Analysis of bull (*Bos taurus*) seminal vesicle fluid proteome in relation to seminal plasma proteome

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ABSTRACT

The existing knowledge on the bull seminal vesicle proteome, a major seminal plasma constituent, and its relationship with seminal plasma is limited. This knowledge is prerequisite for a better understanding of seminal plasma variability, which is linked to semen quality. The objective of this study was to characterize the proteomes of seminal vesicle fluid and seminal plasma and to compare them to better understand the origin of seminal plasma proteins. We collected ejaculates and seminal vesicle fluid postmortem from 6 mature Holstein Friesian bulls. We performed the analysis and identification of proteins using 2-dimensional electrophoresis coupled with matrix-assisted laser desorption/ionization mass spectrometry. We identified 105 proteins in bull seminal vesicle fluid and 88 proteins in seminal plasma. For both seminal vesicles and seminal plasma proteins described in our study, top biological functions were cellular movement, cell death and survival, and cellular growth and proliferation. Additionally, seminal vesicle fluid proteins were involved in protein degradation and synthesis. Seminal plasma proteins were also involved in cellular assembly and organization and cell-to-cell signaling and interactions. Proteins of both fluids were involved in the following canonical pathways: glycolysis, gluconeogenesis, liver X receptor/farnesoid X receptor, and farnesoid X receptor/retinoid X receptor activation. Additionally, seminal vesicle fluid proteins appeared to be involved in oxidative stress response mediated by nuclear factor E2-related factor 2. Our results described the bull seminal vesicle fluid proteome for the first time and allowed for significant expansion of the current knowledge on the bull seminal plasma proteome. Moreover, analysis indicated that both bull seminal vesicle fluid and semi-

nal plasma proteomes contained interconnected protein groups related to protective functions, glycolysis, and the morphology and physiology of the spermatozoa. These proteins and their interactions could be targeted in future research.

Key words: bull, proteome, seminal vesicle, seminal plasma

INTRODUCTION

Semen is a complex mixture consisting of spermatozoa and the liquid known as seminal plasma. The seminal plasma is created during ejaculation by combining epididymal fluid with secretions of accessory sexual glands. In bull, the 4 accessory sex glands consist of seminal vesicles, prostate, bulbourethral glands, and ampulla. Secretion from seminal vesicles makes up the majority of bull seminal plasma (Juyena and Stelletta, 2012). Seminal plasma mainly functions as a nourishment medium for spermatozoa that allows for their transportation, but also plays an important role in major physiological events such as capacitation and spermatozoa-oocyte interaction (Desnoyers and Manjunath, 1992). Seminal plasma constituents include ions, energy substrates (mainly fructose in case of bull sperm), organic compounds, peptides, and proteins. The protein constituent of seminal plasma is vital in efficient fertilization, as it influences spermatozoa plasma membrane stability, motility, capacitation, and sperm-egg interaction (Juyena and Stelletta, 2012).

Research into the bull proteome of seminal plasma and secretions of the reproductive tract has been initiated and allows the description and identification of numerous proteins. To our knowledge, 2 proteomic studies have been published to date concerning comprehensive proteome analysis of seminal plasma. Kelly et al. (2006) described 99 proteins in bull seminal plasma and provided the identification of multiple proteins that had not previously been reported there. Rego et al. (2014) provided a proteomic map of seminal plasma with 108 identified protein spots, corresponding to 46 proteins,

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and proposed putative seminal protein functions during sperm maturation, protection, capacitation, and fertilization. The results of those studies provided a solid foundation to better understand the functions of bull semen.

Despite significant advances in bull reproductive tract proteomic research, knowledge regarding the participation of particular protein components in seminal plasma from different accessory sex gland secretions is not well advanced. Such knowledge is especially important for the characterization of seminal vesicle fluid, because it is the main component of seminal plasma. To the best of our knowledge, only one study has been performed concerning the accessory sexual gland secretion proteome of the bull (Moura et al., 2007). Those authors collected accessory sexual gland secretions separated from sperm cells and epididymal fluid by the catheterization of duct deferens. Proteomic analysis allowed an average of 52 protein spots to be found on 2-dimensional (2-D) gels, which corresponded to 13 proteins. The identification and characterization of those proteins suggested their roles in sperm physiology after ejaculation. Whereas those results shed some light on seminal plasma protein component origins and accessory sex gland functions, they could not, by design, differentiate between particular gland proteomes. In particular, the seminal vesicle fluid proteome was not established; for this reason, its relation with seminal plasma is still not fully understood. Moreover, insight into seminal plasma composition dependence on seminal vesicles and other accessory sex gland fluids could help to explain the variability of seminal plasma. It is especially worth investigating, as recent results on ram seminal plasma show that seminal plasma variability can affect the success of certain biotechniques, such as cryopreservation (Rickard et al., 2016). Recently, seminal plasma proteins bound to spermatozoa membranes, such as binder of sperm protein or calmodulin, have been found to be present in different abundance in X- or Y-bearing bull spermatozoa obtained via flow cytometry (De Canio et al., 2014). This suggests that seminal proteome can influence the outcome of flow cytometry. This idea is supported by the work of de Graaf et al. (2008), who suggested that seminal plasma influence on ram spermatozoa membranes might reflect on sex-sorted spermatozoa. Identifying the seminal vesicle proteome and its relation to seminal plasma proteome might allow for greater insight into efficiency of sperm sorting and storage of sex-sorted spermatozoa, as well as mechanisms differentiating between X and Y spermatozoa characteristics.

In this study, we aimed to compare seminal plasma and seminal vesicle fluid proteomes to better under-

stand the relationship between those 2 fluids. We collected ejaculates and postmortem seminal vesicle fluid from 6 mature Holstein Friesian bulls. Our study led to the identification of 105 proteins from seminal vesicle fluid and 85 proteins from seminal plasma. Additionally, we performed comprehensive functional analysis of the highest number (128) of bull seminal plasma proteins to date by using our data combined with those of Kelly et al. (2006) and Rego et al. (2014).

MATERIALS AND METHODS

Sample Collection and Initial Semen Analysis

All samples were collected from 6 mature Holstein Friesian bulls provided by the Breeding and Insemination Station in Karczew. Semen was collected using an artificial vagina during routine work of the Station. Seminal vesicle fluid was collected from 6 bulls, postmortem, by the Department of Reproduction and Clinic of Farm Animals (Wroclaw, Poland). Initial semen quality assessment was performed on site by the Breeding and Insemination Station according to standard commercial procedures.

Semen Analysis Using Flow Cytometry

In addition to initial analysis, semen quality was assessed with the use of flow cytometry in the Department of Reproduction and Clinic of Farm Animals. Sperm membrane integrity was determined by the double-fluorescent labeling technique, using a Live/Dead Sperm Viability Kit (Life Technologies Ltd., Grand Island, NY). Briefly, 300 μ L of the diluted samples was stained with 5 μ L of SYBR-14 (commercial solution diluted 50-fold) and 5 μ L of propidium iodide (PI). The PI negative and SYBR-14 positive population showing green fluorescence was considered live, with the sperm plasma membrane intact. Sperm acrosome status was assessed using lectin peanut agglutinin from *Arachis hypogaea* Alexa Fluor 488 conjugate (Thermo Fisher Scientific, Waltham, MA). Diluted semen samples were mixed with 10 μ L of lectin peanut agglutinin working solution (1 μ g/mL) and incubated for 5 min at room temperature in the dark. After incubation, the samples were washed and 5 μ L of PI was added before cytometric analysis. Flow cytometric analyses were performed on a FACSCalibur (Becton Dickinson, San Jose, CA) cytometer. The fluorescent probes used in the experiment were excited by an Argon ion 488 nm laser. Acquisitions were performed using the CellQuest 3.3 software (Becton Dickinson). The non-sperm events were gated out based on scatter properties and were

not analyzed. In total, 40,000 events were analyzed for each sample.

Sample Preparation for 2-D SDS-PAGE

Seminal plasma was processed immediately after acquisition of the ejaculate. An aliquot of 0.5 mL of fresh semen was centrifuged at $900 \times g$ for 30 min at 4°C to separate seminal plasma from spermatozoa. Seminal plasma was then transported at 4°C and frozen at -80°C until further use. Vesicular glands were prepared immediately postmortem and their contents were manually expelled directly into the Eppendorf tubes and frozen at -80°C until further use. After thawing, both seminal plasma and seminal vesicle fluid samples were centrifuged at $10,000 \times g$ at 4°C for 60 min to remove any remaining debris from the liquid. After centrifugation, aliquots containing approximately 700 µg of protein were cleaned with a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. The protein concentration before and after the cleaning procedure was measured by the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). No protease inhibitors were used during sample preparation, according to protocols previously used during bull seminal plasma (Kelly et al., 2006; Rego et al., 2014) and accessory gland fluid (Moura et al., 2007) preparation.

2-D SDS-PAGE

Samples containing 500 µg of protein were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 2% immobilized pH gradient buffer, 40 mM dithiothreitol, 0.002% bromophenol blue) to reach a final volume of 450 µL. Samples were then loaded on 24 cm Immobiline DryStrips, 3–10 NL pH range (GE Healthcare), and rehydrated for 10 h. Proteins were then separated by isoelectric focusing on an Ettan IPGphor apparatus (GE Healthcare) operating at 20°C with current limited to 50 µA per strip and the following voltage program: 500 V/5 h, 1,000 V/1 h, 8,000 V/3 h, and 8,000 V/5.5 h. After isoelectric focusing, the strips were equilibrated in SDS equilibration buffer (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, and a trace of bromophenol blue) containing 10 mg/mL of dithiothreitol for 15 min and then in SDS equilibration buffer containing 25 mg/mL of iodoacetamide for 15 min. The equilibrated strips were then transferred to manually cast 12.5% gels (25.5 × 19.6 cm, 1 mm thickness) and sealed with 0.5% agarose. A second dimension of electrophoresis was then

performed at 1 W/gel in an Ettan Dalt-Six apparatus (GE Healthcare) for 16 h.

Image Acquisition and Analysis

After electrophoresis, the gels were stained using Coomassie Brilliant Blue and scanned with an ImageScanner III (GE Healthcare). Acquired images were analyzed using Image Master 2D Platinum 7.0 (GE Healthcare). The software was used to automatically detect the protein spots, and spots consistently occurring in images from every individual were selected for identification. Selected spots were indicated for selection in a spot pick list created by the software.

Excision and Spot Identification Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Tandem Mass Spectrometry

Spots were automatically picked from gels with an Ettan Spot Picker (GE Healthcare), which picked the spots on the basis of a spot pick list generated by Image Master Platinum. Picked spots were put in a 96-well collection plate, and transferred to Ettan Digger (GE Healthcare). Spots were then digested using the following protocol: (1) 3 consecutive washes with 200 µL of 50% methanol, 20 mM NH_4HCO_3 , 25 min each, (2) 2 consecutive washes with 200 µL of 100% acetonitrile, 5 min each, (3) 100 µL wash with 100% acetonitrile, left to dry for over 15 min, and (4) the addition of 10 µL of 0.2 µg/µL modified sequencing grade trypsin (Promega, Madison, WI) solution in 50 mM NH_4HCO_3 followed by incubation for 12 h at 37°C. Peptide mixtures with gel spots were then sonicated briefly with a VCX-130 Ultrasonic Processor (Sonics & Materials Inc., Newtown, CT), put in a Speedvac Concentrator (Thermo Fisher Scientific), and dried in a vacuum. The dried-out gel spots were discarded. Dried-out samples were then placed in an Ettan Spotter robot (GE Healthcare), where they were dissolved in matrix solution (5 mg of α -cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA) in 1 mL of 50% acetonitrile in 0.1% trifluoroacetic acid) and spotted onto the MALDI target plate (MT 34 Target Plate Ground Steel, Bruker Daltonics) using the method "Dissolve Sample in Matrix First – Then Spot" with the following parameters: matrix volume: 3 µL, mix cycles number: 5, mix volume: 3 µL, spot volume: 0.9 µL, and airgap: 3 µL. After spotting, samples were left to dry. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF MS analysis was carried out using a MALDI-TOF tandem mass spectrometer (Autoflex Speed, Bruker Daltonics). Collected

Table 1. Bull semen parameters

Bull no.	Ejaculate volume, mL	Spermatozoa concentration, 10 ⁹ spermatozoa/mL	Quality of movement	Motility, %	Live spermatozoa, %	Spermatozoa with intact acrosome, %
1	15.0	1.450	2/3	>70	58.7	58.9
2	6.5	1.540	2.5/3	>70	66.7	58.1
3	5.0	1.050	2/3	>70	75.8	77.7
4	4.5	1.043	2/3	>70	84.2	67.9
5	3.5	0.864	2/3	>70	89.8	81.3
6	3.5	0.420	1.5/3	>70	90.5	77.6

MS and MS/MS LIFT spectra of selected ions were externally calibrated using monoisotopic [M+H]⁺ ion peptide calibration standards (Bruker Daltonics) and imported to BioTools (Bruker Daltonics). The data were searched using a Mascot Server (Matrix Science, London, UK) in the National Center for Biotechnology Information database, with the following parameters: fixed modifications—carbamidomethyl (C); variable modifications—oxidation (M); cleavage enzyme—trypsin; max missed cleavages—2, peptide mass tolerance—100 ppm, and fragment mass tolerance—0.6 kDa (Perkins et al., 1999).

Ontology, STRING, and Ingenuity Pathway Analyses of the Identified Proteins

Gene Ontology Annotation (GO) was acquired by matching the GI numbers of identified proteins to the UniProtKB database (www.uniprot.org; Huntley et al., 2015). The categories used were “molecular functions” and “biological process.” Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) neighborhood analysis was performed by searching the STRING database to detect possible functional associations (<http://string-db.org>; Szklarczyk et al., 2015). The STRING analysis was performed on the seminal vesicle fluid proteome, as well as on seminal plasma proteins identified by our work, supplemented with data from Kelly et al. (2006) and Rego et al. (2014). On the produced protein networks, we selected groups of proteins based on following criteria: protein groups had to contain at least one protein connected to at least 4 other proteins with the highest confidence bonds (confidence of 0.900, established by STRING software). Ingenuity Pathway analysis (IPA; Qiagen Silicon Valley, Redwood City, CA) of the identified proteins used the Core Analysis function to interpret the identified proteins in the context of biological functions and pathways. The IPA was performed on proteins of seminal plasma, seminal vesicles, and seminal plasma proteins identified by our work, supplemented with data from Kelly et al. (2006) and Rego et al. (2014). The significance of biological functions and canonical pathways was tested auto-

matically by the software, with use of the Fisher exact test *P*-value. Top significant functions and canonical pathways were selected by the software for each tested proteome.

RESULTS

Bull Semen Parameters

All bulls were fertile, as ensured by the Breeding and Insemination Station. Flow cytometry indicated that average of 77.62 (±12.95%) of spermatozoa cells were alive and had intact membranes, and 70.25% (±10.13%) had intact acrosomes. Semen parameters provided by Breeding and Insemination Station and specific flow cytometry results are presented in Table 1.

Characteristic of Bull Seminal Vesicle Fluid Proteome

Analysis of 2-D SDS-PAGE gels of bull seminal vesicles fluid allowed the detection of an average of 450 ± 33 spots (Figure 1). Out of the spots detected on all 3 gels, 227 were identified, corresponding to 105 different proteins (Supplemental Table S1, <https://doi.org/10.3168/jds.2016-11866>). Ten proteins were over the total spot volume of 1% (Figure 1), and therefore recognized as major seminal vesicle fluid proteins (Figure 2A). Binder of sperm protein 1 (BSP1), represented by 4 spots, accounted for the highest percentage of total spot volume detected in gels, followed by spermadhesins (SPHZ1 and SPHZ13) represented by 15 spots, serum albumin precursor (ALB) represented by 17 spots, seminal ribonuclease (SRN) represented by 3 spots, binder of sperm protein 5 (BSP5) represented by 10 spots, metalloproteinase inhibitor 2 (TIMP2) represented by 5 spots, clusterin (CLU) represented by 5 spots, seminal fluid protein A3 (SFPA3) represented by 3 spots, and plasma serine protease inhibitor precursor (SERPINA5) represented by 10 spots. Together, all of the above protein spots accounted for 63.69% of total spot volume detected in gel.

Figure 1. Two-dimensional SDS-PAGE gel of bull seminal vesicles fluid; arrows correspond to spot numbers presented in Supplemental Table S1 (<https://doi.org/10.3168/jds.2016-11866>). Ovals indicate proteins where the total spot volume detected in gels was greater than 1%. ALB = serum albumin precursor; BSP1 = binder of sperm protein 1; BSP5 = binder of sperm protein 5; CLU = clusterin; SERPINA5 = plasma serine protease inhibitor precursor; SRN = seminal ribonuclease; SFPA3 = seminal fluid protein A3; SPHZ = spermadhesin; TIMP = metalloproteinase inhibitor, chain T. Color version available online.

Functional analysis of the identified seminal vesicle fluid proteins by IPA allowed for the detection of functions and canonical pathways associated with proteins. The top functions indicated by IPA were cellular movement, cell death and survival, cellular growth and proliferation, protein degradation, and protein synthesis (Table 2). The top canonical pathways were liver X receptor/farnesoid X receptor (**LXR/FXR**) activation, glycolysis I, farnesoid X receptor/retinoid X receptor (**FXR/RXR**) activation, NRF2-mediated oxidative stress response, and gluconeogenesis I (Table 3). Full information on functions and canonical pathways is presented in Supplemental Tables S2 and S3 (<https://doi.org/10.3168/jds.2016-11866>).

Identified seminal vesicles fluid proteins were also subjected to GO analysis (Figure 2B and 2C). Out of 105 proteins, 100 were successfully categorized according to molecular function and biological process. Based on molecular function analysis, proteins were divided into 7 groups, with the majority having binding, catalytic, and molecular function regulator activity. Based on biological process analysis, proteins were divided into 12 groups, with the majority being involved in metabolic processes, cellular processes, and biological regulation.

The identified proteins were also subjected to STRING neighborhood analysis (Figure 3). Out of 105 proteins, 91 were successfully recognized in the

Figure 2. Pie charts presenting the major proteins of bull seminal vesicle fluid and seminal plasma (A), as indicated by the analysis of electropherograms presented in Figures 1 and 4, respectively, and ontological analysis of identified proteins from bull seminal vesicle fluid and seminal plasma. Classification of the protein set was performed according to the Gene Ontology terms molecular function (B) and biological process (C). BSP1 = binder of sperm protein 1; SPHZ = spermadhesin; ALB = serum albumin precursor; SRN = seminal ribonuclease; BSP5 = binder of sperm protein 5; TIMP2 = metalloproteinase inhibitor 2; CLU = clusterin; SFP3 = seminal fluid protein A3; SERPINA5 = plasma serine protease inhibitor precursor; PLA2G7 = phospholipase A2 group VII; CFH = complement factor H precursor; SERPINE2 = serpin peptidase inhibitor. Color version available online.

Table 2. Top biological functions performed by proteins identified in bull seminal vesicle and seminal plasma

Function name	P-value range	Number of molecules
Seminal vesicle fluid		
Cellular movement	2.10E-03 to 3.27E-15	40
Cell death and survival	1.96E-03 to 7.22E-12	44
Cellular growth and proliferation	1.34E-03 to 1.83E-10	45
Protein degradation	1.87E-03 to 11.4E-09	18
Protein synthesis	1.87E-03 to 11.4E-09	24
Seminal plasma		
Cell death and survival	2.81E-03 to 1.24E-12	48
Cellular assembly and organization	2.55E-03 to 4.02E-11	27
Cell-to-cell signaling and interaction	2.34E-03 to 1.73E-10	28
Cellular movement	2.74E-03 to 8.04E-10	32
Cellular growth and proliferation	2.58E-03 to 1.84E-09	32

STRING database and represented as a network of proteins connected with confidence-based edges. Three groups of proteins were selected: one consisted of 17 proteins, whereas the other 2 consisted of 6 proteins.

Characteristic of Bull Seminal Plasma Proteome

Analysis of 2-D SDS-PAGE gels of bull seminal plasma proteins allowed for the detection of an average of 332 ± 24 spots (Figure 4). Overall, 199 spots were detected on all 3 gels, corresponding to 85 different proteins (Supplemental Table S4, <https://doi.org/10.3168/jds.2016-11866>). Ten proteins were over the total spot volume of 1% (Figure 4), and therefore recognized as major seminal plasma proteins (Figure 2A). Binder of sperm protein 1, represented by 9 spots, accounted for the highest percentage of total spot volume detected in gels, followed by BSP5 represented by 9 spots, isoforms of SPHZ1 and SPHZ13 represented by 10 spots, CLU represented by 15 spots, SRN represented by 4 spots, phospholipase A2 group VII (PLA2G7) represented by 7 spots, SERPINA5 represented by 5 spots, complement factor H precursor (CFH) represented by 7 spots, and

serpin peptidase inhibitor (SERPINE2) represented by 2 spots. Together, all of those protein spots accounted for 57.82% of the total spot volume detected in gels.

Functional analysis of identified seminal plasma proteins by IPA allowed for the detection of functions and canonical pathways associated with proteins. The top functions indicated by IPA were cell death and survival, cellular assembly and organization, cell-to-cell signaling and interaction, cellular movement, cellular growth, and proliferation (Table 2). The top canonical pathways were glycolysis I, gluconeogenesis I, LXR/RXR activation, and FXR/RXR activation (Table 3). Full information on functions and canonical pathways is presented in Supplemental Tables S5 and S6 (<https://doi.org/10.3168/jds.2016-11866>).

Identified seminal plasma proteins were also subjected to GO analysis (Figure 2B and 2C). Out of 85 proteins, 81 were successfully categorized according to the “molecular function” and “biological process.” Based on “molecular function” analysis, proteins were divided into 7 groups, with the majority having binding, catalytic, and enzyme regulator activity. Based on “biological process” analysis, proteins were divided into

Table 3. Top canonical pathways associated with proteins identified in bull seminal vesicle fluid and seminal plasma¹

Pathway name	P-value	Protein overlap, %
Seminal vesicle fluid		
LXR/RXR activation	1.40E-09	7.0 (9/128)
Glycolysis I	1.02E-08	14.6 (6/41)
FXR/RXR activation	5.50E-08	5.8 (8/138)
NRF2-mediated oxidative stress response	4.25E-07	4.4 (8/180)
Gluconeogenesis I	8.52E-07	10.9 (5/46)
Seminal plasma		
Glycolysis I	5.48E-09	16.6 (6/41)
Gluconeogenesis I	1.13E-08	13.0 (6/46)
LXR/RXR activation	9.09E-04	3.2 (4/125)
FXR/RXR activation	1.31E-03	3.1 (4/138)

¹LXR/RXR = liver X receptor/retinoid X receptor; FXR/RXR = farnesoid X receptor/retinoid X receptor activation.

Figure 3. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis of identified bull seminal vesicle fluid proteins; the proteins are connected with confidence network edges: the thicker the line, the greater the confidence of connection; protein groups containing at least one protein connected to at least 4 other proteins with highest confidence bonds are circled; black lines mark proteins involved in liver X receptor/farnesoid X receptor (LXR/FXR) and farnesoid X receptor/retinoid X receptor activation (FXR/RXR) activation pathways; dotted black lines mark proteins involved in glycolysis and gluconeogenesis. Full protein names can be found in the UniProtKB database (www.uniprot.org). Color version available online.

12 groups, with the majority being involved in cellular processes, metabolic processes, and biological regulation.

Comparison of Bull Seminal Plasma and Seminal Vesicles Fluid Electrophoretic Proteins Profile

Comparison of the 2 fluids using 2-D SDS-PAGE gel revealed that 40 proteins were common for seminal plasma and seminal vesicle fluid. Full comparison between both fluids is presented in the form of a Venn diagram in Figure 5A; the list of proteins identified in seminal vesicle fluid only, seminal plasma only, and in both fluids is presented in Tables 4, 5, and 6. Many proteins were present in numerous proteoforms that varied

between the fluids (Supplemental Tables S1 and S4; <https://doi.org/10.3168/jds.2016-11866>). Such protein proteoforms were identified with identical sequences determined by mass spectrometry. For example, albumin in seminal plasma was observed in 5 spots, but in seminal vesicle fluid it was in 17 spots.

DISCUSSION

To the best of our knowledge, in this study we have described the bull seminal vesicle fluid proteome for the first time. Previously, Moura et al. (2007) described the bull accessory sex gland fluid proteome, identifying 13 proteins. When compared with our identification of the seminal vesicle fluid proteome, we were able to confirm

Figure 4. Two-dimensional SDS-PAGE gel of bull seminal plasma; arrows correspond to spot numbers presented in Supplemental Table S4 (<https://doi.org/10.3168/jds.2016-11866>). Ovals indicate proteins where the total spot volume detected in gels was greater than 1%. BSP5 = binder of sperm protein 5; CFH = complement factor H precursor; CLU = clusterin; PLA2G7 = phospholipase A2 group VII; SERPINA5 = plasma serine protease inhibitor precursor; SRN = seminal ribonuclease; SPHZ = spermatidhesin; BSP1 = binder of sperm protein 1. Color version available online.

Figure 5. Venn diagrams comparing (A) the number of proteins identified in bull seminal vesicle proteins with proteins identified in bull seminal plasma in our work; overlapping area shows proteins found in both fluids, and (B) the number of bull seminal plasma proteins identified by our work, Rego et al. (2014), and Kelly et al. (2006). Color version available online.

the identity of 12 out of 13 proteins. Therefore, our results suggest that those 12 proteins identified in both works may originate from seminal vesicles. Osteopontin was the only protein found by Moura et al. (2007) in the mixture of accessory sex gland fluids but not in seminal vesicle fluids by our work. When we inspected images of seminal plasma and seminal vesicle fluid, we found very clear spots of osteopontin in seminal plasma, but not in seminal vesicle fluid, as shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2016-11866>). Cancel et al. (1999) used Western blotting to demonstrate the presence of osteopontin in both seminal vesicles and ampulla fluids, which is contrary to our results. As in-

dicated above, our results suggest that seminal vesicles cannot be the major source of osteopontin in seminal plasma. Therefore, it can be suggested that the bulk of osteopontin originates mostly from the ampulla glands. If results of further studies on osteopontin origin confirm our suggestion, osteopontin would be suggested as a marker of ampulla gland fluid in bull seminal plasma.

We were able to distinguish 3 groups of interconnected proteins in the bull seminal vesicle fluid proteome (Figure 3). The largest group contained multiple proteins with protective capabilities, molecular chaperones such as heat shock proteins (HSPA8, HSP90AA1, and HSP90B1) and the antioxidant enzyme (superoxide

Table 4. Proteins found only in bull seminal vesicle fluid

Protein name	Short name
6-Phosphogluconate dehydrogenase, decarboxylating	PGD
78 kDa glucose-regulated protein precursor	HSPA5
Actin, aortic smooth muscle	ACTA2
Actin-related protein 3	ACTR3
Aldehyde dehydrogenase, mitochondrial precursor	ALDH1B1
α -1-Antiproteinase precursor	SERPINA1
α -1B-Glycoprotein precursor	A1BG
α -2-HS-Glycoprotein precursor	AHSG
Annexin A2	ANXA2
Annexin IV	ANXA4
Annexin V	ANXA5
β -1.4-Galactosyltransferase 4	B4GALT4
β -2-Microglobulin precursor	B2M
Bifunctional purine biosynthesis protein PURH	ATIC
Biliverdin reductase A	BLVRA
Bovine hemoglobin At Ph 5.7	BH
Calreticulin	CALR
Cathepsin B precursor	CTSB
Cofilin-1	CFL1
Creatine kinase B-type	CKB
Cytosolic nonspecific dipeptidase	CNDP2
Dipeptidyl peptidase 4	DPP4
Endoplasmic precursor	HSP90B1
Eukaryotic initiation factor 4A-II	EIF4A2
Glutathione S-transferase M2	GSTM2
Glutathione S-transferase Mu 1	GSTM1
Glutathione S-transferase mu 3	GSTM3
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Glycogen phosphorylase, liver form	PYGL
Heat shock cognate 71 kDa protein	HSPA8
Hemoglobin subunit β	HBB
Hemopexin precursor	HPX
Ig gamma-2 chain C region	IGHG2
IgG2a heavy chain constant region	IGHG
Inhibitor of carbonic anhydrase precursor	ICA
Keratin, type II cytoskeletal 6A	KRT6
Leucine aminopeptidase protein	LAP3
Nucleobindin-2 precursor	NUCB2
Peroxisomal protein 1	PRDX1
Peroxisomal protein 4 precursor	PRDX4
Phosphatidylethanolamine binding protein from calf brain, chain A	PEB1
Phosphoglycerate mutase 1	PGAM1
Plasma PAF acetylhydrolase	PAFAH
PLC α	PLCA
Protein disulfide-isomerase precursor	PDI
Complement C1q tumor necrosis factor-related protein 5	C1QTNF5
Purine nucleoside phosphorylase	PNP
Pyruvate kinase isozymes M1/M2	PKM
Reticulocalbin-2 precursor	RCN2
Retinal dehydrogenase 1	ALDH1A1
Ribonuclease 4 precursor	RNASE4
Seminal plasma protein A3-like	SFPA3L
Serotransferrin precursor	TF
Serpin A3-1 precursor	SERPINA3-1
Serpin A3-2	SERPINA3-2
Serpin A3-2 precursor	SERPINA3-2
Spermadhesin Z13 precursor	SPHZ13
Stefin-C	STEFINC
Transcobalamin-2 precursor	TCN2
Transketolase	TKT
Transthyretin precursor	TTR
Tubulin α -1B chain	TUBA1B
Tubulin α - β dimer, chain A	TUBA4A
Vitamin D-binding protein precursor	GC
WD repeat-containing protein 1	WDR1

Table 5. Proteins found only in bull seminal plasma

Protein name	Short name
Adenylate kinase isoenzyme 1	AK1
α -Centractin	ACNT
α -Mannosidase 2C1	MAN2C1
Angiogenin 1	ANG
Aspartyl aminopeptidase	DNPEP
Bovine lactoferrin	LTF
cAMP-dependent protein kinase type I- α regulatory subunit	PRKAR1A
Cystatin-M precursor	CST6
Dipeptidyl peptidase 2 precursor	DPP2
Double-headed protease inhibitor, submandibular gland-like	IPSG
Epididymis-specific ERBP-like protein; ESP20.5-like	ERBP1
Fructose-1,6-bisphosphatase 1	FBP1
Functional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	DAK
Galectin-3-binding protein precursor	LGALS3BP
Glucose-6-phosphate isomerase	GPI
Glucuronidase, β	GUSB
Glutathione peroxidase 3 precursor	GPX3
Heat shock 70 kDa protein 1-like	HSPA1L
Hemoglobin subunit α	HBA
Hexokinase-1	HK1
Hexosaminidase B	HEXB
Nephronectin isoform 1	NPNT
Osteopontin, partial	SPP1
Phosphoglycerate mutase 2	PGAM2
PRDX5 protein	PRDX5
Proactivator polypeptide	PSAPL1
Prostaglandin-H2 D-isomerase precursor PTGDS	PTGDS
Proteasome subunit α type-6	PSMA6
Seminal plasma protein A3-like	SFPA3L
Serotransferrin-like	LOC525947
Serpin peptidase inhibitor	SERPINB11
Sulfhydryl oxidase 1 precursor	QSOX1
T-complex protein 1 subunit α	CCT1
T-complex protein 1 subunit β	CCT2
T-complex protein 1 subunit delta	CCT4
T-complex protein 1 subunit eta	CCT7
T-complex protein 1 subunit theta	CCT8
T-complex protein 1 subunit zeta	CCT6A
Tripeptidyl peptidase I	TPP1
Tubulin β -4B chain	TUBB4B
Tubulin polymerization-promoting protein family member 2	TPPP2
Vacuolar H ⁺ -ATPase A subunit	ATP6V1A
Vascular noninflammatory molecule 2	VNN2
WAP 4-disulfide core domain protein 2	WFDC2

dismutase). This group was also characterized by the presence of tubulin isoforms (TUBA1B and TUBA4A). The presence of tubulin within this group indicates that those proteins are likely protected by chaperones (Sternlicht et al., 1993), which suggests that the protective action of chaperones is already exerted in seminal vesicle fluid. Some of the remaining proteins in this group, such as calreticulin, cofilin, or protein disulfide-isomerase precursor, are also connected to various aspects of mammalian spermatozoa functioning (Nakamura et al., 1993; Ellerman et al., 2006; Megnagi et al., 2015), but their relation with chaperones present in the described protein group is hard to establish at present. In our opinion, our results suggest that many proteins of seminal vesicles are protected by chaperones

before ejaculation to guarantee their activity in seminal plasma where they interact with spermatozoa.

The second group distinguished in the seminal vesicle fluid proteome is involved in metabolic processes. It consists of 6 proteins involved in glycolysis, gluconeogenesis, and pentose phosphate pathways. Glycolysis is an important part of semen activity, involved in capacitation inhibition and providing a secondary energy source for spermatozoa (Storey, 2008). The presence of glycolytic and gluconeogenic proteins in seminal vesicle fluid is most probably an indication of the metabolic processes taking place in seminal vesicles and its secretions.

The third group distinguished in the seminal vesicle fluid proteome was centered on albumin (**ALB**), and

contained serotransferrin (**TF**) and clusterin (**CLU**). Those 3 proteins are well-known multifunctional proteins, connected with various aspects of homeostasis of seminal plasma and quality of semen (Kragh-Hansen, 1981; Ellison et al., 1988; Jones and Jomary, 2002; Elzanaty et al., 2007; Salehi et al., 2013). Albumin, TF, and CLU, along with the remaining proteins in this group, α -1-antiproteinase, α -2-HS-glycoprotein, and hemopexin, and 2 proteins connected to the group with lower confidence bonds, C-C motif chemokine 2 precursor and transthyrin precursor, were involved in the liver X receptor/farnesoid X receptor (**LXR/FXR**) and farnesoid X receptor/retinoid X receptor (**FXR/RXR**) activation canonical pathways. The LXR/FXR pathway takes part in lipid metabolism, inflammation, and cholesterol catabolism (Calkin and Tontonoz, 2012), and the FXR/RXR pathway is involved in lipid and glucose homeostasis, bile acid regulation, and inflammatory response (Ding et al., 2015). In summary,

it seems that this protein group is characterized by the presence of multifunctional proteins, connected to the quality of semen, lipid metabolism, and inflammatory response.

Our results have shown that some seminal vesicle proteins identified in our study can differentiate between X and Y chromosome bearing bull spermatozoa, because these proteins already been identified by De Canio et al. (2014) as present in different abundance in X or Y bull spermatozoa. These proteins include binder of sperm protein (**BSP**) and glyceraldehyde-3-phosphate dehydrogenase (**GAPDHS**), previously recorded to be more abundant in X spermatozoa (De Canio et al., 2014). This suggests that proteins produced in seminal vesicles can differentially bind to X and Y spermatozoa, which is in accordance with work of de Graaf et al. (2008), who propositioned that seminal plasma proteins influence ram spermatozoa membranes and the sexing process of X and Y spermatozoa by flow cytometry.

Table 6. Proteins found in both bull seminal vesicle fluid and bull seminal plasma

Protein name	Short name
5'-Nucleotidase precursor	NT5E
Actin, cytoplasmic 1	ACTB
Adenosylhomocysteinase	AHCY
Aldose reductase	AKR1B1
α -Enolase	ENO1
ART5 protein	ART5
Bovine Npc2 (Niemann-Pick C2) protein/epididymal secretory protein E1	NPC2
Cathepsin A	CTSA
Cathepsin D	CTSD
Cathepsin L2 precursor	CTSL2
Cathepsin S	CTSS
C-C motif chemokine 2 precursor	CCL2
Clusterin preproprotein	CLU
Complement C1q tumor necrosis factor-related protein 5 precursor	C1QTNF5
Complement C3 preproprotein	C3
Complement factor B precursor	CFB
Complement factor H precursor	CFH
Deoxyribonuclease gamma precursor	DNASE1L3
Ephrin-A1 precursor	EFNA1
Ezrin	EZR
Glia-derived nexin precursor/serpin peptidase inhibitor	SERPINE2
Glucosidase. α ; neutral AB isoform 1	GANAB
Heat shock protein HSP 90- α	HSP90AA1
Inhibitor, acrosin	ACRI
Metalloproteinase inhibitor 2, chain T	TIMP2
Nerve growth factor, partial	NGF
Nucleobindin-1 precursor	NUCB1
Peptidyl-prolyl <i>cis-trans</i> isomerase B precursor	PPIB
Phosphoglycerate kinase 1	PGK1
Plasma serine protease inhibitor precursor	SERPINA5
Platelet-activating factor acetylhydrolase precursor	PLA2G7
Renin receptor	ATP6AP2
Seminal fluid protein A3-bovine	SFPA3
Seminal plasma protein BSP-30 kDa precursor	BSP5
Seminal plasma protein PDC-109 precursor	BSP1
Seminal ribonuclease precursor	SRN
Serum albumin precursor	ALB
Spermadhesin Z13	SPHZ13
Spermadhesin-1 precursor	SPHZ1
Superoxide dismutase (Cu-Zn)	SOD1

etry. It has to be stressed, however, that BSP possesses membrane-binding properties, but such properties have not been described yet for GAPDHS. Further research on seminal vesicles and seminal plasma proteomes may unravel the mechanism for modification of X and Y spermatozoa proteins, and possibly allow for improving the sorting process and storage of sexed semen.

To better understand the functions of seminal plasma proteins, we decided to combine our data with previously published research (Figure 5B) and analyze it using IPA and STRING analysis. The discrepancies in the number of identified proteins observed in all 3 studies are most probably the result of differences in analytical methods and the capabilities of the equipment used. By adding the proteins identified by Kelly et al. (2006) and Rego et al. (2014), we managed to increase the number of analyzed proteins from 85 to 128, producing the most complete data set to date. The STRING analysis allowed the selection of 5 major groups of interconnected proteins (Figure 6), allowing deeper insight into specific functions of seminal plasma.

The protein group with the largest number of connections contained t-complex proteins connected with 2 heat shock proteins, 2 tubulins, and α -centractin. Similar to the largest protein group in seminal vesicles, the biological role of this group of proteins is clearly related to the protection of proteins, as both t-complex and heat shock proteins are molecular chaperones. Because we found tubulins in seminal vesicle secretions, we can assume that their presence in the seminal plasma is not only related to spermatozoa but is also supplied by the accessory sex gland secretions. As mentioned before, tubulins are recognized as targets of protective actions of chaperone molecules. The presence of tubulin in both seminal vesicle fluid and seminal plasma suggests that the protective action of chaperones starts in seminal vesicle secretions and carries over to the semen after ejaculation. It is interesting to note that the protein group is linked to 2 proteasomes, PSMA6 and PSMB4. Our results strongly suggest that protein protection (presumably proteins of spermatozoa and tissues of reproductive system) is an important biological role of seminal plasma, based on mechanisms related to the activity of t-complex, heat shock proteins, and proteasome activity.

Another 2 of the 5 protein groups we found in seminal plasma mirrored the functionality of protein groups we found in seminal vesicle fluid. The proteins from the first group are involved in glycolysis and gluconeogenesis, and its presence both in seminal vesicles and seminal plasma confirms the importance of those metabolic pathways in bull semen. The second group consisted of multifunctional proteins such as ALB, TF, and CLU, and demonstrates varied protective functions and in-

volvement in cholesterol metabolism. This group seems to be positively correlated with the correct morphology and functionality of spermatozoa, as indicated for apolipoprotein A1, lactotransferrin, metalloproteinase inhibitor 1, and gelsolin (Akerlöf et al., 1991; Baumgart et al., 2002; Finkelstein et al., 2010).

Beta-galactosidase (**GLB1**) was in the center of the next distinguished protein group, composed of proteins of presumably epididymal origin—aldose reductase (**AKR1B1**), β -glucuronidase, β -hexosaminidase (**HEXB**), and hexokinase 1, which is also involved in the glycolytic enzyme group described above. The GLB1 is a hydrolytic enzyme located mainly in the epididymis, which is probably involved in sperm maturation and can be an indicator for the function of the epididymal cauda (Jauhiainen and Vanha-Perttula, 1986; Dacheux et al., 2003). The AKR1B1 is associated with bull epididymosomes and epididymal spermatozoa (Frenette et al., 2003). The presence of those proteins in seminal plasma possibly reflects sperm maturation in the epididymis. Interestingly, all proteins from this group with the exception of HEXB can be linked to fertility-related processes, indicating their possible role in the female reproductive tract during capacitation or gamete interaction (Johnson and Hunter, 1972; Nikola-jczyk and O'Rand, 1992; Snell and White, 1996; Katoh et al., 2014).

Immunological protection is an important part of the mechanism involved in protection of spermatozoa within the male and female reproductive system (Dacheux et al., 2003; Rodríguez-Martínez et al., 2011). Complement system proteins identified in our study constituted the majority of last of the distinguished protein groups, consisting of complement factors B and H, complement C3 preprotein, and annexin A1. According to Harris et al. (2006), complement system activation is involved in the “selection of the fittest” spermatozoa in the epididymis (Belleannée et al., 2011), and clearance of dead and dying sperm cells. Both processes require spermatozoa to have complement-regulating proteins on their membranes, which were discovered on the surface of bull spermatozoa by Byrne et al. (2012). As seminal plasma affects female reproductive tract immunological responses (Rodríguez-Martínez et al., 2011), it is possible that seminal plasma complement factor proteins are partially involved in this interaction.

To perform an additional analysis of biological functions of bull seminal plasma proteins, we used IPA software (Supplemental Table S7; <https://doi.org/10.3168/jds.2016-11866>). The results of IPA analysis mostly supported analysis performed with STRING, indicating multiple proteins with various protective functions directed toward other proteins as well as spermatozoa. Most importantly, analysis confirmed the importance of

Contrary to our expectations, we could not identify all seminal vesicle fluid proteins in seminal plasma (Figure 5A). Additionally, seminal vesicle fluid showed abundance of some proteins, such as ALB and AKR1B1, to have dropped significantly, despite the fact that the

abundance of the main proteins of both fluids (BSP1, BSP5, and spermadhesin) did not change significantly. The exact reason for those discrepancies is unknown at present. For some proteins, the lack of the detection in seminal plasma can simply be the result of the low detection limit caused by the dilution of seminal vesicle fluid. We believe that another reason is the adsorption of proteins on the spermatozoa. This suggestion is supported by the results of Byrne et al. (2012), which show the presence of several seminal vesicle fluid proteins unidentified in seminal plasma in our study on the surface of bull spermatozoa and also demonstrated the adsorption of albumin on spermatozoa, which coincides with our data indicating the loss of albumin in seminal plasma.

In summary, the bull seminal vesicle fluid proteome was described for the first time. Analysis of the seminal vesicle proteome suggests that some proteins were adsorbed on the surface of the spermatozoa. Our study has significantly expanded previously available data on bull seminal plasma by both increasing the number of proteins known, as well as providing a more detailed proteomic map. Both in seminal vesicle fluid and seminal plasma, we have indicated multiple protein networks that were involved in protective functions, glycolysis and gluconeogenesis, and lipid metabolism. Our results strongly suggest that protein protection is an important biological role of seminal plasma, based on mechanisms related to the activity of molecular chaperones. Further studies of both bull seminal plasma and seminal vesicle fluid should focus on detailed characteristics of the identified protein groups, to better understand the molecular background of sperm functioning, and to unravel the complex interactions between proteins.

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