REGULAR ARTICLE

Ready for the journey: a comparative proteome profiling of porcine cauda epididymal fluid and spermatozoa

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Abstract

In the present study, we describe the proteome of porcine cauda epididymis fluid and spermatozoa by means of Multidimensional Protein Identification Technology (MudPIT). Ten sexually mature healthy boars were surgically castrated and epididymides were dissected to obtain the cauda epididymal content. Polled protein extracts of cauda epididymal fluid (CEF) and spermatozoa (CESperm) were loaded in an Agilent 1100 quaternary HPLC and peptides eluted from the microcapillary column were electro-sprayed directly into a LTQ Orbitrap XL mass spectrometer. Using bioinformatics, identified proteins were classified by their molecular functions, involvement in biological processes and participation in relevant metabolic pathways associated with spermatozoa physiology, fertility potential and protection. A total of 645 proteins were identified in the CEF, with epididymal-specific lipocalin-5, beta-hexosaminidase subunit beta precursor and phosphatidylethanolamine-binding protein 4 being the most abundant proteins found. A total of 2886 proteins were identified in the CESperm proteome with 81 proteins being considered more abundant (spectral counts > 100). CEF and CESperm data were compared and 345 proteins were present in both proteomes. Phosphatidylethanolamine-binding protein 4 precursor was the only protein found most abundant in both CEF and CESperm proteomes. Based on Gene Ontology analysis, we identified CEF and CESperm proteins associated with sperm protection against ROS and immune mediated response, glycosaminoglycan degradation, ubiquitin-proteasome system, metabolic process and maturation, modulation of acrosome reaction and ZP binding and oocyte penetration. These results provide a better comprehension about the molecular process and biological pathways involved in sperm epididymis maturation and establishment of the cauda epididymis sperm reservoir.

Augusto Weber and Laura Espíndola Argenti contributed equally to this work.

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Introduction

The extensive cellular differentiation processes that occur in the testis, transforming the round spermatid in a highly polarized and specialized cell, are only completed after the epididymal maturation (Dacheux and Paquignon 1980). The epididymis is a very long duct, which receives testicular sperm and fluid via efferent ducts. Divided into three major regions, the caput, corpus and cauda, its function goes beyond the simple storage of spermatozoa prior to ejaculation. During sperm transit through the epididymal lumen, several maturation steps transform an inactive cell into a motile and fertile gamete. These steps remove or modify most of the testicular sperm surface proteins and also add new proteins that have key roles in sperm function (Cowan and Myles 1993; Dacheux et al. 2006). At the end of epididymal transit and maturation, spermatozoa are

stored in the cauda and are ready to be ejaculated and to fertilize an oocyte (Pena Jr. et al. 2015).

The protein composition of the epididymal fluid is directly related to the secretory activity of the epithelium, since almost all testicular proteins are reabsorbed in the initial segment of the organ (Dacheux et al. 2006; Dacheux et al. 2009). Gel-based approaches like 2D-SDS-PAGE demonstrated the secretion of important proteins in the porcine cauda epididymis lumen, such as lactoferrin, epididymal secretory protein E1 (HE1) and epididymal-specific lipocalin-5 (E-RABP) (Park et al. 2012; Syntin et al. 1996; Syntin et al. 1999). The regionalization of the epididymal spermatozoa proteome was described recently (Labas et al. 2015), evidencing protein changes during maturation. In the boar, secretory activity of the epididymis is more prevalent in the proximal regions (Dacheux et al. 2005; Guyonnet et al. 2009). However, evidence also indicates that the cauda epididymal environment is more than just a reservoir of inactive spermatozoa. In fact, sperm storage at the distal epididymal region prevents premature capacitation, maintains metabolic quiescence and protects sperm against oxidative stress and complement-mediated attack (Hinton and Palladino 1995; Moura et al. 2010; Roberts et al. 2003). Therefore, a better understanding of the role of secreted proteins present in this particular environment as well as the protein profile of mature spermatozoa not only could explain the physiological mechanisms associated with semen quality and fertility (Govindaraju et al. 2012) but also may lead to the development of better sperm preservation techniques. Thus, the present experiment was designed to evaluate the cauda epididymal environment by means of Multidimensional Protein Identification Technology (MudPIT) of the cauda epididymal fluid (CEF) and cauda epididymal spermatozoa (CESperm).

Material and methods

Animals and sample preparation

Ten sexually mature healthy boars (Large White), routinely used as semen donors for artificial insemination were assigned for the study. Animals were housed in a boar stud and were normally fed with a commercial corn-soybean meal diet and ad libitum water according to the nutritional requirement guidelines for adult boars (NRC 2012). Animals were surgically castrated and the epididymides were immediately dissected to obtain epididymal fluid samples. Animal experiments were conducted according to protocols approved by the Animal Experimentation Ethics Committee (protocol 001/2015).

Epididymal sections of region 9, corresponding to boar cauda epididymis, (Dacheux et al. 2005) were dissected and the EF (with spermatozoa) was flushed out and collected in tubes. The suspensions were stirred and centrifuged ($800 \times g$

for 10 min) and the supernatant was centrifuged ($12,000 \times g$ for 1 h at 4 °C) to remove remaining spermatozoa. The cauda epididymal fluid (CEF) supernatant was stored at − 80 °C after the addition of protease inhibitors (Protease Inhibitor Cocktail, Sigma, USA). Pellets containing spermatozoa (around 10^7 spermatozoa) were washed three times with 1 mL PBS (140 mM NaCl, 15 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), centrifuged and resuspended in 0.5 mL RIPA Buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) with protease inhibitors. The suspension was gently stirred, lysed and centrifuged at $12,000 \times g$ for 1 h at 4 °C. Total protein content of CEF and cauda epididymal spermatozoa (CESperm) protein extracts were quantified by BCA method using BSA as standard (Pierce, USA).

Preparation of protein extracts and protein digestion

CEF and CESperm samples were polled in equal quantities (300 μg of total protein per sample) and then suspended in digestion buffer (8 M urea, 100 mM Tris-HCl pH 8.5). Proteins were reduced with 5 mM tris-2-carboxyethyl-phosphine (TCEP) for 20 min at RT followed by alkylation with 10 mM iodoacetamide at RT in the dark for 15 min. After the addition of 1 mM $CaCl_2$ (final concentration), proteins were digested using 2 μg of trypsin (Promega, Madison, WI) by incubation at 37 °C for 16 h. Proteolysis was stopped by adding formic acid to a final concentration of 5%. Samples were centrifuged at $10,000 \times g$ for 20 min and the supernatant was collected and stored at − 80 °C.

MudPIT

The protein digest was pressure-loaded into a 250 μm i.d. capillary packed with 2.5 cm of 5 μm Luna strong cation exchanger (SCX) (Whatman, USA), followed by 2 cm of 3 μm Aqua C18 reversed phase (RP) (Phenomenex, USA) with a 1-μm frit. The column was washed with a buffer containing 95% water, 5% acetonitrile and 0.1% formic acid. After washing, a 100-μm i.d. capillary with a 5-μm pulled tip packed with 11 cm of 3 μm Aqua C18 resin (Phenomenex, USA) was attached via a union and placed in line with an Agilent 1100 quaternary HPLC. A modified 12-step separation was used (Santi et al. 2014; Wolters et al. 2001). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B) and 500 mM ammonium acetate, 5% acetonitrile and 0.1% formic acid (buffer C). Step 1 consisted of a 60 min gradient from 0 to 100% (v/v) buffer B. Steps 2–10 had different buffer C percentages, from 10 to 100%. An additional step containing 90% (v/v) buffer C and 10% (v/v) buffer B was used. Three technical replicates were analyzed for CEF and for CESperm.

Mass spectrometry

Peptides eluted from the microcapillary column were electro-sprayed directly into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with the application of a distal 2.5 kV spray voltage. A cycle consisted of one full scan of the mass range (MS) (400–2000 m/z, resolution of 60.000) followed by five data-dependent collision-induced dissociation (CID) MS/MS spectra in the LTQ. Dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 30 s, an exclusion list size of 150 and an exclusion duration of 180 s. Also, the mass window for precursor ion selection was set to 400–1600, unassigned and charge 1 was rejected and the normalized collision energy for CID was 35. Mass spectrometer scan functions and HPLC solvent gradients were controlled through the XCalibur data system. All MS/MS spectra were analyzed using the following software analysis protocol, as proposed by Santi et al. (2014). Briefly, protein identification and quantification analysis were done with Integrated Proteomics Pipeline (www.integratedproteomics.com/). Tandem mass spectra were extracted into ms2 files from raw files using RawExtract 1.9.9 (McDonald et al. 2004) and were searched using the ProLuCID algorithm as described by Xu et al. (2006) against the *Sus scrofa* reference sequence database from NCBI, downloaded on July 2015. The peptide mass search tolerance was set to 3 Da and carboxymethylation (+ 57.02146 Da) of cysteine was considered to be a static modification. ProLuCID results were assembled and filtered using the DTASelect program (Tabb et al. 2002) resulting in a data set with a false discovery rate of 1% for protein.

Data analysis and bioinformatics

The Blast2GO 5 tool (Consea et al. 2005) was used to categorize the proteins detected by Gene Ontology (GO) annotation (Ashburner et al. 2000) according to biological process and molecular function. Also, the metabolic pathways were assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) map module. Other bioinformatics tools were used to determine the subcellular location (TargetP 1.0; Emanuelsson et al. 2007) and to predict transmembrane protein topology with a hidden Markov model (TMHMM 2.0; Krogh et al. 2001). SignalP 4.1 was used for prediction of secreted proteins (using cutoff default) (Petersen et al. 2011). TargetP, TMHMM and SignalP programs are available at http://www.cbs.dtu.dk/services.

In addition to protein data analysis, tissue specificity and gene set enrichment analyses were carried out with GENE2FUNC module implemented in the FUMA (Functional Mapping and Annotation of Genome-Wide Association Studies) platform (Watanabe et al. 2017). All proteins identified by MudPIT in CEF and CSperm were mapped in the Uniprot database using the Retrieve/ID mapping tool. This step allowed the identification of the genes related to the proteins found in CEF and CSPerm. A first analysis (2×2 enrichment tests) included all the genes retrieved from Uniprot setting only protein-coding genes as a background gene set. The GTEx v7 30 general tissue type data set was used for tissue specificity analyses. Differentially expressed gene (DEGs) sets were pre-calculated in the GENE2FUNC by means of two-sided t test for any one of the tissues against all others. For this, expression values were normalized (zero-mean) following a $\log^2$ transformation of expression values (transcripts per million). After Benjamini-Hochberg correction and absolute log fold change ≥ 0.58, genes with $p \leq 0.05$ were defined as DEGs in a given tissue compared to others. Finally, only the genes, expressed in the testis (epididymis data are not available in FUMA), were reanalyzed by GENE2FUNC for tissue expression, gene ontology and hallmarks based in the Molecular Signatures Database (MSigDB) (Liberzon et al. 2015).

Expression of testicular and epididymal genes related to proteins identified in CEF and CSperm

To validate proteomics results, genes related with proteins with high and low abundance in CEF and CSperm were selected for expression analysis by qPCR. For this purpose, samples from testicular parenchyma and regions 1, 5 and 9 of the epididymides were excised, rinsed in ice-cold PBS and treated with RNALater® (Invitrogen, USA). Then, tissues were snap frozen in liquid nitrogen and stored at − 80 °C until RNA extraction. Total RNA was extracted from 100 mg of tissue using the GE Healthcare Illustra Spin® kit. Synthesis of cDNA was carried out from 1.5 μg of RNA using M-MLV Reverse Transcriptase® (Invitrogen). The integrity of the RNA was verified by polymerase chain reaction followed by agarose gel electrophoresis. SYBR Green qPCR assays were performed in a StepOne Plus thermocycler (Applied Biosystems, USA) to evaluate the expression of the genes CALR, LCN5, PTGDS, TR and NUCB2 (primers available as Supplementary Table 1). Reactions of 25 μL were performed in quadruplicates. Each reaction contained 10 μL cDNA (diluted 1:50), 4.14 μL water, 2 μL 10× buffer ([200 mM Tris-HCl (pH 8.4), 500 mM KCl]), 3.31 mM $MgCl_2$, 0.11 mM dNTPs, 0.22 mM of each primer, 2 μL SYBR green (diluted 1:10,000, Molecular Probes, USA) and 0.625 μL of Platinum Taq DNA Polymerase (5 U/μL, Invitrogen). qPCR reaction parameters were as follows: a pre-run at 95 °C for 15 min, 45 cycles with a 15-s denaturation step at 95 °C, followed by a 56 °C annealing step for 30 s and a 72 °C extension step for 30 s and a final extension step at 72 °C for 10 min. Melting curves were performed with a 0.3 °C increase every 30 s. Fluorescence detection was performed immediately at the end of each annealing step and the

Table 1 Most abundant proteins identified in porcine cauda epididymal fluid (CEF) by Multidimensional Protein Identification Technology (MudPIT)

Protein ID (*Sus scrofa*)	NCBI accession number	SC (%SC)	SignalP	TargetP (RC)*	Gene Ontology terms
Epididymal-specific lipocalin-5	XP_003122377.1	1465 (14.6)	Yes	Secretory pathway (1)	Lipid metabolic process; small molecule binding; response to hormone; extracellular region; transport;
Beta-hexosaminidase subunit beta precursor	NP_999086.1	1346 (13.4)	No	Secretory pathway (1)	Male courtship behavior; oligosaccharide catabolic process; beta-N-acetylhexosaminidase activity; penetration of zona pellucida; phospholipid biosynthetic process; cellular protein metabolic process; oogenesis; membrane
Phosphatidylethanolamine-binding protein 4 precursor	NP_001156360.1	367 (3.7)	Yes	Secretory pathway (1)	Extracellular exosome; protein binding
Lactotransferrin precursor	NP_999527.1	226 (2.3)	Yes	Secretory pathway (1)	Iron ion homeostasis; positive regulation of I-kappaB kinase/NF-kappaB signaling; positive regulation of NF-kappaB transcription factor activity; ion transport; iron ion binding; protein serine/threonine kinase activator activity; negative regulation of ATPase activity; positive regulation of toll-like receptor 4 signaling pathway; serine-type endopeptidase activity; protein complex; negative regulation of apoptotic process; proteolysis positive regulation of protein serine/threonine kinase activity; negative regulation of lipopolysaccharide-mediated signaling pathway
Brain acid–soluble protein 1 isoform 2	XP_003483854.1	134 (1.3)	No	—	cytoplasm; transcription corepressor activity; nuclear speck; transcription regulatory region DNA binding; plasma membrane
Di-N-acetylchitobiase, partial	XP_003356466.3	115 (1.1)	Yes	Secretory pathway (2)	Chitinase activity; carbohydrate metabolic process; extracellular space; extracellular exosome; chitin catabolic process
Epididymis-specific alpha-mannosidase precursor	NP_999014.1	114 (1.1)	Yes	Secretory pathway (3)	Protein deglycosylation; alpha-mannosidase activity; zinc ion binding; extracellular exosome; mannose metabolic process; carbohydrate binding
Epididymal secretory glutathione peroxidase precursor	NP_999051.1	112 (1.1)	Yes	Secretory pathway (1)	Extracellular space; glutathione peroxidase activity; sperm plasma membrane; cellular oxidant detoxification; cellular response to oxidative stress; oxidation-reduction process
Reticulocalbin-1 isoform 2	XP_003353965.2	103 (1.0)	No	—	In utero embryonic development; calcium ion binding; endoplasmic reticulum
Alkaline phosphatase, tissue-nonspecific isozyme	XP_005658515.1	101 (1.0)	No	—	Membrane; extracellular membrane-bounded organelle; response to organic cyclic compound; dephosphorylation; alkaline phosphatase activity

The 5 reliability classes, defined as follows: 1: diff > 0.800; 2: 0.800 > diff > 0.600; 3: 0.600 > diff > 0.400; 4: 0.400 > diff > 0.200; 5: 0.200 > diff

[a] Spectral counts with percentage of total spectral counts

[b] Prediction of the presence and location of signal peptide cleavage sites in amino acid sequences

[c] RC: reliability class, from 1 to 5, where 1 indicates the strongest prediction
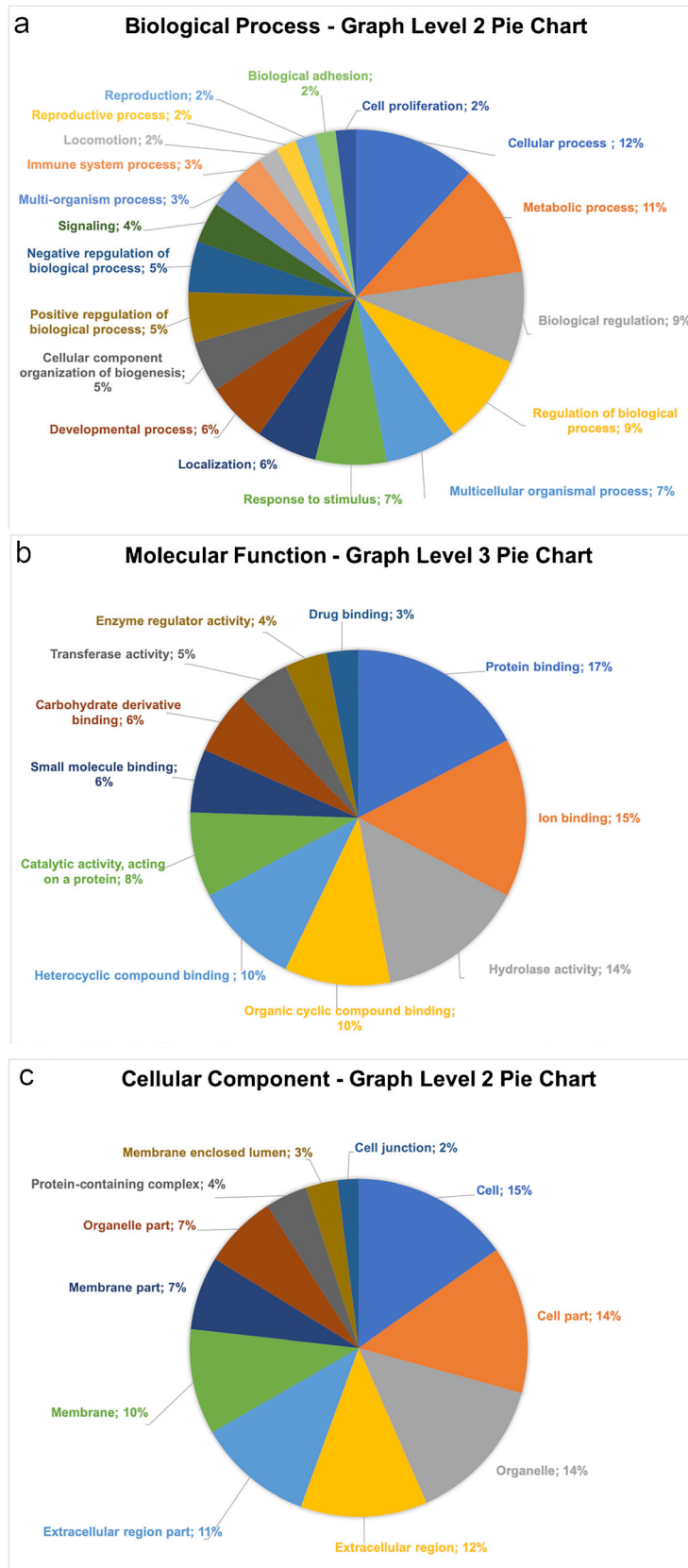
Fig. 1 Gene Ontology classification of proteins identified in porcine cauda epididymal fluid (CEF) by MudPIT. **a** Biological process. **b** Molecular function. **c** Cellular component. Protein data were analyzed using Blast2GO 5 software

Table 2 Most abundant proteins identified in porcine cauda epididymal spermatozoa (CESperm) by MudPIT

Protein ID (Sus scrofa)	NCBI accession number	SC (%SC)[a]	SignalP[b]	TargetP (RC)[c]	TMHMM[d]	Gene Ontology terms
Heat shock 70 kDa protein 1-like	NP_001116600.1	2646 (3.9)	No	–	Outside	ATP binding; heat shock protein binding; binding of sperm to zona pellucida; ubiquitin protein ligase binding; cell body; blood microparticle; cytosol; unfolded protein binding; COP9 signalosome; protein refolding; zona pellucida receptor complex; positive regulation of protein targeting to mitochondrion
Acrosin-binding protein	XP_003126581.1	1039 (1.5)	Yes	s	Outside	Nucleus; zinc ion binding; protein binding; sperm capacitation; acrosomal vesicle; integral component of membrane; extracellular region
Heat shock 70 kDa protein 1b-like	NP_998931.1	820 (1.1)	No	–	Outside	ATP binding; cytoplasm
Beta-enolase isoform ×1	NP_001037992.1	741(1.1)	No	–	Outside	Magnesium ion binding; phosphopyruvate hydratase activity; extracellular space; extracellular exosome; glycolytic process; phosphopyruvate hydratase complex; plasma membrane
Gamma-enolase	XP_005652652.1	725 (1.0)	No	–	Outside	Magnesium ion binding; phosphopyruvate hydratase activity; gluconeogenesis; extracellular space; myelin sheath; extracellular exosome; protein binding; photoreceptor inner segment; perikaryon; canonical glycolysis; phosphopyruvate hydratase complex; plasma membrane
Phosphatidylethanolamine-binding protein 4 isoform ×1	NP_001156360.1	693 (1.0)	Yes	s	Outside	Extracellular exosome
Zona pellucida-binding protein 1	NP_999271.1	642 (0.7)	No	m	Outside	Nucleus; acrosome assembly; binding of sperm to zona pellucida; acrosomal vesicle; cell body; extracellular region; zona pellucida receptor complex
Leucine-rich repeat-containing protein 37a-like	XP_005668767.1	498 (0.7)	Yes	s	TMhelix, inside	Integral component of membrane
Camp-dependent protein kinase catalytic subunit alpha isoform ×1	XP_003123401.1	496 (0.7)	No	–	Outside	Regulation of proteasomal protein catabolic process; activation of protein kinase A activity; water transport; calcium channel complex; sperm capacitation; regulation of protein binding; cellular response to glucose stimulus; cellular response to glucagon stimulus; protein kinase binding; gluconeogenesis; positive regulation of protein export from nucleus; negative regulation of meiotic cell cycle process involved in oocyte maturation; germinal vesicle; regulation of ryanodine-sensitive calcium-release channel activity; calcium-mediated signaling using intracellular calcium source; extracellular exosome; protein serine/threonine/tyrosine kinase activity; G2/M transition of mitotic cell cycle; sperm midpiece; neural tube closure; cellular response to epinephrine stimulus; ciliary base; positive regulation of cell cycle arrest; regulation of bicellular tight junction assembly; cell; regulation of cytosolic calcium ion concentration; cAMP-dependent protein kinase activity; centrosome; innate immune response; mitochondrion;
Alpha-mannosidase 2c1 isoform ×2	XP_001924224.1	491 (0.7)	No	–	Outside	Protein deglycosylation; alpha-mannosidase activity; zinc ion binding; mannose metabolic process; carbohydrate binding; vacuole
L-lactate dehydrogenase a-like 6b	XP_003361852.2	489 (0.7)	No	m	Outside	Nucleus; carbohydrate metabolic process; L-lactate dehydrogenase activity; mitochondrion; oxidation-reduction process; carboxylic acid metabolic process

The 5 reliability classes, defined as follows: 1: diff>0.800; 2: 0.800>diff>0.600; 3: 0.600>diff>0.400; 4: 0.400>diff>0.200; 5: 0.200>diff

[a] Spectral counts and percentage of total spectral counts

[b] Prediction of the presence and location of signal peptide cleavage sites in amino acid sequences

[c] RC: reliability class, from 1 to 5, where 1 indicates the strongest prediction
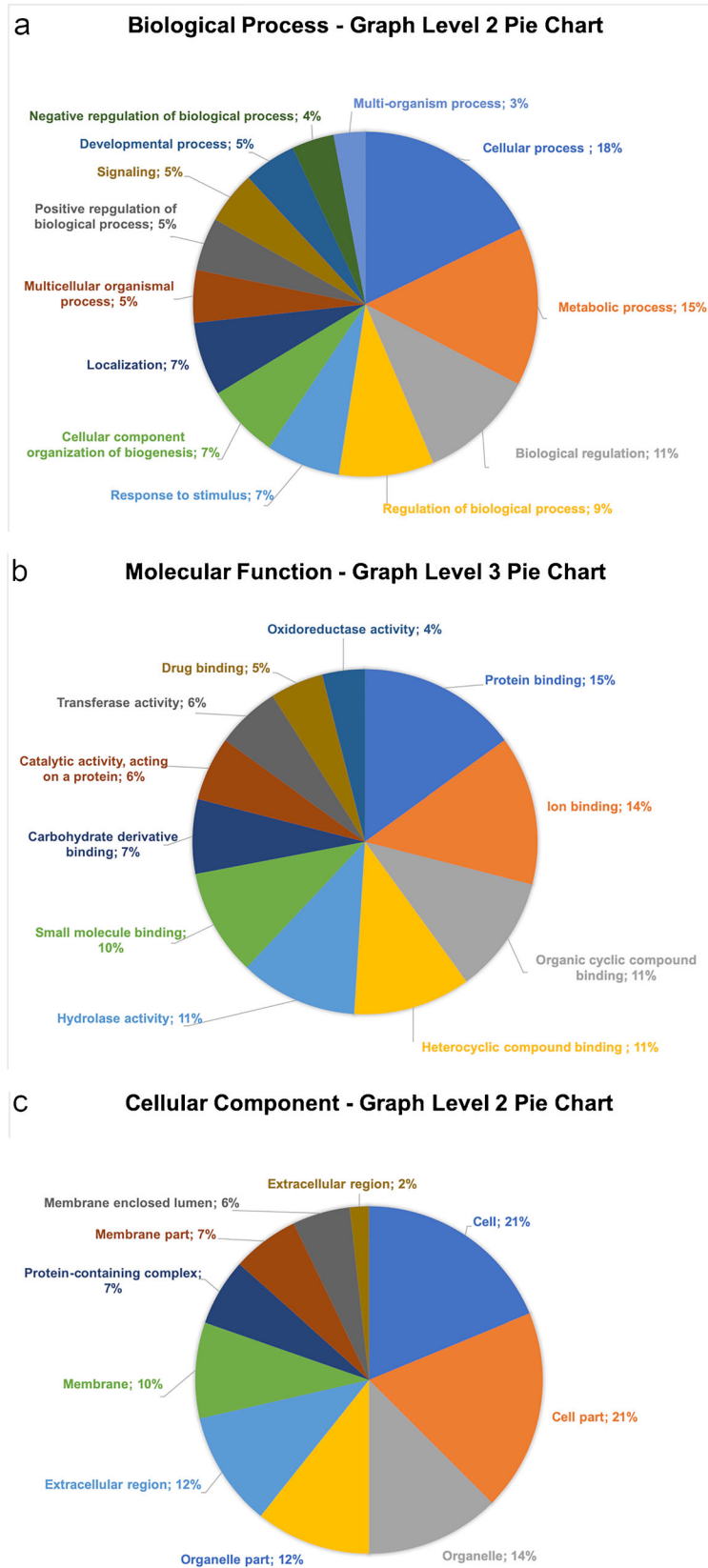
[d] Prediction of transmembrane helices in proteins

Fig. 2 Gene Ontology classification of proteins identified in porcine cauda epididymal spermatozoa (CESperm) by MudPIT. **a** Biological process. **b** Molecular function. **c** Cellular component. Protein data were analyzed using Blast2GO 5 software

specificity of the amplification was confirmed by analyzing the melting curves. A no-template control was also included in each assay. PCR efficiency was assessed with LinRegPCR software (Ramakers et al. 2003; Ruijter et al. 2009). The relative expression ratio was calculated compared to the arithmetic mean expression of the reference gene ACTB as previously described (Pfaffl 2001).

Statistical analysis

Data normal distribution was accessed by the D'Agostino-person test. Differences in gene expression between testis and epididymal regions were verified by one-way ANOVA, followed by the Tukey post-hoc test. All calculations were performed using GraphPad Prism 6 software and significance was considered when $p < 0.05$.

Results

A total of 645 proteins were identified in the CEF. Based on the spectral count, proteins with higher relative abundance (> 100) counted for 1.5% ($n = 10$) of all proteins found in the CEF. On the other hand, 75.4% ($n = 500$) of all proteins identified had a spectral count under 10, demonstrating the high complexity of the cauda epididymis luminal content.

Interesting, a significant number of proteins were found for the first time in boar epididymis. Epididymal-specific lipocalin-5 and beta-hexosaminidase subunit beta precursor were the most abundant proteins found in boar CEF. With 1465 and 1346 spectral counts respectively, both proteins have 3 times more spectral counts than the third more abundant, phosphatidylethanolamine-binding protein 4 precursor. The proteins with higher spectral counts are described in Table 1. The complete list of proteins found in CEF is presented as Supplementary Table 2.

The Gene Ontology analysis showed that the proteins found in swine CEF are associated with numerous biological processes, such as the cellular and metabolic process, biological regulation, reproduction, locomotion and developmental process (Fig. 1a). Several molecular functions were described for the identified proteins, mainly involving binding to different molecules like proteins, ions, organic cyclic compounds and macromolecular complexes. Hydrolase and transferase activities and enzyme regulators were also described (Fig. 1b). Regarding the cellular component, 22% proteins are associated to cell membrane, 50% as part of the cell and organelles and 22% described as present at the extracellular region (Fig. 1c).

MudPIT analysis of the spermatozoa retrieved from cauda epididymis resulted in the identification of 4.3 times more proteins in comparison to CEF. Due the occurrence of different isoforms presenting similar spectral counts, some proteins

Fig. 3 Venn diagram representing the distribution of all proteins identified in the cauda epididymal fluid (CEF) and spermatozoa (CESperm). Most abundant proteins (spectral counts above 100) are also represented as CEF > 100 and CESperm > 100. Data analyzed and data generated by InteractVenn software (Heberle et al. 2015)
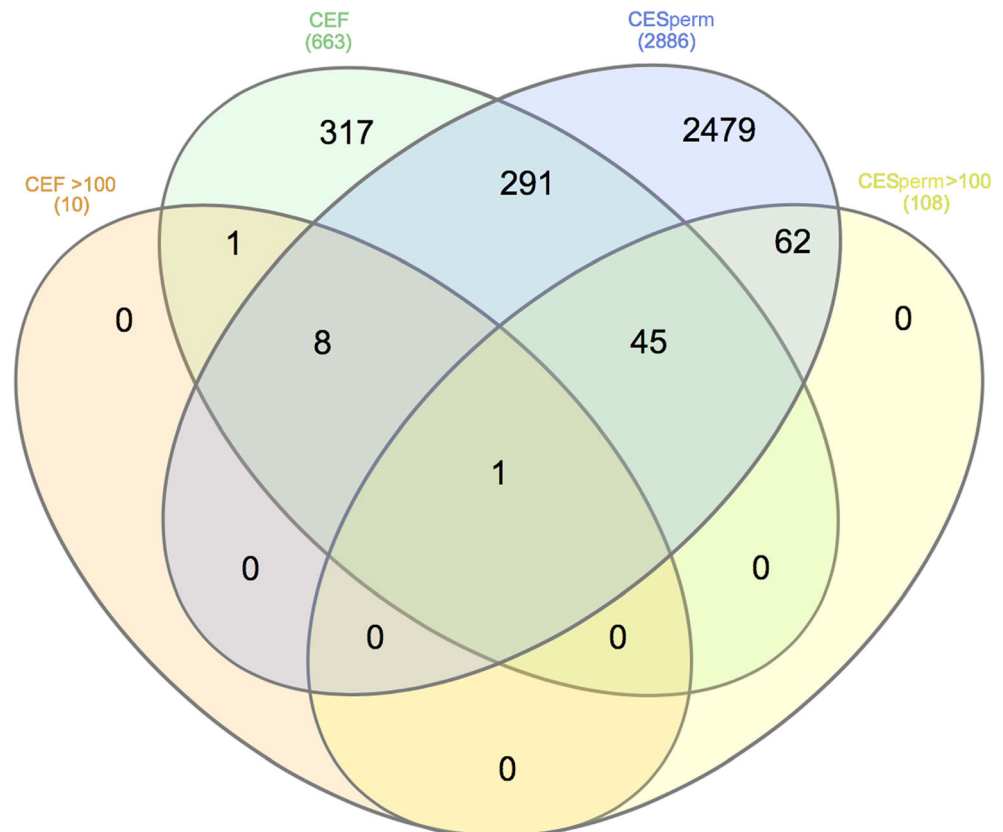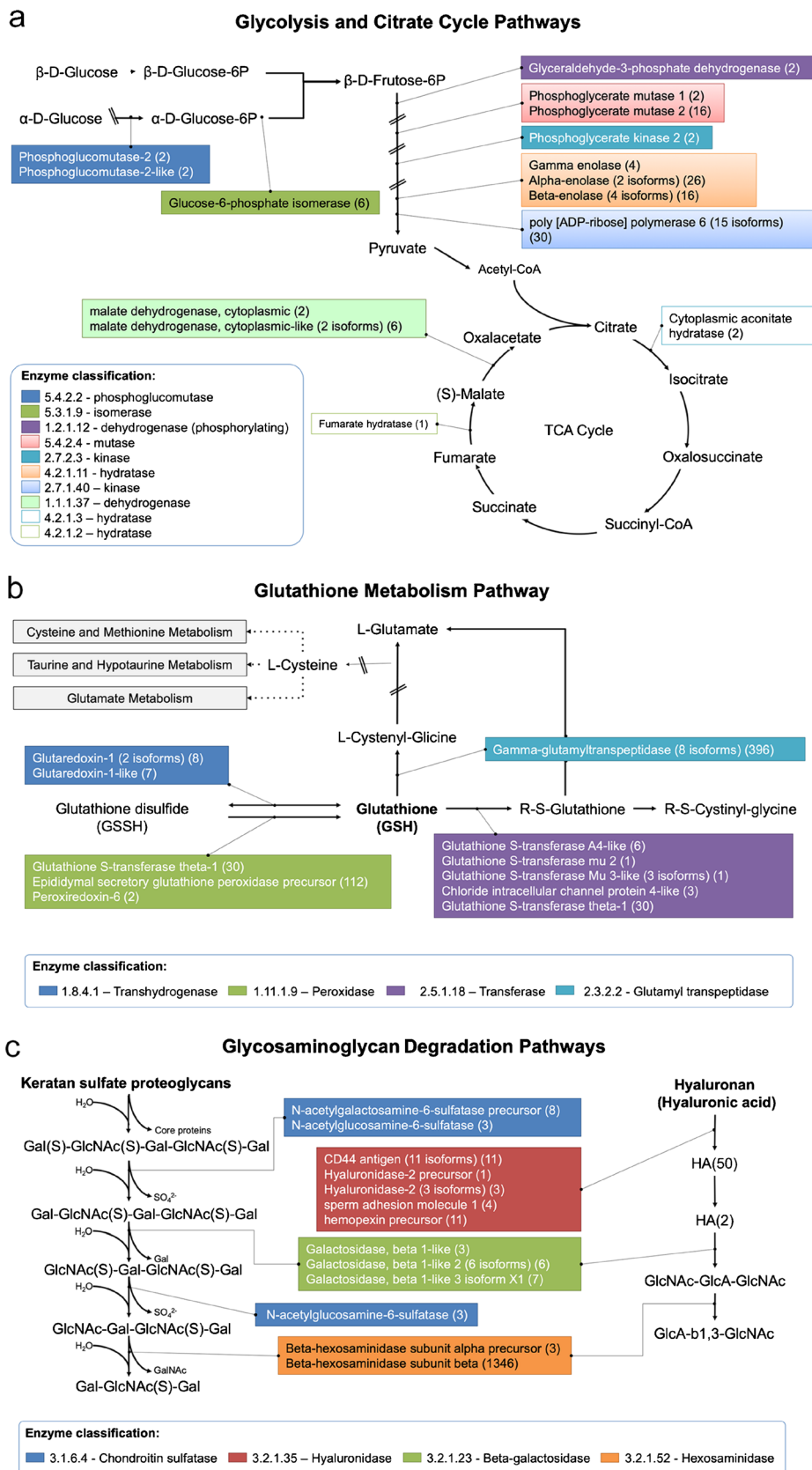
Fig. 4 Metabolic pathways associated with proteins from porcine cauda epididymal fluid and spermatozoa identified by MudPIT. Maps created based on KEGG pathways, provided by Blast2GO 5 software. **a** Glycolisis and citrate acid pathways. **b** Glutathione metabolism pathway. **c** Glycosaminoglycan degradation pathways

Fig. 5 Venn diagram showing the distribution of the genes associated with the proteins identified by MudPIT in the cauda epididymal fluid (CEF) and sperm (CESperm) of adult boars

CEF genes (253)

CSperm genes (955)

99   154   801
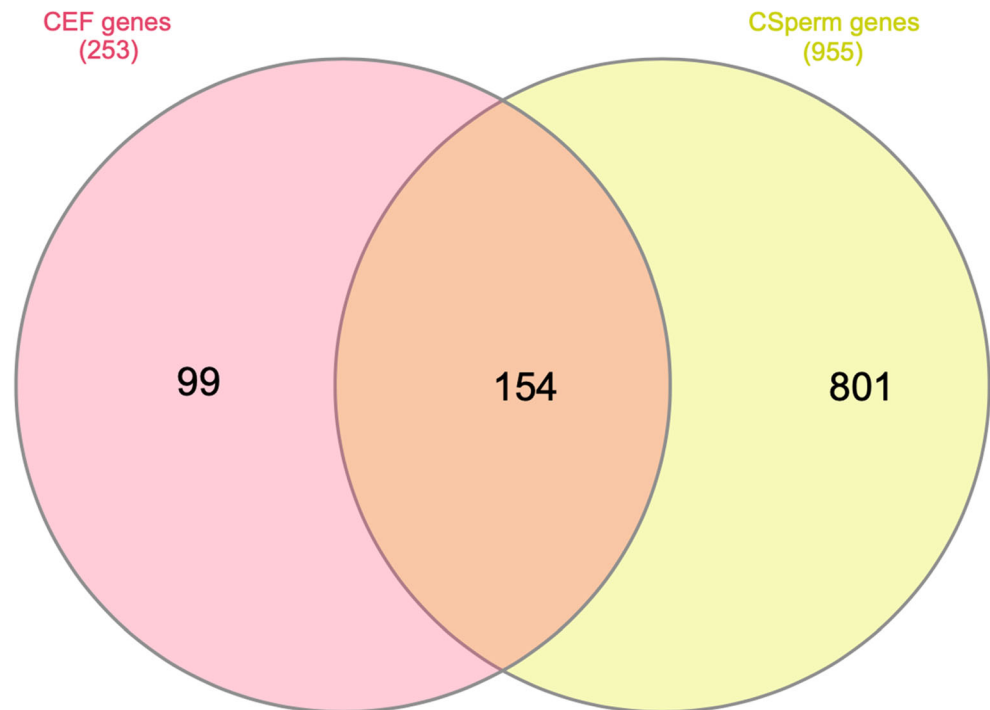
were excluded in the subsequent analysis. From a total of 2886 proteins, 81 had over 100 spectral counts, representing 4.8% of the total proteins identified. A total of 566 proteins had between 10 and 100 spectral counts, while 1044 proteins had up to 10 spectral counts. Table 2 presents the most abundant CESperm proteins according to their spectral counts. The complete list of proteins identified in spermatozoa from cauda epididymis is presented in Supplementary Table 3.

The molecular functions of proteins identified in boar spermatozoa were compiled by Blast2GO software and are presented in Fig. 2. The analysis revealed that 985 proteins are related with protein binding, 752 proteins bind to the organic cyclic compound and 747 proteins bind with heterocyclic compounds. Also, 27% of the proteins of CESperm present enzymatic activities, acting as oxidoreductases, transferases and hydrolases (Fig. 2b). The cellular component analysis identified 1021 proteins related with cells, 1019 with portions of cells, 952 related with organelles and 578 with parts of organelles (Fig. 2c). The biological processes analysis identified 1844 proteins related with the cellular process and 1551 related with the metabolic process. Among these, 477 and 441 proteins are related to positive and negative regulation of the biological process, respectively, while 459 are related to the developmental process, such as the reproductive process and reproduction (Fig. 2a).

The comparison between CEF and CESperm proteomes evidenced that 345 proteins were identified in both samples. Forty-six of the most abundant proteins present in CESperm were also present in the CEF. Differently, 9 out of 10 of the most abundant proteins found in the CEF were present in CESperm (Fig. 3). Phosphatidylethanolamine-binding protein 4 precursor

(NP_001156360.1) was the only protein found most abundant (SC > 100) in both CEF and CESperm proteomes, presenting 367 and 693 spectral counts, respectively.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to categorize in which pathways the proteins identified in porcine CEF and CESperm were involved. Thirty-three different pathways were associated with both proteomes, being the following pathways with a higher number of enzymes involved: glutathione metabolism, glycosaminoglycan degradation, arachidonic acid metabolism, other glycan degradations and energy metabolism (Fig. 4a–c). These pathways are directly related to sperm protection, membrane composition and metabolism control, which not only are essential tasks for spermatozoa preservation prior ejaculation but also play key roles in sperm transit in the female genital tract and fertilization.

Gene enrichment analysis was performed and resulted in 955 and 253 genes associated with CSperm and CEF proteins, respectively (Fig. 5). Testis-expressed genes counted for 371 in CSperm and 84 in CEF. Tissue enrichment analysis with the GENE2FUNC tool demonstrated a high expression of the prioritized genes in testis (Figs. 6a and 7a). Also, gene ontology analysis confirms the association of the testis-expressed genes to biological processes and hallmarks linked to reproduction, fertilization and cell metabolism (Figs. 6 b and 7 b).

The analysis of the expression of genes that code for proteins found in the CEF and CSperm showed different expression levels in testis and a marked regional distribution along the epididymis (Fig. 8a–f). PTGDS and LCN5 had higher expression in the caput epididymis ($p < 0.01$), while NUCB2
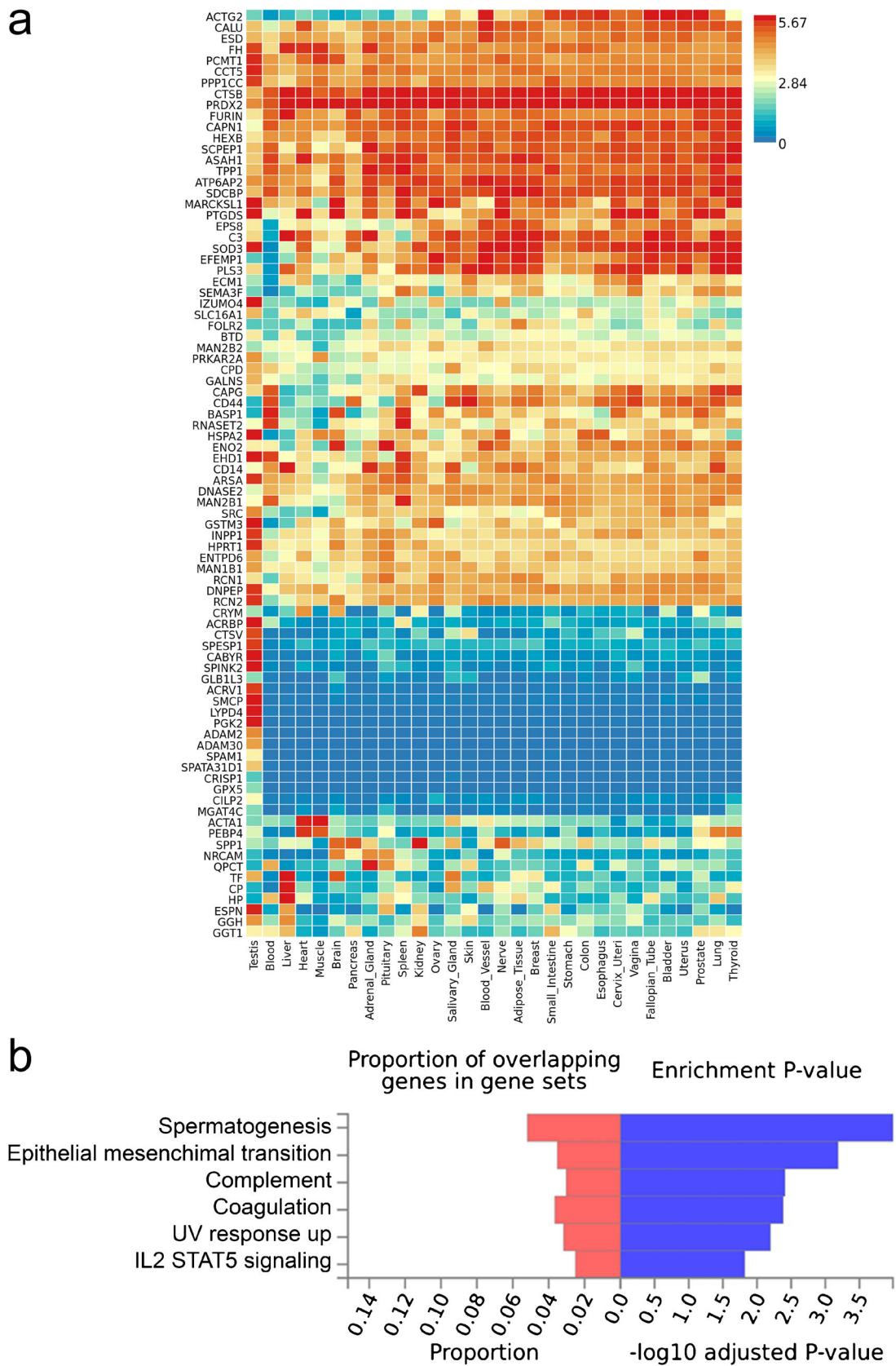
Fig. 6 Analysis of the genes expressed in the testis related to the proteins identified in the fluid from cauda epididymis (CEF). **a** Tissue enrichment heatmaps indicating the level of expression of the genes related to CEF and CSperm proteins. **b** Hallmark pathways identified using hypergeometric tests indicating overrepresentation of testis-expressed genes within gene sets from MsigDB. Analysis performed using GENE2FUNC tool from FUMA platform
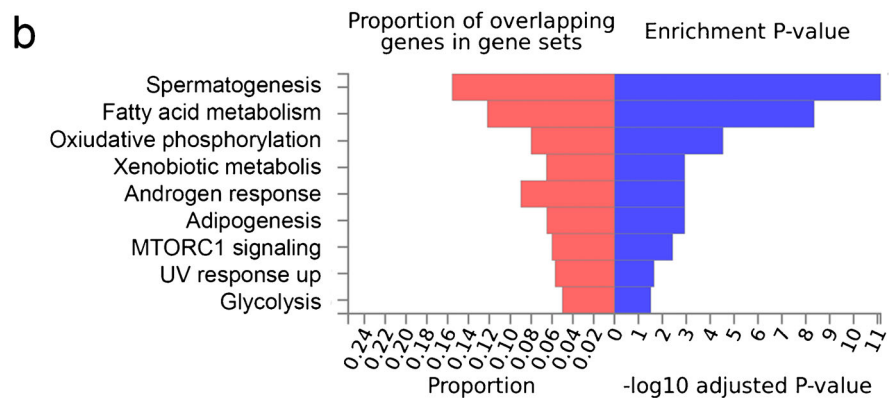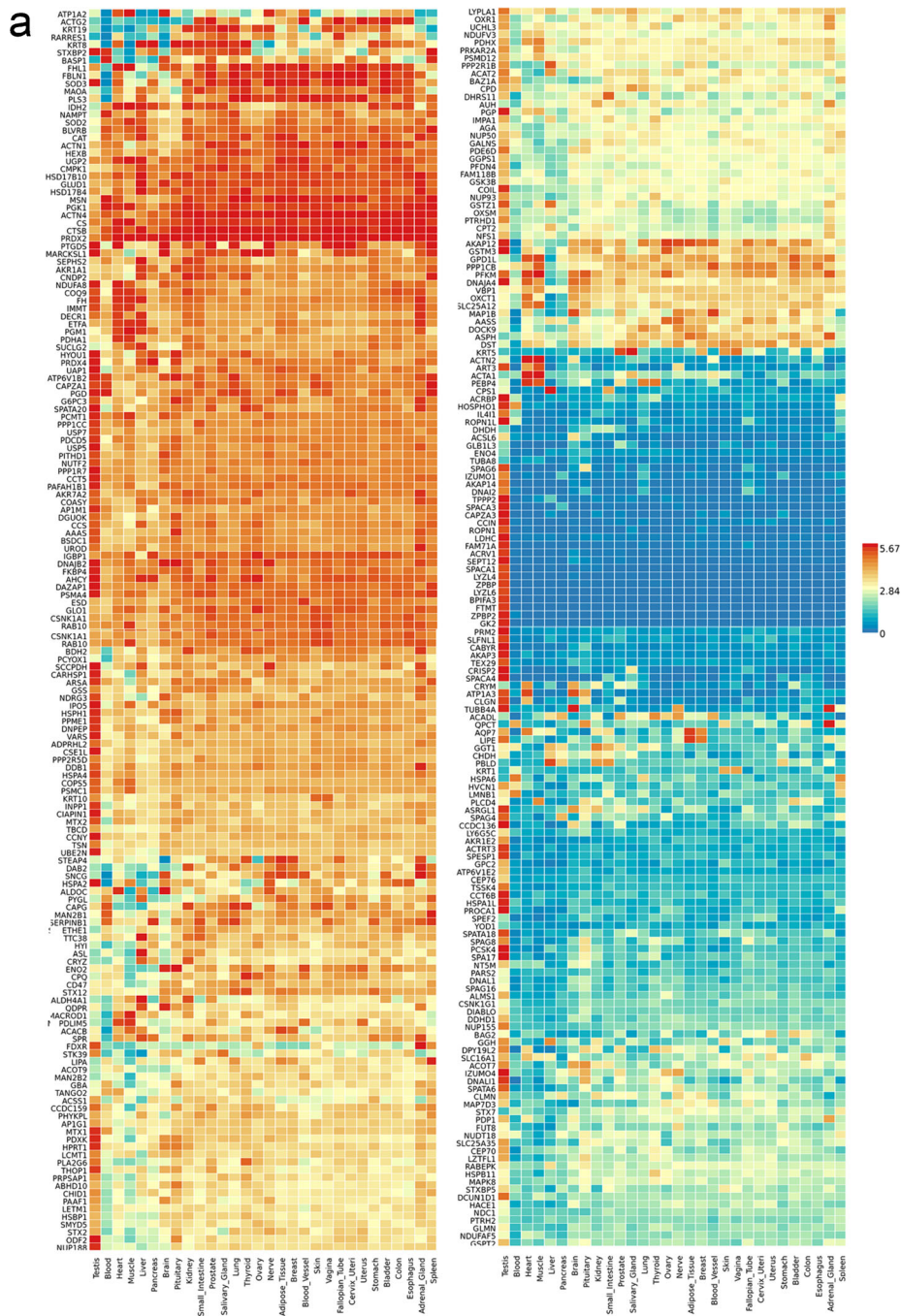
showed a high expression in the cauda region ($p < 0.001$). Testicular expressions of target genes were higher or similar to at least one of the epididymal regions except for the genes CALR and LCN5. The CALR gene did not differ between testis and epididymis and LCN5 presented the lower expression level in the testis ($p < 0.001$).

Discussion

In this study, we characterized the proteomes of cauda epididymal spermatozoa (CESperm) and fluid (CEF) using MudPIT. This approach made possible the identification of 2886 proteins in CESperm associated with different cellular processes, more than 1000 proteins described in a recent study (Perez-

Patino et al. 2018). Also, 663 proteins were found in the CEF, a notably higher number in comparison with previous reports using gel-based techniques (Syntin et al. 1996). These results provide a better comprehension about the molecular process and biological pathways that are needed to preserve the epididymal boar spermatozoa for their full fertilizing potential.

The comparison between sperm and fluid cauda epididymal proteome shows that 2541 proteins are exclusively of spermatozoa, whereas 318 are present only in the fluid. Due to the methodology used, a high number of low abundance proteins were detected, adding more information about the cauda epididymal environment. Proteins shared by fluid and spermatozoa (10.8%) are associated with important biological functions for sperm physiology as well as cell structural and function preservation, such as energy metabolism and antioxidant protection.

Using different bioinformatics tools, we were able to identify 1208 genes associated with the CEF and CSperm proteins. This difference is explained due to high occurrence of protein isoforms and to database annotation. Gene Ontology analysis by FUMA platform retrieved same molecular functions, biological processes and pathways identified by Blast2GO
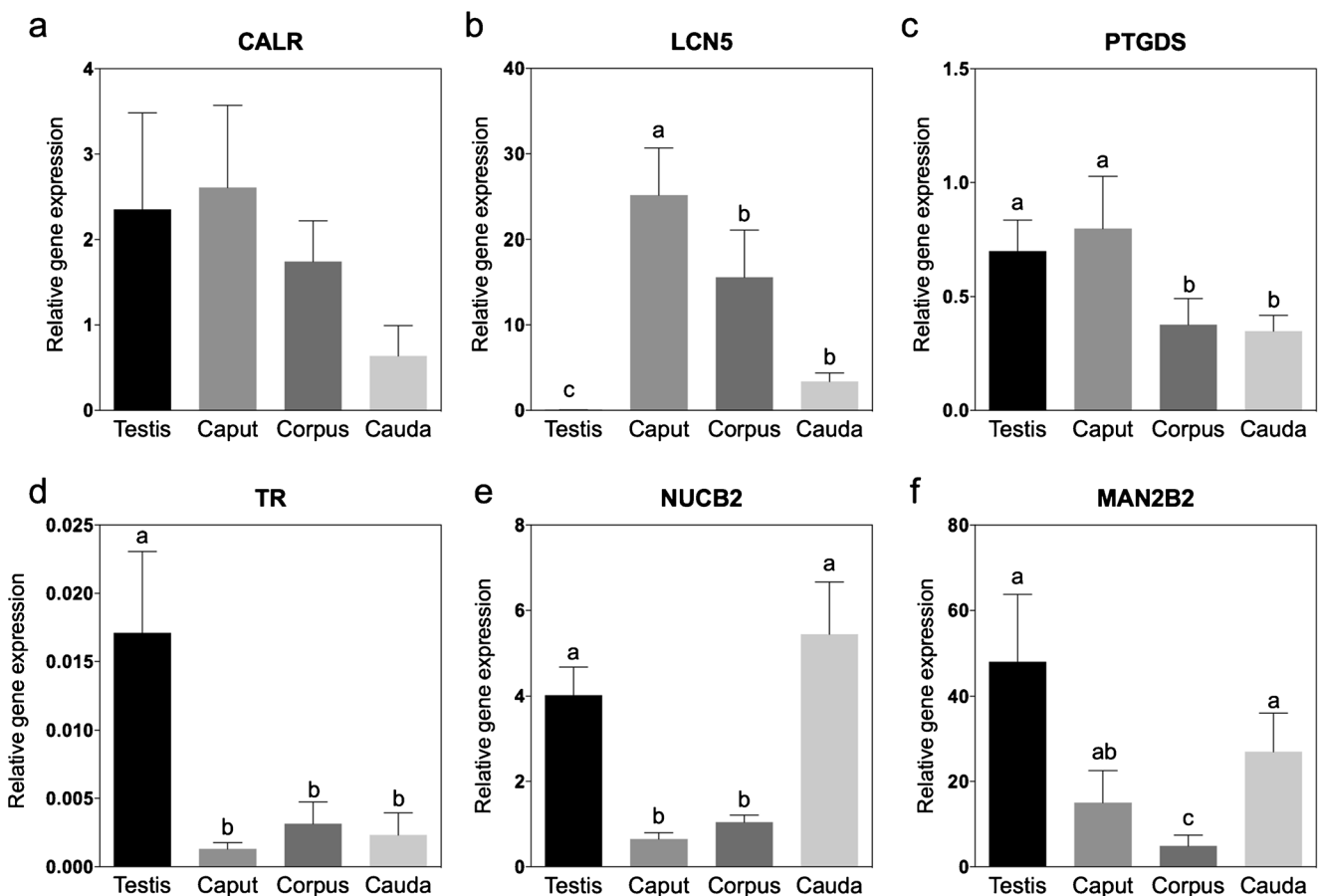

Fig. 8 mRNA expression of genes related to the synthesis of the proteins. **a** Calreticulin (CALR). **b** Epididymis-specific lipocalin 5 (LCN5). **c** Prostaglandin D synthase (PTGDS). **d** Serotransferrin (TR). **e** Nucleobindin-2 (NUCB2). **f** Epididymis-specific alpha-mannosidase (MANB2). Values represent mean ± SD. Letters denote significant differences between regions ($p < 0.05$)

software for proteomic data. This approach provides reliability to data analysis and interpretation.

In the boar cauda epididymal fluid, 10 proteins were identified as highly abundant based on spectral counts (higher than 100). Previous results demonstrated that about 15–20 proteins make up more than 60–80% of the total protein concentration of epididymal fluid in most part of animals (Dacheux et al. 2009; Dacheux and Dacheux 2014). However, in this study, we observed that cauda epididymis fluid is more complex than previously observed. Among the highly abundant proteins, several were associated to sperm metabolic processes and maturation like epididymal retinoic acid-binding protein, epididymal-specific lipocalin-5, epididymal specific α-mannosidase and reticulocalbin-1. Other proteins are involved in sperm protection, such as lactotransferrin and di-N-acetylchitobiase, while others play a role in sperm fertilizing ability, like glutathione peroxidase 5 and B-hexosaminidase. Except di-N-acetylchitobiase, these proteins were already described not only in the porcine CEF but also in the seminal plasma. For example, epididymal-specific lipocalin-5 and B-hexosaminidase were found more abundant in the seminal plasma from sperm rich fractions (Perez-Patino et al. 2016).

The epididymal fluid has a crucial importance in sperm maturation inside the epididymis. In this tube, the media surrounding sperm will be completely renewed. Its protein and other chemical compositions are modified by the uptake and secretion activities of the tubular epithelium (Yanagimachi 1994). At the end of sperm maturation, spermatozoa are immotile, a process dependent on an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) and calcium and changes in the phosphorylation status of specific proteins (Dacheux and Dacheux 2014). Our data provide evidence that the CEF has a role avoiding the activation of phosphorylation-dependent pathways with the presence of several phosphatases such as alkaline phosphatase, tissue-nonspecific isozyme, prostatic phosphatase, ectonucleotide pyrophosphatase/phosphodiesterase family member 2 and acid phosphatase-like 2, among others. Also, CEF has different proteins that modulate cAMP (e.g., cAMP-dependent protein kinase type II-alpha regulatory subunit, cAMP-dependent protein kinase catalytic subunit alpha) and calcium (e.g., nucleobindin 2, calmodulin, calreticulin, 45 kDa calcium-binding protein), dependent events.

Cauda epididymis is the final stage before ejaculation and is characterized by a relatively large lumen and its surrounding epithelial cells exhibit strong absorptive activity (Hermo and Robaire 2002). Such attributes align with the dominant function of the cauda epididymis in terms of the formation of a sperm storage reservoir (Zhou et al. 2018). Antioxidant protection of mature spermatozoa is a key feature in the epididymis (Chabory et al. 2010; El-Taieb et al. 2009; Vernet et al. 2004), contributing for the immotile state as well regulating ROS inside the epididymal duct that facilitates sperm

chromatin compaction (Chabory et al. 2009). Together with enzymatic scavengers already described in the porcine epididymis, such as glutathione peroxidase, superoxide dismutase and glutathione S-transferase (Koziorowska-Gilun et al. 2011; Zaja et al. 2016), other proteins with antioxidant activity were found (Fig. 4b). Peroxiredoxin-6 and peroxiredoxin-2 are thiol-specific peroxidases that catalyze the reduction of H_2O_2 and organic hydroperoxides to water and alcohol, respectively. By regulating the intracellular concentrations of H_2O_2 (Conrad et al. 2015), cauda epididymis peroxiredoxins could play an important role avoiding premature sperm capacitation and acrosome reaction, events regulated by ROS (Rivlin et al. 2003).

The protein composition of cauda epididymal spermatozoa is the final signature of a long process of maturation and the amount of proteins found in this study expands the complexity of sperm proteome. The majority (62.1%) of the proteins had very low abundance (spectral counts < 10) and this figure is a result of the high-resolution power of MudPIT shotgun proteomics comparing to gel-based techniques (Fränzel and Wolters 2011). On the other hand, the 10 most abundant proteins represented 0.59% of proteins identified and 20.52% of total spectral counts. These proteins are involved in sperm protection (Heat Shock Protein 70 kDa), gamete interaction (acrosin-binding protein, zona pellucida-binding protein 1, alpha-mannosidase), sperm metabolism (beta-enolase, gamma-enolase, cAMP-dependent protein kinase, lactate dehydrogenase), cell signaling (phosphatidylethanolamine-binding protein 4) and cell function (leucine-rich repeat-containing protein).

As previously mentioned, these highly abundant proteins correspond to less than 1% of the identified proteins, evidencing the complexity of the sperm proteome. Due to the high number of proteins identified, bioinformatics tools are very helpful in order to identify processes and pathways relevant to sperm physiology and epididymal function. The identification of several enzymes involved with glycosaminoglycan (GAG) degradation pathways (e.g., beta-hexosaminidase, hyaluronidase-2, N-acetylgalactosamine-6sulfatase, galactosidase, CD44 antigen and hemopexin; Fig. 4c) supports the importance of the processing of molecules like heparan sulfate, keratin sulfate, chondroitin sulfate, hyaluronic acid and dermatan sulfate (Binette et al. 1996; Therien et al. 2005). In fact, GAGs play key roles in sperm capacitation and interaction with female genital tract epithelia, oviductal sperm reservoir formation and sperm-egg interaction (Sostaric et al. 2005; Talevi and Gualtieri 2001). One of the main sources of GAG is the follicular fluid, which has in important contribution in promoting sperm capacitation and acrosome reaction, as demonstrated in bovine (Handrow et al. 1982; Lenz et al. 1982) and swine (DAPINO et al. 2006) spermatozoa. Also rich in GAG, the oviductal fluid is essential to establish the oviduct for a specific purpose: sustain and regulate gamete

preparation, fertilization and the first steps of zygote development (Rodriguez-Martinez et al. 2001). Therefore, spermatozoa must be equipped with proper enzymes in order to manage all events dependent on GAG, such as the spermatozoa release from the sperm reservoir (Liberda et al. 2006; Rodriguez-Martinez 2007). The individual evaluation of GAG-related enzymes found in this work allows a better understanding of the interaction of spermatozoa and the oviductal environment and associated events.

The identification of more than 100 proteins (including isoforms) associated with the ubiquitin-proteasome pathway in the spermatozoa collected from cauda epididymis brings new evidence that the epididymal ubiquitin proteasome system (UPS) is an active system of sperm quality control (Baska et al. 2008). Ubiquitin is secreted in the epididymal lumen by the principal cells of the epididymal epithelium via epididymosomes, transferring epididymis-secreted cytosolic and integral membrane proteins to spermatozoa (Sullivan et al. 2007; Sullivan and Saez 2013). There is a growing body of evidence that the epididymis has a proteolytic sperm quality control mechanism based on surface ubiquitination of the defective spermatozoa (Sutovsky 2003; Sutovsky et al. 2001; Tengowski et al. 2005). Therefore, UPS specificity would ensure that only damaged spermatozoa are ubiquitinated and possibly degraded.

Composed by the ubiquitin-activating enzyme E1 (not found in the CEF and CESperm proteomes), the ubiquitin-conjugating enzyme E2 and the ubiquitin-ligase E3, the ubiquitin-substrate-ligation pathway is required for the ubiquitination of proteinaceous substrates prior to substrate degradation by the 26S proteasome (Baska et al. 2008; Glickman and Ciechanover 2002). Interestingly, not only the cytoplasmic UPS is involved but also an extracellular ubiquitination mechanism is present and active in the epididymal fluid (Baska et al. 2008). In the present work, the number of UPS related proteins in the CEF was 10 times lower than in CESperm. Nevertheless, the intense apocrine secretion, with direct delivery of proteins by extracellular vesicular transport (Martin-DeLeon 2015; Sullivan et al. 2007), could explain this difference in protein number. In fact, the importance of this mechanism in swine reproduction was recently demonstrated. UPS participates in the de-aggregation of boar spermadhesins (most abundant porcine seminal plasma proteins) and DQH protein (also known as Binder of Sperm protein 1, BSP1) from the sperm surface during capacitation, with a possible involvement in sperm detachment from the oviductal sperm reservoir and/or sperm-zona pellucida interactions (Zigo et al. 2019). This new evidence shows the importance of proteomic studies in reproductive biology in order to allow a better understanding of the role of proteins in key pathways involved in gamete physiology.

Conclusion

In summary, the use of MudPIT shotgun proteomics allowed the identification of over three thousand proteins in porcine cauda epididymal fluid and spermatozoa. Together with bioinformatics, identified proteins were classified by their molecular functions, involvement in biological processes and participation in relevant metabolic pathways associated with spermatozoa physiology, fertility potential and protection. These results provide a better understanding of the mature spermatozoa and the interactions with the cauda epididymis fluid, an important part of the seminal plasma.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures with animals described in this article were previously approved by the Local Animal Experimentation Ethics Committee (protocol 001/2015) and according to the Brazilian Animal Welfare legislation.

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