining sperm pellet and the sample was incubated for 5 min at 4 °C and was then vortexed and left overnight in a buffer [95,98] with modifications. Protease Inhibitor Cocktail (Sigma-Aldrich/P8340, St. Louis, MO, USA) was added both to the SIPs and the remaining sperm pellet. The samples were then centrifuged at 8000× *g* for 10 min, 4 °C, to obtain a clear lysate of the sperm membrane-associated protein fraction (SMAPs). The clear lysate was collected into another Eppendorf tube and mixed together with SIPs to get a whole protein set of an epididymal sperm cell (sperm extracts—SE) and was then frozen and kept at −80 °C until further analyses.

4.6.2. Total Protein Content Measurement

The total protein content was measured using Bradford Reagent (Sigma-Aldrich/B6916) in SE.

4.7. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For proteomic analysis, the SE that were isolated from the epididymal spermatozoa of individual dogs were pooled according to age group (G1, G2, G3). Each pool was run in triplicate (technical replicate). Each well was loaded with 50 µg of protein in solution. The SDS-PAGE procedure was previously described by Mogielnicka-Brzozowska et al. [95]. The molecular weight (MW) and the optical density (OD) of the stained protein bands (PB) were determined using MultiAnalyst 1.1 software (BioRad, Laboratories, Hercules, CA, USA). In the current experiment, proteins in the gel were not analyzed by mass spectrometry.

4.8. Western Blotting Analysis

Sperm extract protein samples containing exactly 50 µg were separated by 12% SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Electroblotting was carried out for 1 h at 300 mA, according to the previously described method [99].

After blocking non-specific binding sites with 5% nonfat milk in Tris-buffered saline, TBS (1 M Tris, 5 M NaCl, pH 8.0), containing 0.05% (*v*/*v*) Tween 20, TBST (MP Biomedicals LLC, Santa-Ana, CA, USA), the blots were incubated with one of the following primary antibodies (Thermo Fisher Scientific, Waltham, MA, USA): beta Actin rabbit polyclonal antibody (PA5-85271; 1:500), Bcl-10 rabbit polyclonal antibody (PA5-85359; 1:250), NPC2 rabbit polyclonal antibody (PA5-51463; 1:500), and CRISP rabbit polyclonal antibody (PA5-97621; 1:1000). The gene name Bcl-10 was used as the alias name for CE10. According to the manufacturer's information, it is an antibody that recognizes CE10. Following incubation with the primary antibodies overnight at 4 °C, the membranes were washed and incubated for 1 h at room temperature with Peroxidase AffiniPure Goat Anti-Rabbit secondary antibody (111-035-003; 1:20,000; Jackson ImmunoResearch, Baltimore Pike, PA, USA), developed with enhanced chemiluminescence ServaLight CL EOS Substrate kit (Serva, Heidelberg,

Germany), and scanned with the ChemiDoc™ Touch Imaging System (BioRad Laboratories). The molecular weights of the protein were determined using the molecular weight standard (PageRuler™ Prestained Protein Ladde, Catalog Number 26,617, Thermo Fisher Scientific). Protein bands from the scanned images were quantified using MultiAnalyst 1.1 software (BioRad Laboratories). The signal intensities were normalized to the total protein by staining membranes with Coomassie Blue. The values were expressed as the total signal intensity inside the boundary of a band that was measured in pixel intensity units/mm^2—optical density (OD).

4.9. Identification of Proteins in Liquid Samples by Mass Spectrometry

4.9.1. Sample Preparation for LC-MS Analysis

Sperm cell samples were extracted as follows. The frozen samples were put on ice, and 800 µL of MTBE: MeOH mixture (3:1) was added to each Eppendorf tube. The extraction mixture was pre-cooled to −18 °C and dispensed as quick as possible. Next, the samples were sonicated in an ice-water-filled sonic bath for 15 min. To separate the polar and non-polar metabolites, 400 µL of water and a MeOH mixture was added and was well vortexed. The extracted samples were placed in a pre-cooled centrifuge (4 °C) and spun for 7 min at 21,000× *g*. The upper non-polar phase for analysis of lipids was collected into a new tube. The interphase containing residual upper and some of the lower phase was discarded. After a short centrifugation (5 min), the lower methanol-water phase was transferred into a new tube. Finally, the residual methanol-water phase was discarded, and the pellets were frozen at −80 °C. The collected liquid extracts were dried in a speed-vac and stored at −80 °C prior to the LC-MS analysis. The MTBE: MeOH mixture contained 12.5 µM D7-Arginine and 100 ng/mL D70-Phosphatidylcholine (36:0) as internal standards for the polar and non-polar phases, respectively.

4.9.2. In Solution Digestion

The protein pellets were dissolved in 10 µL 6M Guanidine-HCl in a 25 mM bicarbonate ammonium solution (pH 8.0). A total of 1 µL of 200 mM DTT in 25 mM bicarbonate ammonium solution (pH 8.0) was then added to each sample and incubated for 30 min at 37 °C. After protein reduction, 10 µL of 200 mM iodoacetamide in 25 mM bicarbonate ammonium solution (pH 8.0) was added for alkylation. Each sample was gently vortexed and incubated in the dark for 1 h at room temperature. A total of 25 mM bicarbonate ammonium solution (pH 8.0) was then added in order to reduce the guanidine-HCl concentration to 0.6 M. Protein digestion was performed by adding a trypsin solution with a final ratio of 1:25 (trypsin:protein), and the samples were incubated overnight in 37 °C with gentle vortexing. After trypsinolysis, 1% formic acid was added to adjust the pH to 3–4. The samples were then desalted on Pierce™ C18 Spin Columns (Thermo Fisher Scientific), vacuum-dried, and resuspended in acetonitrile.

4.9.3. NanoUPLC-Q-TOF/MS Analysis

Waters Acquity liquid chromatography M-Class system (Waters Corp., Milford, MA, USA) equipped with a Peptide BEH C18 analytical column (150 mm × 75 µm; 1.7 µm, Waters Corp.), and Symmetry C18 precolumn (180 µm x 20 mm; 1.7 µm, Waters Corp.) was performed to separate the digested samples.

Each sample was injected into the precolumn and then washed with 99% solvent A (0.1% formic acid in water) at a flow rate of 5 µL/min for 5 min. After washing, the peptides were transferred to an analytical column and separated. The flow rate of the mobile phase was 300 nL/min. The total run time of the analytical gradient, including the column equilibration step, was set at 75 min. The elution gradient steps were as follows: from 0 to 2 min, 5% B (0.1% formic acid in acetonitrile); from 2 to 15 min, 5% to 30% B; from 15 to 45 min, 30% to 60% B; from 45 to 48 min, 60% to 85% B; 10 min, 85% B; and from 58 to 58.5 min, the B concentration dropped from 85% to 5%.

A mass spectrometry (MS) analysis was performed using Synapt G2-Si (Waters Corp., Milford, MA, USA) with a nano-electrospray ionization (nESI) source, operating under a positive ion mode. The capillary voltage was set at 3.0 kV, and the cone voltage was set at 40 V. The cone gas flow was set at 40 L/h, and the source temperature was set at 100 °C. The nanoflow gas flow was set at 0.2 Bar. Data were acquired for m/z 70 to m/z 1800 using data-independent mode (MSE). Leucine enkephalin (m/z 556.2771) was used as a Lockspray. The lock mass was acquired every 45 s, and a mass correction was applied automatically during acquisition.

Raw chromatography files were analyzed with Byonic software (Protein Metrics, Cupertino, CA, USA). The following settings were used for peak picking and identification: trypsin digestion, max. two miss-cleavages, max. three charges, possible modifications: carbamidomethylated Cys; oxidation of Met and Trp; dioxidation of Trp; pyro-Glu; de-carbamidomethylated Cys; oxidation of Pro; phosphorylation of Ser, Tyr, Thr; (di)methylation of Lys and Arg; acetylation of Lys; trimethylation at Lys; sulfation of Cys, Ser, Thr, Tyr. The detected peptides were compared to the SWISSPROT dog proteome (CANLF)—that was downloaded April 2021. False identifications were limited by comparison with common contaminants and decoys that were obtained by reverse amino acid sequencing in silico-cleavage peptide models. The total intensity was a sum of all the peak intensities over all MS/MS spectra. The protein *p*-value is the likelihood of the peptide-spectrum matches (PSMs) to this protein (or protein group) arising by random chance, according to a simple probabilistic model. A log *p*-value of −3.0 corresponds to a protein *p*-value of 0.001, or one chance in a thousand.

4.10. Gene Ontology and Functional Annotation

The functional enrichment of proteins that were present in the dog (*Canis lupus familiaris*) epididymal spermatozoa according to age groups (G1, G2, G3) in Gene Ontology (GO) categories: molecular function (GO:MF), biological process (GO:BP), and cellular component (GO:CC), was performed with the g:Profiler online tool, (https://biit.cs.ut.ee/gprofiler/gost, accessed on 26 April 2022). Annotations were performed with the *Canis lupus familiaris* database, using the false discovery rate (FDR) of the Benjamini–Hochberg (BH) method for the significance threshold. The user threshold was 0.05. The significance of the dog (*Canis lupus familiaris*) epididymal spermatozoa proteins were analyzed in groups (G1, G2, G3) with different male ages in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and the KOBAS 3.0 (http://kobas.cbi.pku.edu, accessed on 10 February 2022) protein functional annotation tool [100]. A Venn diagram was constructed using a web tool (http://bioinformatics.psb.uugent.be/webtools/Venn, accessed on 23 November 2021).

Heatmaps, GO plots, and morphological characteristic plots were performed using GraphPad Prism software (GraphPad Prism v.9.2.0. for Windows, GraphPad Prism software, San Diego, CA, USA).

4.11. Statistical Analysis

The data analysis was carried out using Statistica version 13.1 (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA). The results are presented as the means and standard error (mean ± SE). The sperm motility, morphology, protein intensity, and comparison of the OD values were analyzed with Tukey's HSD test to detect the significant differences among the age groups. The values were considered to differ significantly at $p < 0.05$.

5. Conclusions

In conclusion, the present study demonstrated that aging in dogs was associated with diminished functional quality of the cauda epididymal spermatozoa. Differences in the sperm proteome composition were shown for the young, middle-age, and old dogs, which was followed by changes in the sperm metabolic pathways, which might influence the sperm fertilizing ability. The findings of the current study involving maturation and

aging protein markers may be applicable to clinical andrology and to improving canine reproductive technologies.

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I hereby declare that my personal contribution in the first publication which is the basis of my doctoral thesis:

1. Zmudzinska, A.; Bromke, M.A.; Strzezek, R.; Zielinska, M.; Olejnik, B.; Mogielnicka-Brzozowska, M. Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa and Sperm Structure Separation. *Animals* **2022**, *12*, 772

involved conceptualizing research, developing methodology, acquiring and describing research material, adapting the software for analyzes, conducting the research process, validating, analyzing and interpreting the results, writing an original draft of the publication, conducting the editorial process and visualizing the work, acquiring financial support and it was 65%.

Contribution in the second publication which is the basis of my doctoral thesis:

2. Zmudzinska, A.; Wisniewski, J.; Mlynarz, P.; Olejnik, B.; Mogielnicka-Brzozowska, M. Age-Dependent Variations in Functional Quality and Proteomic Characteristics of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa. *Int. J. Mol. Sci.* **2022**, *23*, 9143

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