The Proteome of Pig Spermatozoa Is Remodeled During Ejaculation

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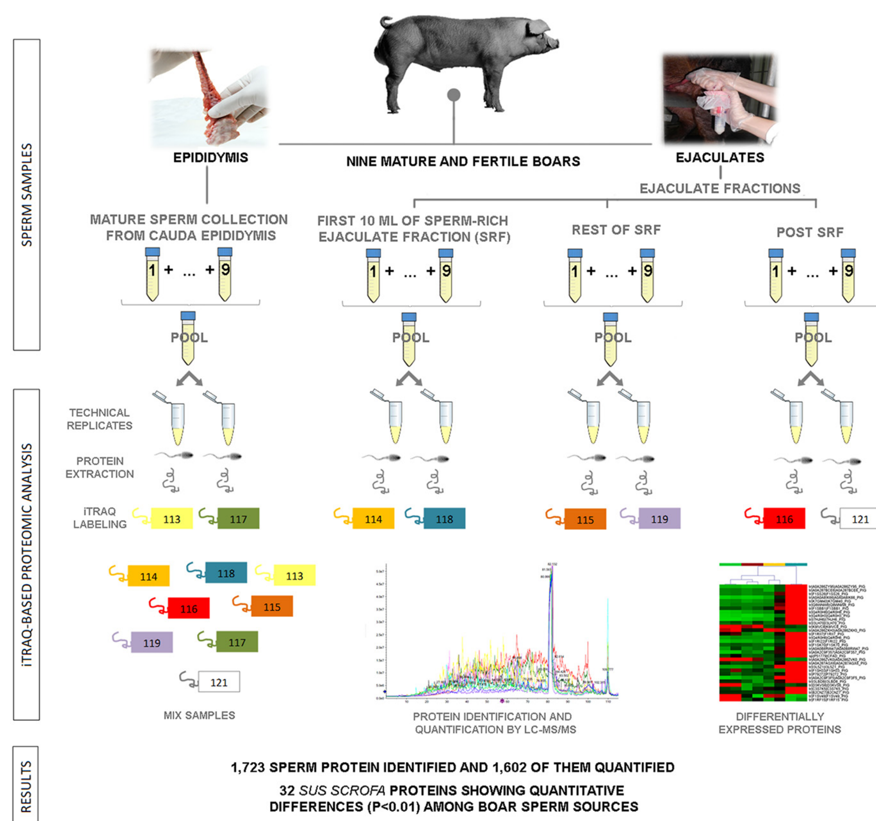
In Brief

The present study identified and quantified using iTRAQ-based LC-MS/MS, 1,723 and 1,602 proteins, respectively, in mature boar spermatozoa retrieved from cauda epididymis and three distinct fractions of the ejaculate. A total of 974 of the identified and 960 of the quantified proteins belonged to the *Sus scrofa* taxonomy. An ANOVA test revealed that 32 *Sus scrofa* proteins implicated in sperm function showed quantitative differences among sperm sources. The findings demonstrated that the proteome of boar spermatozoa is remodeled during ejaculation.

Graphical Abstract



Highlights

- Proteome of mature boar spermatozoa from cauda epididymal and ejaculated sources were analyzed by iTRAQ-based LC-MS/MS.
- 1,723 sperm proteins identified (974 of *Sus scrofa* taxonomy).
- 1,602 sperm proteins quantified (960 of *Sus scrofa* taxonomy).
- 32 *Sus scrofa* sperm proteins were differentially expressed among sperm sources.
- The proteome of boar spermatozoa is remodelled during ejaculation.

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Research

The Proteome of Pig Spermatozoa Is Remodeled During Ejaculation*

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Proteins are essential for sperm function, including their fertilizing capacity. Pig spermatozoa, emitted in well-defined ejaculate fractions, vary in their functionality, which could be related to different sperm protein composition. This study aimed (i) to update the porcine sperm proteome and (ii) to identify proteins differentially expressed in mature spermatozoa from cauda epididymis and those delivered in separate ejaculate fractions. Ejaculates from nine mature and fertile boars were manually collected in three separate portions: the first 10 ml of the sperm-rich ejaculate fraction (SRF), the rest of the SRF and the post-SRF. The contents of cauda epididymides of the boars were collected post-mortem by retrograde duct perfusion, generating four different semen sources for each boar. Following centrifugation, the resulting pellets of each semen source were initially pooled and later split to generate two technical replicates per source. The resulting eight sperm samples (two per semen source) were subjected to iTRAQ-based 2D-LC-MS/MS for protein identification and quantification. A total of 1,723 proteins were identified (974 of *Sus scrofa* taxonomy) and 1,602 of them were also quantified (960 of *Sus scrofa* taxonomy). After an ANOVA test, 32 *Sus scrofa* proteins showed quantitative differences ($p < 0.01$) among semen sources, which was particularly relevant for sperm functionality in the post-SRF. The present study showed that the proteome of boar spermatozoa is remodeled during ejaculation involving proteins clearly implicated in sperm function. The findings provide valuable groundwork for further studies focused on identifying protein biomarkers of sperm fertility. ***Molecular & Cellular Proteomics 18: 41–50, 2019. DOI: 10.1074/mcp.RA118.000840.***

Spermatozoa are highly differentiated, structurally complex and dynamic cells that deliver the paternal genome/epigenome to mature oocytes. To achieve this goal, the spermatozoon must attain full fertilization capacity, which implies they underwent molecular and/or functional maturational changes in the epididymis to later display an active forward movement, undergo capacitation, zona binding and the acrosome reaction, and chromatin decondensation during fertilization (1). Most of these essential functional processes involve modifications in the expression of proteins through sperm interaction with the surrounding environment, as evidenced in spermatozoa from rodents, humans and livestock species, including pigs (2–7).

Because the ejaculate contains a heterogeneous suspension of spermatozoa, sperm sub-populations of different quality and functionality are present (8). D'Amours *et al.* (9) suggested that qualitative and/or quantitative differences in protein expression among these sperm subpopulations probably resulted in an unbalanced interaction between spermatozoa and the changing surrounding environment during ejaculation. This noticeable hypothesis, derived from empirical studies of ejaculated bull sperm sub-populations artificially generated through cell separation methods, needs testing. The porcine is an excellent model for such testing because, as in human, the ejaculate is emitted in fractions, where the cauda epididymal spermatozoa are sequentially exposed to the differential secretion of the sexual accessory glands, building separate ejaculate fractions (10). These different fractions/environments apparently impose remarkable differences in the capability of spermatozoa to withstand technologies such as cryopreservation (11–13). A recent study (14) suggested differences in the proteome of spermatozoa in the different boar ejaculate fractions. The present study aimed to update the porcine sperm proteome (15), taking advantage of the increased annotation of protein-coding genes (16). In addition, it aimed to identify and measure, using isobaric tags for relative and absolute quantification (iTRAQ)[1], eventual putative differences in protein composition between mature spermatozoa retrieved from the cauda epididymis and from the most representative ejaculate fractions, specifically the sperm peak (first 10 ml of the sperm-rich ejaculate fraction, SRF), the remaining SRF and the post-SRF. This approach allowed the measurement of the exchange in proteins experienced by the spermatozoa during ejaculation and provided evidence for the relevance of the different sexual accessory glands in such exchange.

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EXPERIMENTAL PROCEDURES

Boars and Collection of Ejaculate and Cauda Epididymal Contents—All procedures involving animals followed international guidelines (Directive 2010/63/EU), including the approval of the Bioethics Committee of Murcia University (research code: 639/2012) and of the Local Ethical Committee for Experimentation with Animals at Linköping, Sweden (permit nr ID-1400).

Semen and cauda epididymal contents were retrieved from nine mature and fertile boars (*Sus scrofa*) of different breeds and crossbreds. The boars were housed in an artificial insemination (AI) center (Topigs Norsvin España) located in Murcia (Spain) and regularly used in conventional AI programs (two ejaculates collected per week). The boars were kept under the same management conditions, housed in individual pens under controlled regimens of temperature (15–25 °C) and light (16 h per day), with free access to water, and fed with commercial feedstuff for mature boars.

One ejaculate per boar was manually collected in separate portions, specifically the first 10 ml of the SRF (P1), the remaining SRF (P2), and the post-SRF (P3) using the gloved-hand method. A proportional mixture of each ejaculate fraction was used to verify that all collected ejaculates fulfilled the standard thresholds of sperm quantity and quality needed for the preparation of AI semen doses, specifically more than 200×10^6 sperm/ml (SP-100 NucleoCounter; ChemoMetec A/S, Allerød, Denmark) with more than 70 and 75% of them motile (objectively evaluated using ISASV1® CASA; Proiser R+D, Valencia, Spain) and viable (cytometrically evaluated after staining using Hoechst 33342 and propidium iodide; BD FACSCanto II cytometer; Becton Dickinson Co, Franklin Lakes, NJ), respectively. The boars, still healthy and fit to deliver semen, were eventually removed from the AI center because of genetic replacement reasons and slaughtered (Slaughterhouse Agroalimentaria de Teruel, Teruel, Spain). Immediately post-mortem, the scrotal contents were collected and transported in insulated containers (5 °C) to the Andrology Laboratory of Veterinary Teaching Hospital of University of Murcia (VTH) within 4 h.

Sperm Sample Preparation—Immediately after ejaculation, the semen samples of each ejaculate fraction were centrifuged twice ($1{,}500 \times g$ at rt for 10 min, Rotofix 32 A, Hettich Zentrifuge, Tuttlingen, Germany) to separate the seminal plasma (SP) from a sperm pellet. The pellets were washed twice with PBS ($1{,}500 \times g$ at rt, 10 min) with a final suspension in PBS (1/3, v/v). These PBS-suspended sperm pellets were transported in thermal containers to the VTH, arriving within 2 h of ejaculate collection. Once in the VTH-laboratory, the PBS-suspended pellets were again centrifuged ($2{,}400 \times g$, rt, 3 min; Megafuge 1.0 R, Heraeus, Hanau, Germany) and cytometrically checked for sperm content after staining with Hoechst 33342. As expected for the species (17), the pellets contained >97% of spermatozoa. Finally, the sperm suspensions were diluted with PBS to a final concentration of $1{,}000 \times 10^6$ spermatozoa/ml, aliquoted in 1 ml volumes and stored at −80 °C (Ultra Low Freezer; Haier, Schomberg, Ontario, Canada) until use.

The contents of the epididymal caudae were collected following the procedure described by Alkmin *et al.* (12) with slight modifications. The cauda epididymides were carefully dissected, a needle was placed in the ductus deferens, and air was retrogradely infused. The luminal fluid was collected at a section of the ductus made at the corpus-cauda limit. The harvested fluid of the two caudae of each boar was pooled and microscopically evaluated to confirm that more than 75% of viable, mature spermatozoa were present. The cauda epididymal sperm pellets were processed and stored following the same protocol for ejaculated spermatozoa described above. A total of four sperm samples from different semen sources were generated by each of the 9 boars.

PROTEOME ANALYSIS

Protein Extraction—The proteomic analyses were carried out in the Proteomics Unit of the University of Valencia, Valencia, Spain (member of the PRB2-ISCIII ProteoRed Proteomics Platform). The sperm pellets were thawed at rt and centrifuged at $14{,}000 \times g$ at 10 °C for 10 min (Eppendorf 5424R, Eppendorf AG, Hamburg, Germany) to obtain protein-enriched fractions. Two extraction cycles, which involved dilution in 200 μl of U/T/C lysis buffer (7 M Urea, 2 M thiourea and 4% CHAPS) and constant rotation at 5 °C during 1 h, were performed. Thereafter, the results of the two extraction cycles were combined and treated with 10% (final concentration) of TCA (Fisher Scientific, Madrid, Spain) and stored overnight at 5 °C to achieve complete sperm lysis. Thereafter, samples were diluted in 200 μl of MilliQ water (Merck Millipore, Darmstadt, Germany) and centrifuged at $14{,}000 \times g$ for 2 h.

A TCA/Acetone protocol was used for protein precipitation; the TCA-treated samples were diluted (1:4, v/v) in a TCA-cold acetone solution, stirred and stored on ice for 15 min. The samples were then centrifuged ($14{,}000 \times g$, at 4 °C for 20 min; Eppendorf 5424R) and the resulting pellets were washed twice with 1 ml ice-cold acetone and centrifuged ($14{,}000 \times g$, at 4 °C for 20 min; Eppendorf 5424R). The pellets were incubated overnight at rt to volatilize the residual acetone. The dried pellets were lysed by dilution in 200 μl of protein extraction reagent (8 M urea and 0.5 M triethylammonium bicarbonate buffer [TEAB]), sonicated and constantly rotated at rt for 1 h. Protein concentration was measured using the Lowry modified RC DC™ Protein Assay Kit (Bio-Rad, Richmond, CA). A total of 100 μg of final protein extract per sample was used for iTRAQ analysis.

iTRAQ Labeling—The iTRAQ labeling was performed using the AB SCIEX kit (Framingham, MA). Cysteine residues were blocked by incubation in 4 μl of 50 mM 2-carboxyethyl phosphine (TCEP) at 37 °C for 180 min to avoid undesirable secondary urea reactions. Sulfhydryl groups were alkylated with 1 μl of 200 mM S-methyl methanethiosulfonate (MMTS) at rt for 10 min. Urea was diluted to 2 M with 0.5 M of TEAB buffer to a final volume of 100 μl.

The protein samples were digested with 10 μg of sequencing-grade modified trypsin (Promega Corporation, Madison, WI) diluted in 0.5 M of TEAB buffer, and incubated at 37 °C overnight. The digested protein samples were dried in a centrifuge vacuum concentrator (ISS 110 SpeedVac System, Thermo Savant, ThermoScientific, Langenselbold, Germany), dissolved in 80 μl of TEAB buffer in ethanol solution (3:7, v/v) and sonicated for 10 min. Then, the resulting peptide mixtures were labeled with the appropriate iTRAQ reagents following the protocol of the 8-plex iTRAQ labeling kit (AB SCIEX). The iTRAQ-labeled peptides were then incubated at rt for 3 h, mixed, aliquoted in 250 μg portions and dried by vacuum centrifugation.

Peptide Fractionation by Isoelectric Focusing—The dried iTRAQ-labeled peptides were subjected to fractionation by IEF separation following the protocol of Krijgsveld *et al.* (18) with minor modifications. Briefly, 250 μg of the peptide mixture was brought up to 8 M urea in the presence of IPG buffer, 3–11 NL (GE Healthcare Life Sciences,

[1] The abbreviations used are: iTRAQ, isobaric tags for relative and absolute quantification; SRF, sperm-rich ejaculate fraction; AI, artificial insemination; VTH, veterinary teaching hospital; SP, seminal plasma; TEAB, triethylammonium bicarbonate buffer; TCEP, 2-carboxyethyl phosphine; MMTS, S-methyl methanethiosulphonate; FA, formic acid; FDR, false discovery rate; PCA, principal component analysis; MeV, multi-experiment viewer; FC, fold change; GO, gene ontology; DDI1, DNA damage inducible 1 homolog 1; CES1, Carboxyl ester hydrolase; pB1, porcine B1; BSP, bovine seminal plasma; FN1, Fibronectin 1; TSP1, Thrombospondin-1; ROS, reactive oxygen species; WGA, wheat germ agglutinin; COL18A1, type XVIII Collagen α1 chain.

Little Chalfont, United Kingdom) and applied to 13 cm IPG dry strips, 3–11 NL (GE Healthcare), which were isoelectrofocused with 5,000 V up to 25,000 Vh. Thereafter, strips were washed with MilliQ-grade water and cut into 11 equal pieces. The isoelectrofocused peptides were extracted from the strips with 120 μl of the following sequential five extracted solutions (Fisher Scientific): (1) 5% aqueous ACN 0.1% TFA, (2) 20% ACN 0.1% TFA, (3) 50% ACN 0.1% TFA, (4) 70% ACN 0.1% TFA and (5) 99.9% ACN 0.1% TFA. All peptide fractions were combined, dried by vacuum centrifugation and redissolved with 40 μl of 2% ACN 0.1% TFA. The samples were cleaned and concentrated by C18 silica homemade "in tip" columns, dried by speed vacuum and resuspended to a concentration of ca. 0.30 μg/μl in 2% ACN 0.1% TFA (the peptide concentration was determined assuming 100% performance).

Liquid Chromatography and Tandem Mass Spectrometry Analysis (LC-MS/MS)—The labeled peptides were analyzed by LC using a NanoLC Ultra 1-D plus Eksigent (Eksigent Technologies, Dublin, CA), which was directly connected to a TripleTOF 5600 mass spectrometer (AB SCIEX). Briefly, 5 μl from each sample was loaded onto a trap column (NanoLC column, Chrom XP C18–3 μm, 350 μm × 0.5 mm; Eksigent Technologies) and desalted with 0.1% TFA at 3 μl/min for 5 min. Then, the peptides were eluted from the trap column and separated using an analytical LC-column (3 μm particle size C18-CL, 75 μm x 12 cm, Nikkyo Technos Co®, Tokyo, Japan) equilibrated in 5% ACN and 0.1% formic acid (FA) (Fisher Scientific). Peptide elution was performed by applying a linear gradient of solvents A (0.1% FA in water) and B (0.1% FA in ACN) from 5% to 35% of solvent B in A at a constant flow rate of 300 nL/min over 90 min. The eluted peptides were thereafter ionized using an ESI Nanospray III ion source (AB SCIEX) for analysis with a TripleTOF 5600 mass spectrometer coupled to the NanoLC system. The samples were ionized by applying 2.8 kV to the spray emitter and the TripleTOF was operated in an information-dependent acquisition mode, in which a TOF MS scan was made from 350 to 1250 *m/z*, accumulating for 250 ms TOF followed by 75 product ion scans from 100–1500 m/z; the 25 most abundant multiply charged (2+, 3+, 4+ or 5+) precursor peptide ions were automatically selected. Ions with 1+ and unassigned charge states were rejected from the MS/MS analysis. The collision energy was automatically set by the instrument rolling collision energies for iTRAQ labeled peptides.

Data Processing: Protein Identification, Validation, and Quantification—The generated SCIEX.wiff data-files were processed using the ProteinPilot v5.0 search engine (AB SCIEX) for protein identification and quantification with a peptide confidence threshold of 95% and a false discovery rate (FDR) less than 1% at the protein level. The Paragon algorithm (4.0.0.0, 4767) of ProteinPilot was used to search against the Uniprot_mammalia database (version 20180307 with 3,810,720 proteins searched) with the following parameters: iTRAQ quantitation, trypsin specificity, cys-alkylation (MMTS), no taxonomy restrictions, and the search effort set to throughout. The identified proteins were grouped by the Pro Group™ algorithm (ProteinPilot™ Software) following the Pro Group Report recommendation (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_040586.pdf). Protein groups were exclusively made from observed peptides, and the grouping was guided by the spectral usage. Consequently, unobserved regions of protein sequence were not considered to explain the data.

Gene Ontology Analysis and Bioinformatics—Bioinformatics of all identified and differentially expressed sperm-proteins was manually performed using the comprehensive bioinformatics tool for functional annotation UniProt KB database (www.uniprot.org) downloaded 28/03/2018, containing 111,425,245 total entries with 40,710 of them encoded in the *Sus scrofa* taxonomy. This analysis allowed the elucidation of the different functions and processes in which the differentially expressed sperm proteins were putatively involved. Three independent sets of ontology were used in the annotation: "molecular function," "biological processes" in which the proteins participate, and their "cellular components." A total of 130 proteins were not considered for collation, as they showed no similarity with database entries.

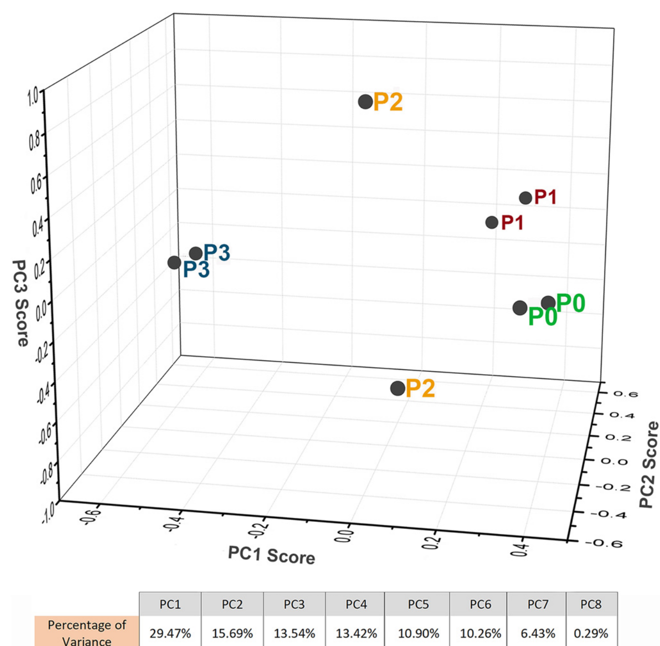
Experimental Design and Statistical Rationale—Spermatozoa of nine mature and fertile boars were used, obtained from four different sources per boar; (P0) cauda epididymis, (P1) the first 10 ml of the SRF, (P2) the remaining SRF and (P3) the post-SRF. Before proteomic analysis, the sperm pellets derived from the same source per boar (either cauda epididymis or fractions of the ejaculate) were mixed together to diminish individual effects. Consequently, four single sperm pools were built (P0-P3) to study the proteome of pre- and post-ejaculation mature boar spermatozoa. To validate analytical reliability, each sperm pool was in turn split into two aliquots to generate two technical replicates. Samples labeled with the appropriate iTRAQ reagents corresponded to the first and second technical replicate, respectively: spermatozoa from P0: 113 and 117; spermatozoa from P1: 114 and 118; spermatozoa from P2: 115 and 119 and spermatozoa from P3: 116 and 121.

The relative quantification of sperm proteins was achieved by comparison of the relative intensities of reporter ions of different sperm samples (P0, P1, P2, and P3), dividing the iTRAQ reporter groups (114, 115, 116, 117, 118, 119, and 121) by the peak intensity of 113. Principal component analysis (PCA) was performed to evaluate the discriminative ability of sperm proteins in the four sperm samples using the Origin Software (OriginLab, Northampton, MA). Thereafter, the Multiexperiment Viewer (MeV) software (version 4.8) (http://www.tm4.org/mev.html) was used for statistical normalization following software instructions. An ANOVA test was used to identify the differentially expressed sperm proteins among the four sperm samples. Proteins were considered differentially expressed with an adjusted p value < 0.01, and those with a fold change (FC) ≥ 1.5 after $\log_2$ transformation were highlighted. The results of the hierarchical clustering analysis of the proteome profile of the different sperm samples were shown with a heat map after z-score normalization, using Euclidean distances. Quantitative analysis was done only on proteins identified in all sperm samples. Differences in the gene ontology (GO) distribution between total and differentially expressed proteins were analyzed using a Chi-square analysis.

RESULTS

Sperm Proteome Profile—A total of 1,723 proteins were identified with a cutoff of unused prot score > 1.3 (corresponding to a confidence limit of 95% and an FDR < 1%), 974 of them belonging to the *Sus scrofa* taxonomy. Among the identified proteins, 1,602 were successfully quantified, 960 of them belonging to the *Sus scrofa* taxonomy. All the identified proteins were present in the spermatozoa of the four sperm sources. The complete list of identified and quantified proteins and the ratio of the peak area of the iTRAQ reporter ion displaying the relative abundance of each protein is shown in supplemental Data S1.

Differentially Expressed Proteins—The PCA analysis of all identified proteins showed proportions of variance of 24.9%, 15.1% and 14.5% for PC1, PC2 and PC3, respectively (supplemental Fig. S1). The PCA of identified proteins encoded in the *Sus scrofa* taxonomy showed similar distributions, explaining 29.5%, 15.7% and 13.5% of the variance in PC1, PC2 and PC3, respectively (Fig. 1). PC1 had the highest



	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Percentage of Variance	29.47%	15.69%	13.54%	13.42%	10.90%	10.26%	6.43%	0.29%

FIG. 1. **Principal component analysis (PCA) showing the separation among the four sperm source for the sperm proteins identified in the *Sus scrofa* taxonomy: (P0) mature spermatozoa from the cauda epididymis, (P1) spermatozoa from the first 10 ml of the sperm-rich ejaculate fraction (SRF), (P2) the remainder of the SRF and (P3) the post-SRF.** The points represent two technical replicates for each sperm source and are based on the relative amounts quantified in each.

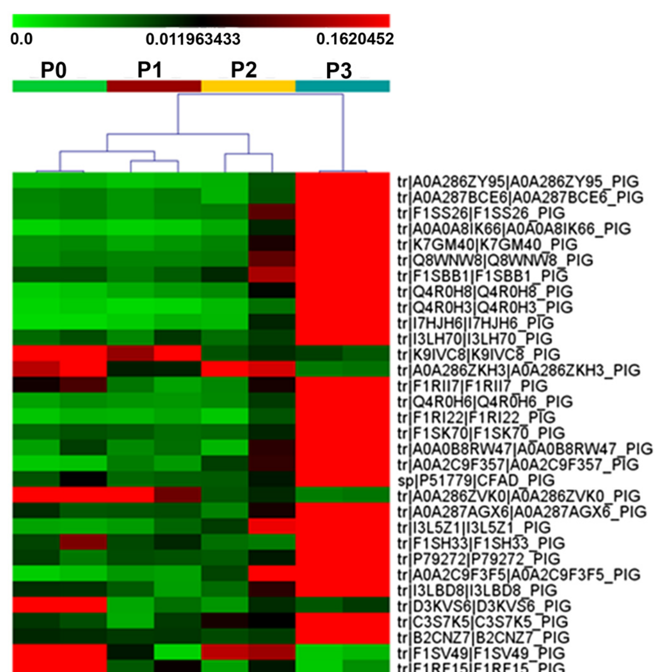


FIG. 2. **Heat map with dendrograms representing the differentially expressed proteins belonging to the *Sus scrofa* among the four sperm samples: (P0) mature spermatozoa from the cauda epididymis, (P1) spermatozoa from the first 10 ml of the sperm-rich ejaculate fraction (SRF), (P2) the remainder of the SRF and (P3) the post-SRF.** The data were obtained from two technical replicates of each sperm source. The hierarchical clustering tree of sperm sources is shown at the top. The relative expression level of each differentially expressed protein is shown on a color scale from red (highest level) to green (lowest level).

discrimination among sperm sources. Specifically, the sperm samples were grouped into three distinct branches; the first one included the P0 and P1 sources, whereas the second and third included the P2 and P3, respectively. The ANOVA test revealed a total of 43 proteins differentially expressed ($p < 0.01$) among the sperm sources, 32 of them belonging to the *Sus scrofa* taxonomy. The quantitative value of the 43 differentially expressed proteins, following data normalization per source and the FC estimation among sources after $\log_2$ transformation, is shown in supplemental Data S2. The expression pattern of the differentially expressed sperm proteins of the two technical replicates of each sperm source is graphically presented as a heat map in Fig. 2 (proteins encoded in *Sus scrofa* taxonomy) and supplemental Fig. S2 (all differentially expressed proteins). The dendrograms of the two heat-maps showed that the technical replicates merged into a close cluster, highlighting the robustness of the analysis carried out. The dendrograms also showed that the distance between the cluster grouping P0-P2 source and P3 source samples was large, showing that the greatest differences in protein expression were between these two clusters. Twenty-eight of the 32 differentially expressed *Sus scrofa* proteins showed an FC $\geq$ 1.5 among sources (Table I). Only three of these proteins were differentially expressed between spermatozoa of the epididymis (P0) and the P1-P2 ejaculate fractions (SRF), being these three proteins overexpressed in P0. In contrast, a larger number of proteins ($n = 20$), were differentially expressed between the post-SRF (P3) and the other sperm sources (epididymis -P0-, first 10 ml of SRF -P1- and rest of SRF -P2-). Notably, these 20 differentially expressed proteins were overexpressed in the spermatozoa of the post-SRF.

Bioinformatics—The identified sperm proteins including those encoded in the *Sus scrofa* taxonomy followed a similar pattern of distribution among the functional GO categories. The GO distribution of all identified proteins and of those differentially expressed is shown in supplemental Fig. S3. The results presented here focus on the proteins encoded in the *Sus scrofa* taxonomy whose GO distribution (all and those differentially expressed) is shown in Fig. 3*A*–3*C*. Regarding all the identified *Sus scrofa* proteins (outer circles in Fig. 3), a total of 808 hits were linked to molecular function (Fig. 3*A*), and most of them were involved in catalytic (420 proteins, 52%) and binding (354 proteins, 44%) activities. A total of 751 hits were related to biological processes (Fig. 3*B*), and most of them were involved in cellular (381, 34%) and metabolic (293, 27%) processes. Remarkably, only 58 proteins (5%) found were linked to reproductive processes, with 39 of them belonging to sperm function (Table II). Finally, a total of 947 hits were associated with cellular components (Fig. 3*C*) mainly

TABLE I
Sus scrofa proteins differentially expressed with a fold change >1.5 among boar sperm samples from the cauda epididymis (P0), the first 10 ml of the sperm-rich ejaculate fraction (SRF) (P1), the remainder of the SRF (P2) and the post-SRF (P3)

PROTEIN NAME	ACCESSION	SEMINAL PLASMA (27)	FOLD CHANGE					
			P0 vs P1	P0 vs P2	P0 vs P3	P1 vs P2	P1 vs P3	P2 vs P3
Fibronectin 1	A0A286ZY95	Yes	0.20	−0.74	**−3.43**	−0.94	**−3.63**	**−2.69**
Fc fragment IgG binding protein	A0A287BCE6	Yes	0.11	0.00	**−2.47**	−0.10	**−2.58**	**−2.48**
Thrombospondin 1	F1SS26	Yes	0.09	−0.75	**−2.31**	−0.85	**−2.40**	**−1.55**
Heparin-binding protein WGA16	A0A0A8IK66	Yes	0.04	−1.28	**−4.00**	−1.32	**−4.04**	**−2.42**
Apolipoprotein A-I	K7GM40	No	0.25	−0.76	**−2.53**	−1.01	**−2.78**	**−1.77**
Nexin-1	Q8WNW8	Yes	−0.03	−0.80	**−2.34**	−0.77	**−2.32**	**−1.54**
Ca-binding phosphorylation	F1SBB1	No	0.23	−0.62	**−1.48**	−0.85	**−1.71**	−0.86
Spermadhesin AWN	Q4R0H8	Yes	−0.76	−1.14	**−3.75**	−0.97	**−2.99**	**−2.01**
Spermadhesin AQN1	Q4R0H3	Yes	0.22	−1.17	**−4.21**	−1.39	**−4.43**	**−3.05**
Spermadhesin AQN3	I7HJH6	Yes	−0.79	−1.35	**−4.45**	−1.16	**−3.66**	**−2.50**
Collagen type XVIII alpha 1 chain	I3LH70	Yes	0.02	−0.06	**−1.81**	−0.08	**−1.84**	**−1.76**
Hemoglobin subunit beta	F1RII7	Yes	1.24	0.50	−0.84	−0.74	**−2.08**	−1.35
Spermadhesin PSPI	Q4R0H6	Yes	−0.02	−0.61	**−2.77**	−0.60	**−2.75**	**−2.15**
Spermadhesin PSPII	F1RI22	Yes	−0.30	−0.73	**−3.55**	−0.43	**−3.26**	**−2.83**
Protein S	F1SK70	No	0.05	−0.19	**−1.80**	−0.25	**−1.85**	**−1.60**
Adenosine deaminase	A0A0B8RW47	No	0.14	−0.25	**−2.10**	−0.39	**−2.24**	**−1.86**
Seminal pB1	A0A2C9F357	Yes	−0.99	−1.00	**−3.50**	−1.01	**−2.51**	**−1.50**
Complement factor D	P51779	Yes	0.44	0.10	−1.22	−0.34	**−1.66**	−1.32
Glutathione S-transferase alpha 1	A0A287AGX6	No	0.11	−0.03	**−1.58**	−0.14	**−1.52**	**−1.48**
Cadherin 1	I3L5Z1	Yes	−0.51	−1.29	**−2.71**	−1.08	**−2.21**	**−1.52**
Serpin family I member 1	F1SH33	Yes	0.28	0.82	−1.00	0.53	−1.28	**−1.82**
Vitronectin	P79272	Yes	−0.10	−0.27	**−1.68**	−0.17	**−1.58**	**−1.51**
Acrosin inhibitor	A0A2C9F3F5	Yes	−0.77	−1.24	**−3.47**	−1.27	**−2.70**	**−1.63**
Semaphorin-3C	I3LBD8	Yes	0.69	0.00	**−1.51**	−0.69	**−1.99**	**−1.51**
16kDa secretory protein	D3KVS6	Yes	**2.40**	**1.94**	**1.74**	−0.46	−0.66	−0.20
Protein S100 A8	C3S7K5	No	0.44	−0.45	−1.32	−0.89	**−1.76**	−0.87
DNA damage inducible 1	F1SV49	No	**1.97**	0.79	**3.11**	−1.18	1.13	1.32
Carboxylic ester hydrolase	F1RF15	No	**1.52**	**1.90**	**2.88**	0.38	1.36	0.98

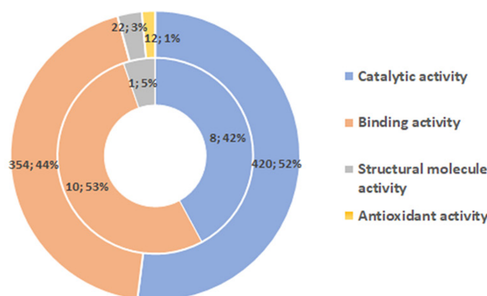
distributed in the cell part (461, 49%), organelle (229, 24%) and membrane (207, 22%) regions.

The functional distribution of the differentially expressed *Sus scrofa* proteins is shown in the inner circles of Fig. 3. The 19 hits associated with molecular function followed a pattern of distribution like that of all identified *Sus scrofa* proteins, with 10 (53%) proteins involving binding and 8 (42%) involving catalytic activities (Fig. 3*A*). However, the distribution pattern was different ($p < 0.001$) for biological process and cellular component (Fig. 3*B*–3*C*). Most of the 23 hits associated with biological processes were involved in metabolic (7, 19%), reproductive (6, 16%) and cellular (6, 16%) processes or biological regulation (6, 16%). Regarding the cellular components (Fig. 3*C*), the 21 hits found were mainly attributed to the extracellular region (8, 38%) and cell parts (7, 34%).

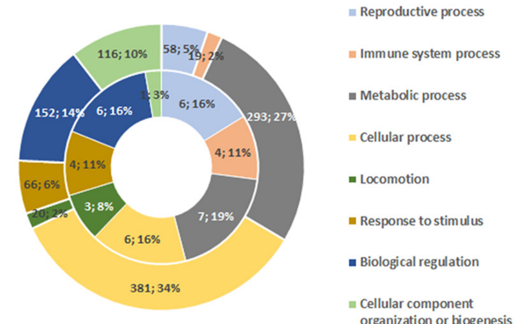
DISCUSSION

Sperm proteomes have been reported for mouse (19), human (20), monkey (21), equine (22) and most relevant livestock species including pig (15, 23, 24). However, to improve the understanding of complex regulate mechanisms involved in sperm function or to identify molecular causes of male infertility, the sperm proteome should be updated as the annotation of the protein-coding genome of each species has increased over time (3), as has recently been the case for pigs (16). Accordingly, the first contribution of the present study was the generation, using iTRAQ, of an updated proteome of mature porcine spermatozoa that includes 1,723 proteins identified and 1,602 of them quantified, albeit only 60% of them encoded in the *Sus scrofa* taxonomy. This shortage evidences the yet incomplete functional and structural annotation of the *Sus scrofa* protein-coding genes (16). Surprisingly, the total number of identified proteins was substantially lower than the 2,728 proteins identified recently by Feugang *et al.* (15) using a shotgun approach. The difference in the number of identified proteins between both studies is related to the methodological differences used for peptide detection and data processing for protein identification and validation, including cutoff data acquisition, algorithms and search databases. For instance, the iTRAQ is more restrictive in identifying proteins than the shotgun approach, as iTRAQ requires high collision energies that cause a loss of sequence-informative fragments (25). In addition, the FDR differed substantially between the studies. Feugang *et al.* (15) set the FDR <10% while we used an FDR <1%, which substantially improved the confidence for the identified proteins. For the sake of comparing results between studies, if we had used an FDR <10%, the number of identified proteins in our study would have been 2,203, near the number found by Feugang *et al.* (15). The quantitative approach used, together with the opportunity to access the latest updates of the porcine proteome, allowed us to identify and quantify a proportionally higher number of

A) MOLECULAR FUNCTION



B) BIOLOGICAL PROCESS



C) CELLULAR COMPONENT

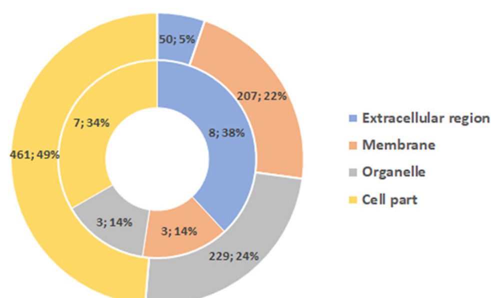


FIG. 3. **Functional distribution of *Sus scrofa* sperm proteins into the three categories available at the UniProtKB/Swiss-Prot Web site (www.uniprot.org) for total identified (outer circles) and differentially expressed (inner circles) sperm proteins belonging to the *Sus scrofa* taxonomy.**

proteins encoded in the *Sus scrofa* taxonomy. Specifically, 256 of the 974 proteins identified and encoded in the *Sus scrofa* taxonomy have been incorporated into the UniProt KB database since November 2017, the date when more than 20,000 new proteins encoded in the *Sus scrofa* taxonomy were included in the data set.

Recently, D'Amours *et al.* (9), using frozen-thawed bovine sperm samples subjected to Percoll-based gradient centrifugation enrichment suggested the possibility that the mammalian ejaculated sperm population could be heterogeneous in protein composition, a bold consideration considering they used a species where the ejaculate is delivered as a single volume without fractions. The present study used mature spermatozoa retrieved from three classically recognizable portions of the porcine ejaculate and, post-mortem, from the cauda epididymis, to discern relevant proteomic changes before and after ejaculation. Because the porcine (or human) ejaculate is delivered in clearly identifiable fractions, which can be separately collected, the findings might have comparative value.

Focusing on the identified proteins, the first noticeable result was that all of them were present in all spermatozoa, independently of the source considered. Pini *et al.* (24) reported similar results in ovine spermatozoa where only 0.5% of the proteins identified in epididymal spermatozoa were absent in ejaculated spermatozoa. These findings were somewhat expected knowing that mature spermatozoa have a very limited capacity to generate new proteins (2, 26). However, there were quantitative differences in the proteome of cauda epididymal spermatozoa compared with those ejaculated, retrieved from three specific ejaculate portions. iTRAQ revealed 43 proteins differentially expressed, with 32 of them encoded in the *Sus scrofa* taxonomy and the remaining 11 encoded in other mammalian species functionally far from domestic species. These findings clearly indicate that the proteome of boar spermatozoa is remodeled during ejaculation, as differences between cauda epididymal and ejaculated spermatozoa were evident. The findings also demonstrate that this protein remodeling during ejaculation was not equal in all spermatozoa, as quantitative differences among spermatozoa of the three ejaculate fractions were found. This latter finding is especially relevant as it showed for the first time that mature, ejaculated spermatozoa are heterogeneous in protein composition as they are in other parameters such as sperm motility, morphology or fertilizing capacity, variables previously used to define sperm sub-populations (8).

The distribution of total identified proteins into the GO categories followed a similar pattern to that observed in the sperm proteome of other mammalian species, like humans (20) or equines (22). Among the proteins encoded in the *Sus scrofa* taxonomy, *i.e.* the only ones validated for porcine, the GO distribution of those differentially expressed differed of that of the totality of identified proteins, particularly for biological processes and cellular components. Compared with the totality of identified proteins, the proportion of differentially expressed proteins related to reproductive processes and those located in the extracellular region were significantly higher. The differentially expressed sperm proteins related to reproductive processes were involved in sperm function, including fertilizing capacity, and those located in the extracellular region were also identified in boar seminal plasma (SP, 27). This dual identification suggests that the differences in protein expression among mature boar spermatozoa affected sperm functionality and that the interaction between spermatozoa and the surrounding media during ejaculation, *i.e.* the different fractions of the SP, could explain such differ-

TABLE II

Proteins identified in boar spermatozoa belonging to the Sus scrofa taxonomy linked to the sperm function categories listed in UniProt KB database (www.uniprot.org). The only protein differentially expressed among the four sperm sources is highlighted in bold

SPERM FUNCTIONALITY RELATED PROTEINS	
ACCESION	PROTEIN NAME
ACROSOME REACTION	
P08001	Acrosin
A0A287B423	Sperm equatorial segment protein 1
F1RFQ6	Syntaxin 2
F1SRY6	Phosphoinositide phospholipase C
I3LT46	Proprotein convertase subtilisin/kexin type 4
BINDING TO ZONA PELLUCIDA	
F1SDH8	Phospholipid-transporting ATPase
D0G0C8	Chaperonin containing TCP1, subunit 2
F1SQN1	T-complex protein 1 subunit delta
I3LR32	Chaperonin containing TCP1 subunit 5
I3LCA2	Chaperonin containing TCP1 subunit 8
I3LQ01	Calmegin
A5A8V7	Heat shock 70 kDa protein 1-like
I3LT46	Proprotein convertase subtilisin/kexin type 4
F1S7B1	Sperm surface protein Sp17
F1SB63	t-complex 1
F1SF28	Zona pellucida-binding protein 1
C8C4M8	Zona pellucida binding protein 2 transcript variant 1
CAPACITATION	
I3LT05	Rhophilin associated tail protein 1
F1SAF0	Dihydrolipoyl dehydrogenase
A0A2C9F357	**Seminal plasma protein pB1**
I3LT46	Proprotein convertase subtilisin/kexin type 4
SPERMATOGENESIS	
F1S2A8	B-cell receptor associated protein 31
P36968	Phospholipid hydroperoxide glutathione peroxidase
F1RRW5	Angiotensin-converting enzyme
F1RZG8	Meiosis specific nuclear structural 1
I3LTK6	Ornithine decarboxylase antizyme 3
D5K8A6	Sperm associated antigen 4-like protein
F1RR24	Spermatogenesis associated 32
F1S5Q9	Theg spermatid protein
F1STE2	Testis specific 10
F1SND1	Thioredoxin domain containing 8
SPERM MOTILITY	
F1SFX0	L-lactate dehydrogenase
F1SGC2	Coiled-coil domain containing 39
I3LAH7	Coiled-coil domain containing 40
F1SLL4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
F1S6P4	Growth arrest specific 8
X4ZHK0	Phosphoglycerate kinase
I3LT05	rhophilin associated tail protein 1
F1RGN9	Tektin 1
F1SV60	Tektin 2
F1SDE8	Tektin 3
F1RL08	Tektin 5

ences. Either the SP protein coating was kept, or the SP caused relevant differences in the extracellular region of the spermatozoa.

During ejaculation in pigs, mature spermatozoa stored in the caudal portion of the epididymis duct are propelled through the vas deferens and urethra where they are resuspended with secretions from the seminal vesicles, prostate and bulbourethral glands (10). The spermatozoa present in the SRF (first 10 ml of SRF and remainder of the SRF portion) are mainly exposed to epididymal fluids and prostate secretion; neither one is especially rich in proteins (28). This particularity of the initial extracellular milieu exposure would explain the similarity in the protein profile between the mature spermatozoa collected from the caudal region of the epididymis (P0) and those ejaculated in the SRF (P1: 10 first ml of SRF and P2: the remainder of the SRF), as they only show differences in 3 proteins with an FC $\geq$ 1.5, which were found to be overexpressed in epididymal mature spermatozoa. Specifically, these proteins were 16-kDa secretory protein, DNA damage inducible 1 homolog 1 (DDI1) and carboxyl ester hydrolase (CES1), which are involved in sperm maturation. The 16-kDa secretory protein is involved in the regulation of membrane lipids during epididymal sperm maturation (29). The DDI proteins attenuate apoptosis during sperm maturation (30, 31)

and the CES proteins, present in the epididymal fluid (32), are considered a safeguarding mechanism for sperm maturation when overexpressed in the male reproductive tract (33).

In contrast to "SRF-spermatozoa," those spermatozoa present in the post-SRF are mainly resuspended in secretions from the vesicular and bulbourethral glands (34, 35). The post-SRF is the ejaculate fraction proportionally containing the largest amount of SP, and in turn is the most protein rich (36). Therefore, the spermatozoa of the post-SRF fraction are proportionally more exposed to absorbable SP proteins. This explain the fact that the spermatozoa of the post-SRF contained more differentially expressed proteins with an FC $\geq$ 1.5. Specifically, 25 proteins were differentially overexpressed, and they were mainly provided by the SP, as 19 of them have also been identified in boar SP by our previous study (27). The SP proteins bind to the sperm surface at ejaculation and many of them remodel the protein domains of sperm membranes and consequently influence sperm function (37). Jonakova *et al.* (38) suggested that boar SP proteins bind to the sperm surface mostly in aggregates rather than individually. These authors highlighted an aggregate that included the five spermadhesins identified in boar SP (PSPI, PSPII, AWN, AQN1 and AQN3) together with porcine B1 (pB1), a protein of bovine seminal plasma (BSP) family. These six SP proteins were among those overexpressed in the spermatozoa of the post-SRF portion. Spermadhesins are low molecular weight glycoproteins with multifunctional properties that are mainly secreted by the seminal vesicles (39–42). Once bound to the sperm plasma membrane, they are involved in regulating some of the most relevant sperm attributes, such as motility and the ability for capacitation, acrosome reaction or sperm-zona pellucida binding (43–45). However, if overexpressed, they may also be detrimental to sperm functionality. For instance, the PSPI/PSPII heterodimer showed decapacitating properties in highly extended boar spermatozoa (46), mimicking the high extension spermatozoa would experience in the post-SRF fraction where a relatively low number of spermatozoa are surrounded by a large volume of protein-rich SP. Similarly, Dyck *et al.* (47) found that the SP-PSPI was negatively correlated with sperm motility in liquid-stored boar semen AI-doses. Spermadhesins can even impair boar sperm fertility if overexpressed in spermatozoa or when spermatozoa are exposed to them in excess. Novak *et al.* (48) showed that boar ejaculates with high SP-concentration of AWN1 or PSPI had lower fertility outcomes in artificially inseminated sows whereas Kwon *et al.* (49) reported that boar spermatozoa overexpressing AQN3 yielded small litters. The other protein of the aggregate, pB1, is a heparin-binding glycoprotein also secreted by the seminal vesicles (50). The role of SP-pB1, once bound to the sperm membrane, is yet unclear. More is known about the DQH protein, a homolog of pB1 (51), which once attached to the sperm surface, plays a crucial role in the ability of boar spermatozoa to bind to the oocyte zona pellucida, an ability that would be substantially reduced if the protein was overexpressed (42). It is also interesting that long exposures of bull spermatozoa to BSP proteins increase their sensitivity to cooling stress (52, 53). Only some of the remaining proteins overexpressed in post-SRF have shown clear evidence of their involvement in sperm functionality. One of them is the fibronectin 1 (FN1), a cell adhesion glycoprotein overexpressed in the SP of the post-SRF (27) and positively correlated with the percentage of spermatozoa with abnormal morphology (54) but also with good sperm freezability (55). Regarding FN1 bound to the sperm membrane, Pinke *et al.* (56) showed that human spermatozoa rich in FN1 were less functional and more cryosensitive. Furthermore, Martínez-León, *et al.* (57) demonstrated that human spermatozoa incubated with FN1 are more likely to undergo capacitation and acrosome reaction and are less prone to the zona pellucida binding. The SP IgG-binding protein was also positively correlated with the proportion of boar spermatozoa depicting abnormal morphology (54) and with low sperm motility in human ejaculates (58). Thrombospondin-1 (TSP1) is a matricellular protein that binds to cell surface receptors, modulating cellular function (59). Although a specific role for sperm function is not known, the TSP1 contributes to the production of reactive oxygen species (ROS) that -when in excess- can lead to cellular dysfunction (60). Moreover, SP concentration of TSP1 was recently correlated with boar ejaculates generating the smallest litter sizes in artificially inseminated sows (61). The wheat germ agglutinin (WGA)-reactive protein on lipid rafts (WGA16) is a heparin-binding protein secreted by the prostate gland that binds to the sperm surface and that must then be removed so they can undergo the capacitation process (62). Accordingly, WGA16 could hinder the capacitation process when overexpressed in the sperm membrane. Nexin-1, a seminal vesicle-derived protein, was also recently correlated with small litters in artificially inseminated sows (61). Type XVIII Collagen α1 chain (COL18A1) is an extracellular matrix protein whose overexpression has been related to spermatogenic dysfunction in human (63). COL18A1 is elevated in the SP of men with non-obstructive azoospermia and is therefore considered to be a potential biomarker for infertility (64). Overall, the overexpression of the abovementioned proteins exerts deleterious effects on sperm functionality, which explains why post-SRF spermatozoa are proportionally less functional and more sensitive to cryopreservation than those of the other boar ejaculate portions and even of those of the cauda epididymis (12, 13). Finally, it is striking that many of the differentially expressed proteins among sperm sources, regardless of the value of the FC, showed the same sign change. Specifically, a total of 16 proteins were overexpressed in spermatozoa from the SRF (first 10 ml of SRF and rest of SRF portions) compared with spermatozoa from the cauda epididymis. The same proteins were underexpressed in spermatozoa from the post-SRF compared with cauda epididymis spermatozoa,

which suggested a coordinated process with respect to the changes measured in this study.

In summary, the present study presents an updated proteome of the porcine spermatozoa, revealing for the first time that the proteome of boar spermatozoa is remodeled during ejaculation, with ejaculated pig spermatozoa showing quantitative differences in proteins implicated in sperm function. The interaction between spermatozoa with the specific secretions of well-defined fractions they bathe in during ejaculation could cause such differences in protein composition. This, in turn, could explain the documented differences between spermatozoa of the same ejaculate in their response to certain sperm biotechnologies, such as cryopreservation, or in achieving successful fertilization. These findings warrant further studies to disclose the inner details of these differentially expressed proteins.

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DATA AVAILABILITY

The raw mass spectrometry data have been deposited in the ProteomeXchange database with the identifier PXD010062 and are publicly accessible at: http://www.ebi.ac.uk/pride.

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[S] This article contains supplemental Figures and Data. The authors declare no conflicts of interest regarding this study.

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REFERENCES

1. Gadella, B. M. (2017) Reproductive tract modifications of the boar sperm surface. *Mol. Reprod. Dev.* **84,** 822–831
2. Park, Y. J., Kim, J., You, Y. A., and Pang, M. G. (2013) Proteomic revolution to improve tools for evaluating male fertility in animals. *J. Proteome Res.* **12,** 4738–4747
3. Mohanty, G., Swain, N., and Samanta, L. (2015) Sperm Proteome: What is on the horizon? *Reprod. Sci.* **22,** 638–653
4. Dostàlovà, Z., Calvete, J. J., Sanz, L., and Töpfer-Petersen, E. (1994) Quantitation of boar spermadhesins in accessory sex gland fluids and on the surface of epididymal, ejaculated and capacitated spermatozoa. *Biochim. Biophys. Acta* **1200,** 48–54
5. Ensslin, M., Vogel, T., Calvete, J. J., Thole, H. H., Schmidtke, J., Matsuda, T., and Töpfer-Petersen, E. (1998) Molecular cloning and characterization of P47, a novel boar sperm-associated zona pellucida-binding protein homologous to a family of mammalian secretory proteins. *Biol. Reprod.* **58,** 1057–1064
6. Li, J., Liu, F., Liu, X., Liu, J., Zhu, P., Wan, F., Jin, S., Wang, W., Li, N., Liu, J., and Wang, H. (2011) Mapping of the human testicular proteome and its relationship with that of the epididymis and spermatozoa. *Mol. Cell. Proteomics* **10,** M110.004630
7. Skerget, S., Rosenow, M. A., Petritis, K., and Karr, T. L. (2015) Sperm Proteome Maturation in the Mouse Epididymis. *PLoS ONE* **10,** e0140650
8. Roca, J., Parrilla, I., Gil, M. A., Cuello, C., Martinez, E. A., and Rodriguez-Martinez, H. (2016) Non-viable sperm in the ejaculate: Lethal escorts for contemporary viable sperm. *Anim. Reprod. Sci.* **169,** 24–31
9. D'Amours, O., Frenette, G., Bourassa, S., Calvo É Blondin . P, Sullivan, R. (2018) Proteomic markers of functional sperm population in bovines: Comparison of low- and high-density spermatozoa following cryopreservation. *J. Proteome Res.* **17,** 177–188
10. Rodríguez-Martínez, H., Kvist, U., Saravia, F., Wallgren, M., Johannisson, A., Sanz, L., Peña, F. J., Martínez, E. A., Roca, J., Vázquez, J. M., and Calvete, J. J. (2009) The physiological roles of the boar ejaculate. *Soc. Reprod. Fertil. Suppl.* **66,** 1–21
11. Saravia, P., Wallgren, M., Johannisson, A., Calvete, J. J., Sanz, L., Peña, F. J., Roca, J., and Rodríguez-Martínez, H. (2009) Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. *Theriogenology* **71,** 662–675
12. Alkmin, D. V., Perez-Patiño, C., Barranco, I., Parrilla, I., Vazquez, J. M., Martinez, E. A., Rodriguez-Martinez, H., and Roca, J. (2014) Boar sperm cryosurvival is better after exposure to seminal plasma from selected fractions than to those from entire ejaculate. *Cryobiology* **69,** 203–210
13. Li, J., Barranco, I., Tvarijonaviciute, A., Molina, M. F., Martinez, E. A., Rodriguez-Martinez, H., Parrilla, I., and Roca, J. (2018) Seminal plasma antioxidants are directly involved in boar sperm cryotolerance. *Theriogenology* **107,** 27–35
14. Li, J., Roca, J., Perez-Patiño, C., Barranco, I., Martinez, E. A., Rodriguez-Martinez, H., and Parrilla, I. (2018) Is boar sperm freezability more intrinsically linked to spermatozoa than to the surrounding seminal plasma? *Anim. Rep. Sci.* (in press).
15. Feugang, J. M., Liao, S. F., Willard, S. T., and Ryan, P. L. (2018) In-depth proteomic analysis of boar spermatozoa through shotgun and gel-based methods. *BMC Genomics* **19,** 62
16. Marx, H., Hahne, H., Ulbrich, S. E., Schnieke, A., Rottmann, O., Frishman, D., and Kuster, B. (2017), Annotation of the domestic pig genome by quantitative proteogenomics. *J. Proteome Res.* **16,** 2887–2898
17. Ford, W. C. (2004) Regulation of sperm function by reactive oxygen species. *Hum. Reprod. Update* **10,** 387–399
18. Krijgsveld, J., Gauci, S., Dormeyer, W., and Heck, A. J. (2006) In-gel isoelectric focusing of peptides as a tool for improved protein identification. *J. Proteome Res.* **5,** 1721–1730
19. Baker, M. A., Hetherington, L., Reeves, G. M., and Aitken, R. J. (2008) The mouse sperm proteome characterized via IPG strip prefractionation and LC-MS/MS identification. *Proteomics* **8,** 1720–1730
20. Martínez-Heredia, J., Estanyol, J. M., Ballescà, J. L., and Oliva, R. (2006) Proteomic identification of human sperm proteins. *Proteomics* **6,** 4356–4369
21. Skerget, S., Rosenow, M., Polpitiya, A., Petritis, K., Dorus, S., and Karr, T. L. (2013) The Rhesus macaque (Macaca mulatta) sperm proteome. *Mol. Cell. Proteomics* **12,** 3052–3067
22. Swegen, A., Curry, B. J., Gibb, Z., Lambourne, S. R., Smith, N. D., and Aitken, R. J. (2015) Investigation of the stallion sperm proteome by mass spectrometry. *Reproduction* **149,** 235–244
23. Peddinti, D., Nanduri, B., Kaya, A., Feugang, J. M., Burgess, S. C., and Memili, E. (2008) Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst. Biol.* **2,** 19
24. Pini, T., Leahy, T., Soleilhavoup, C., Tsikis, G., Labas, V., Combes-Soia, L., Harichaux, G., Rickard, J. P., Druart, X., and de Graaf, S. P. (2016) Proteomic investigation of ram spermatozoa and the proteins conferred by seminal plasma. *J. Proteome Res.* **15,** 3700–3711
25. Wiese, S., Reidegeld, K. A., Meyer, H. E., and Warscheid, B. (2007) Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics* **7,** 340–350
26. Baker, M. A. (2011) The 'omics revolution and our understanding of sperm cell biology. *Asian J. Androl.* **13,** 6–10
27. Perez-Patiño, C., Barranco, I., Parrilla, I., Valero, M. L., Martinez, E. A., Rodriguez-Martinez, H., and Roca, J. (2016) Characterization of the

porcine seminal plasma proteome comparing ejaculate portions. *J. Proteomics* **142,** 15–23
28. Rodríguez-Martínez, H. (2007) State of the art in farm animal sperm evaluation. *Reprod. Fertil. Dev.* **19,** 91–101
29. Okamura, N., Kiuchi, S., Tamba, M., Kashima, T., Hiramoto, S., Baba, T., Dacheux, F., Dacheux, J. L., Sugita, Y., and Jin, Y. Z. (1999) A porcine homolog of the major secretory protein of human epididymis, HE1, specifically binds cholesterol. *Biochim. Biophys. Acta* **1438,** 377–387
30. Bhat, G. K., Sea, T. L., Olatinwo, M. O., Simorangkir, D., Ford, G. D., Ford, B. D., and Mann, D. R. (2006) Influence of a leptin deficiency on testicular morphology, germ cell apoptosis, and expression levels of apoptosis-related genes in the mouse. *J. Androl.* **27,** 302–310
31. Gong, X., Xie, H., Li, X., Wu, J., and Lin, Y. (2017) Bisphenol A induced apoptosis and transcriptome differences of spermatogonial stem cells in vitro. *Acta Biochim. Biophys. Sin.* **49,** 780–791
32. Ecroyd, H., Belghazi, M., Dacheux, J. L., Miyazaki, M., Yamashita, T., and Gatti, J. L. (2006) An epididymal form of cauxin, a carboxylesterase-like enzyme, is present and active in mammalian male reproductive fluids. *Biol. Reprod.* **74,** 439–447
33. Mikhailov, A. T., and Torrado, M. (1999) Carboxylesterase overexpression in the male reproductive tract: a universal safeguarding mechanism? *Reprod. Fertil. Dev.* **11,** 133–145
34. Einarsson, S. (1971) Studies on the composition of epididymal content and semen in the boar. *Acta Vet. Scand.* **36,** 1–80
35. Mann, T., and Lutwak-Mann, C. (1981) Male reproductive function and semen. Berlin/Heidelberg/New York: Springer Verlag.
36. Strzezek, J. (2002) Secretory activity of boar seminal vesicle glands. *Reprod. Biol.* **2,** 243–266
37. Caballero, I., Parrilla, I., Almiñana, C., del Olmo, D., Roca, J., Martínez, E. A., and Vázquez, J. M. (2012) Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. *Reprod. Domest. Anim.* **47,** 12–21
38. Jonáková, V., Manásková, P., Kraus, M., Liberda, J., and Tichá, M. (2000) Sperm surface proteins in mammalian fertilization. *Mol. Reprod. Dev.* **56,** 275–277
39. Calvete, J. J., Mann, K., Schäfer, W., Raida, M., Sanz, L., and Töpfer-Petersen, E. (1995) Boar spermadhesin PSP-II: location of posttranslational modifications, heterodimer formation with PSP-I glycoforms and effect of dimerization on the ligand-binding capabilities of the subunits. *FEBS Lett.* **365,** 179–182
40. Töpfer-Petersen, E., Romero, A., Varela, P. F., Ekhlasi-Hundrieser, M., Dostàlovà, Z., Sanz, L., and Calvete, J. J. (1998) Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* **30,** 217–224
41. Kwok, S. C., Yang, D., Dai, G., Soares, M. J., Chen, S., and McMurtry, J. P. (1993) Molecular cloning and sequence analysis of two porcine seminal proteins, PSP-I and PSP-II: new members of the spermadhesin family. *DNA Cell Biol.* **12,** 605–610
42. Manásková, P., and Jonáková, V. (2008) Localization of porcine seminal plasma (PSP) proteins in the boar reproductive tract and spermatozoa. *J. Reprod. Immunol.* **78,** 40–48
43. Dostálová, Z., Calvete, J. J., Sanz, L., and Töpfer-Petersen, E. (1995) Boar spermadhesin AWN-1. Oligosaccharide and zona pellucida binding characteristics. *Eur. J. Biochem.* **230,** 329–336
44. Boerke, A., Tsai, P. S., Garcia-Gil, N., Brewis, I. A., and Gadella, B. M. (2008) Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology* **70,** 1188–1196
45. Kwon, W. S., Rahman, M. S., Lee, J. S., Kim, J., Yoon, S. J., Park, Y. J., You, Y. A., Hwang, S., and Pang, M. G. (2014) A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. *BMC Genomics* **15,** 897
46. Caballero, I., Vazquez, J. M., Mayor, G. M., Almiñana, C., Calvete, J. J., Sanz, L., Roca, J., and Martinez, E. A. (2009) PSP-I/PSP-II spermadhesin exert a decapacitation effect on highly extended boar spermatozoa. *Int. J. Androl.* **32,** 505–513
47. Dyck, M. K., Foxcroft, G. R., Novak, S., Ruiz-Sanchez, A., Patterson, J., and Dixon, W. T. (2011) Biological markers of boar fertility. *Reprod. Domest. Anim.* **46,** 55–58
48. Novak, S., Ruiz-Sánchez, A., Dixon, W. T., Foxcroft, G. R., and Dyck, M. K. (2010) Seminal plasma proteins as potential markers of relative fertility in boars. *J. Androl.* **31,** 188–200
49. Kwon, W. S., Rahman, M. S., Lee, J. S., Yoon, S. J., Park, Y. J., and Pang, M. G. (2015) Discovery of predictive biomarkers for litter size in boar spermatozoa. *Mol. Cell. Proteomics* **14,** 1230–1240
50. Calvete, J. J., Raida, M., Gentzel, M., Urbanke, C., Sanz, L., and Töpfer-Petersen, E. (1997) Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1. *FEBS Lett.* **407,** 201–206
51. Bezouska, K., Sklenár, J., Novák, P., Halada, P., Havlícek, V., Kraus, M., Tichá, M., and Jonáková, V. (1999) Determination of the complete covalent structure of the major glycoform of DQH sperm surface protein, a novel trypsin-resistant boar seminal plasma O-glycoprotein related to pB1 protein. *Protein Sci.* **8,** 1551–1556
52. Manjunath, P., and Thérien, I. (2002) Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. *J. Reprod. Immunol.* **53,** 109–119
53. Manjunath, P., Bergeron, A., Lefebvre, J., and Fan, J. (2007) Seminal plasma proteins: functions and interaction with protective agents during semen preservation. *Soc. Reprod. Fertil. Suppl.* **65,** 217–228
54. González-Cadavid, V., Martins, J. A., Moreno, F. B., Andrade, T. S., Santos, A. C., Monteiro-Moreira, A. C., Moreira, R. A., and Moura, A. A. (2014) Seminal plasma proteins of adult boars and correlations with sperm parameters. *Theriogenology* **82,** 697–707
55. Vilagran, I., Yeste, M., Sancho, S., Castillo, J., Oliva, R., and Bonet, S. (2015) Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker. *Andrology* **3,** 345–356
56. Pinke, L. A., Swanlund, D. J., Hensleigh, H. C., McCarthy, J. B., Roberts, K. P., and Pryor, J. L. (1997) Analysis of fibronectin on human sperm. *J. Urol.* **158,** 936–941
57. Martínez-León, E., Osycka-Salut, C., Signorelli, J., Pozo, P., Pérez, B., Kong, M., Morales, P., Pérez-Martínez, S., and Díaz, E. S. (2015) Fibronectin stimulates human sperm capacitation through the cyclic AMP/protein kinase A pathway. *Hum. Reprod.* **30,** 2138–2151
58. Chan, C. C., Shui, H. A., Wu, C. H., Wang, C. Y., Sun, G. H., Chen, H. M., and Wu, G. J. (2009) Motility and protein phosphorylation in healthy and asthenozoospermic sperm. *J. Proteome Res.* **8,** 5382–5386
59. Murphy-Ullrich, J. E., and Iozzo, R. V. (2012) Thrombospondins in physiology and disease: new tricks for old dogs. *Matrix Biol.* **31,** 152–154
60. LeBlanc, A. J., and Kelm, N. Q. (2017) Thrombospondin-1, free radicals, and the coronary microcirculation: The aging conundrum. *Antioxid. Redox Signal* **27,** 785–801
61. Pérez-Patiño, C., Parrilla, I., Barranco, I., Vergara-Barberán, M., Simó-Alfonso, E. F., Herrero-Martínez, J. M., Rodriguez-Martínez, H., Martínez, E. A., and Roca, J. (2018) New in-depth analytical approach of the porcine seminal plasma proteome reveals potential fertility biomarkers. *J. Proteome Res.* **17,** 1065–1076
62. Garénaux, E., Kanagawa, M., Tsuchiyama, T., Hori, K., Kanazawa, T., Goshima, A., Chiba, M., Yasue, H., Ikeda, A., Yamaguchi, Y., Sato, C., and Kitajima, K. (2015) Discovery, primary, and crystal structures and capacitation-related properties of a prostate-derived heparin-binding protein WGA16 from boar sperm. *J. Biol. Chem.* **290,** 5484–5501
63. Dobashi, M., Fujisawa, M., Naito, I., Yamazaki, T., Okada, H., and Kamidono, S. (2003) Distribution of type IV collagen subtypes in human testes and their association with spermatogenesis. *Fertil. Steril.* **80,** 755–760
64. Batruch, I., Lecker, I., Kagedan, D., Smith, C. R., Mullen, B. J., Grober, E., Lo, K. C., Diamandis, E. P., and Jarvi, K. A. (2011) Proteomic analysis of seminal plasma from normal volunteers and post-vasectomy patients identifies over 2000 proteins and candidate biomarkers of the urogenital system. *J. Proteome Res.* **10,** 941–953