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Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams☆

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ABSTRACT

The present study is focused on the proteome of reproductive tract fluids from tropically-adapted Santa Ines rams. Seminal plasma, cauda epididymal (CEF) and vesicular gland fluid (VGF) proteins were analyzed by 2-D electrophoresis and mass spectrometry. Seminal plasma maps contained 302±16 spots, within the 4–7 pH range. From these maps, 73 spots were identified, corresponding to 41 proteins. Ram Seminal Vesicle Proteins (RSVP) 14 and 22 kDa and bodhesins 1 and 2 represented the most abundant seminal components. Other seminal proteins included clusterin, angiotensin-converting enzyme, matrix metalloproteinase-2, tissue-inhibitor of metalloproteinase-2, plasma glutamate carboxypeptidase, albumin, lactoferrin, alpha enolase, peroxiredoxin, leucine aminopeptidase, β-galactosidase, among others. Later, seminal plasma gels were run within narrow pH intervals (3.9–5.1; 4.7–5.9; 5.5–6.7), allowing the additional identification of 21 proteins not detected in 4–7 pH maps. Major proteins of CEF and VGF were albumin and transferrin, and RSVPs, respectively. Western blots confirmed that RSVPs were mainly present in VGF while bodhesins, in VGF and CEF. Based on RT-PCR, RSVP and bodhesin genes were primarily expressed in the vesicular glands. In summary, the reproductive tract fluids of Brazilian hairy rams contain several categories of proteins, with potential roles in sperm protection, capacitation, acrosome reaction and sperm–oocyte interaction. This article is part of a Special Issue entitled: Farm animal proteomics.

1. Introduction

Spermatozoa are produced in the germinative epithelium of the testis, matured during epididymal transit and stored in the cauda epididymal region. Following ejaculation, spermatozoa and cauda epididymal fluid are mixed with secretions from the accessory sex glands and deposited in the female reproductive tract, where they are capacitated and start their journey to the fertilization site [1]. In this context, we suggest that constituents of the sperm themselves and fluids of the

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male and female reproductive tracts potentially modulate each of these single steps that precede conception. For example, sperm proteins [2], DNA integrity [3] and mRNA profile [4] are among the components that influence fertility. The seminal plasma is the media surrounding sperm cells immediately after ejaculation and it is basically composed by secretions from the epididymis and accessory sex glands, in addition to some proteins detached from sperm cells [5]. Seminal plasma proteins are associated with aspects of the reproductive potential in several species, such as the ability to penetrate oocytes in vitro [6] and fertility of sires [7–10]. Such relationships, according to those authors, are probably due to the ability of seminal proteins to modulate several functions, including sperm capacitation, acrosome reaction and sperm–oocyte interaction, among others.

Proteomics-related techniques, such as 2-D PAGE (polyacrylamide gel electrophoresis), liquid chromatography and mass spectrometry constitute a powerful arsenal to study protein composition as well as their biological functions in cells, tissues and their secretions. This is especially useful in the study of the mechanisms by which reproductive tract proteins affect sperm function. Knowledge about the composition of seminal plasma of bulls is far more advanced than for other species of farm animals, especially those in tropical areas of the world. In vast areas of Brazil, sheep are predominantly without wool and, among these breeds of hair sheep, the Santa Ines is the most common. It originally evolved in a semi-arid region of the Brazilian Northeast and, over the last three decades, spread to practically all regions of the country. The Santa Ines represents an important genotype because of its performance in medium-to-low external input production systems, their capacity to graze local pastures and adaptability to heat stress [11]. Thus, the present study was conducted to establish the identity of the major protein composition of the reproductive tract fluids from adult Santa Ines rams. We also evaluated how major seminal plasma proteins interact with membranes of ejaculated and epididymal sperm.

2. Material and methods

2.1. General procedure

Semen samples were collected from ten Santa Ines rams with normal reproductive status. At a commercial slaughter house, we obtained cauda epididymal and vesicular gland secretions from ten animals with the same phenotype. The seminal plasma and cauda epididymal fluid were separated from sperm by centrifugation. Vesicular gland fluid samples were also centrifuged to remove debris. All samples were subjected to 2-D electrophoresis and Coomassie blue-stained gels analyzed with PDQuest software (Bio-Rad, Rockville, MD, USA). Spots of interest were excised from gels, digested with trypsin and prepared for protein identification by mass spectrometry. Western blots and RT-PCR were carried out to confirm identities of specific proteins and evaluate gene expression, respectively. We relied on indirect immunofluorescence coupled with confocal microscopy to evaluate how major seminal fluid proteins interact with sperm. All rams used in the present study were managed in accordance to International Guiding Principles for Biomedical Research Involving Animals.

2.2. Collection of semen and seminal plasma

For collection of semen, we used ten yearling Santa Ines rams with average body weight and scrotal circumference of 58.0 ± 1.8 kg and 34.3 ± 1.8 cm, respectively. Semen samples were obtained three times from each animal, every other day, and analyzed as previously reported [12], with modifications. Briefly, semen was harvested using an artificial vagina, immediately placed in a water bath (37 °C) and aliquots taken for assessment of sperm counts and the percentages of motile sperm and sperm with progressive motility. Spermatozoa were counted with the aid of a Neubauer hemocytometer, diluting semen samples (1:400) in a buffered saline-formol solution. Total number of ejaculated sperm was determined by multiplying sperm concentration by ejaculated volume [12]. The remaining of semen samples were initially centrifuged at 700 × *g* for 10 min (4 °C) to separate the seminal plasma from sperm. Seminal plasma was then transferred to a clean tube and centrifuged once more (10,000 × *g*, 60 min, 4 °C). After the last centrifugation, seminal plasma was divided into aliquots and stored at −80 °C.

2.3. Collection of cauda epididymal and vesicular gland fluid

To obtain cauda epididymal and vesicular gland fluid, we selected ten other Santa Ines animals with similar phenotype at a local slaughterhouse. Right after slaughter, the entire epididymis was dissected from the testis and cauda epididymal fluid (CEF) was obtained by micro-perfusion with PBS (at 37 °C), followed by centrifugation at 700 × *g* for 10 min (at 4 °C) to separate fluid from sperm. The supernatant was further centrifuged at 10,000 × *g* for 60 min, at 4 °C, divided into aliquots and kept at −80 °C. Vesicular gland fluid (VGF) was collected by gentle massage of the vesicular glands right after animals were slaughtered. The fluid obtained as such was mixed (1:1) with PBS, centrifuged, aliquoted and stored as above.

2.4. Two-dimensional electrophoresis

Aliquots from seminal plasma, cauda epididymal and vesicular fluids were used to determine total protein concentration [13]. This protocol was run in triplicates and used bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA) as standards. Electrophoresis of seminal plasma, cauda epididymal and vesicular fluid proteins was carried out as described before [12], with modifications. In summary, one sample from each fluid, containing 400 μg of total protein, was mixed with re-hydration buffer (7 M urea, 2 M thiourea, 65 mM DTT (dithiothreitol), 0.5% free ampholytes (IPG buffer, 4–7), 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and traces of bromophenol blue) sufficient to make 250 μL. Then, samples were loaded in the reswelling tray, incubated with 13 cm IPG (immobilized pH gradient) strips (pH 4 to 7, linear; GE Lifesciences, Piscataway, NJ, USA), and allowed to rehydrate for 20 h. Isoelectric focusing was carried out in Ettan™ IPGphor 3™ apparatus (GE Lifesciences,

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Piscataway, NJ, USA) at 20 °C, according to the following program: 200 V (200 V h), 500 V (1000 V h), 5000 V (10,000 V h) and 10,000 V (22,000 V h), with a total of 33,200 V h. After focusing, IPG strips were incubated (15 min) in equilibration buffer I (6 M urea, 50 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 1% DTT) and re-equilibrated for additional 15 min in buffer II (similar to equilibration buffer I, but containing 2.5% iodoacetamide instead of DTT). After equilibration, strips were fixed with agarose (5% in SDS-PAGE running buffer) on the top of homogeneous SDS-PAGE gels (15%) and run at 250 V, with 30 mA per gel (Hoefer SE 600; GE Lifesciences, USA).

After all seminal plasma maps were analyzed, other aliquots of the same samples were subjected again to new 2-D electrophoresis runs. In these cases, each sample (containing 750 μg of total protein) was mixed with re-hydration buffer as before, and loaded in the reswelling tray with 18-cm IPG strips covering three different linear pH ranges (3.9–5.1; 4.7–5.9 and 5.5–6.7; GE Lifesciences, USA). Then, isoelectric focusing was carried according to the following program: 200 V (200 V h), 500 V (500Vh), 1000 V (600Vh), 5000 V (5000 V h) and 10,000 (60,000 V h), with a total of 60,325 V h. After electrofocusing, strips with seminal plasma proteins were subjected to the second dimension as detailed above, in homogeneous SDS-PAGE gels (15%), run at 300 V, with 40 mA per gel, using an Ettan™ DALTsix Large Vertical System (GE Lifesciences, USA).

2.5. Gel staining

All gels described in the present study were stained in colloidal Coomassie blue, adapting a protocol described by Candiano et al. [14]. In summary, after the second phase of the 2-D electrophoresis, gels were washed three times (20 min each) in a solution containing phosphoric acid (2%) and ethanol (30%). Then, three other washes in 2% phosphoric acid were followed and gels were then immersed for 72 h in a solution with phosphoric acid (2%), ethanol (18%) and ammonium sulfate (15%), added with 2 mL of a Coomassie blue G-250 solution (2%). Reagents used for electrophoresis and gel staining were purchased from Bio-Rad Laboratories (USA), GE Lifesciences (USA) and/or Sigma-Aldrich (USA).

2.6. Analysis of gel images

Two-dimensional gels were scanned at 300 dpi (ImageScanner II; GE Lifesciences, USA), saved as tagged image file format (.tiff) files and analyzed using PDQuest software, version 7.3.0 (Bio-Rad, USA). According to a strategy previously reported in detail [9,10,12,15,16], maps of seminal plasma, cauda epididymal and vesicular gland fluid were evaluated in separate match sets. In the analysis, each match set contained 10 gels from each sample type. We also analyzed in separate match sets each group of seminal plasma gels obtained with strips of narrow pH range (3.9–5.1; 4.7–5.9 and 5.5–6.7). For each match set, a unique master gel was generated based on a representative gel for each type of sample. In addition, spots consistently present in the remaining gels were added to the master, so that they could be matched to all samples. We used proteins in key regions of the master gel as landmarks and achieved the matching of spots after several rounds of extensive comparisons. Also, final spot matches were organized by checking each spot in each gel with the respective pattern in the master. Protein quantities were given as parts per million of the total integrated optical density of spots in the gels, according to PDQuest.

2.7. In-gel protein digestion

Proteins of interest separated by 2-D electrophoresis and marked as spots in the seminal plasma, CEF and VGF gels were destained and subjected to in-gel trypsin digestion [15,17]. Briefly, each spot was excised individually from 3 gels of each type of sample, cut into 1-mm^3 pieces and transferred to clean tubes. Dye and SDS were removed from the spots after three washes in 400 μL of a solution containing acetonitrile (50%) and ammonium bicarbonate (50%; 25 mM at pH 8.0). Gel pieces were then dehydrated after two washes with 200 μL of absolute acetonitrile for 5 min and air-dried at room temperature. We incubated the resulting material for 20 h (37 °C) with trypsin (166 ng/spot; Promega, cat. # v5111, Madison, WI, USA). Peptides were extracted from gel pieces by washing with 5% trifluoroacetic acid, 50% acetonitrile, in ammonium bicarbonate (50 mM) during 30 min, in three washes. Supernatants were transferred to micro tubes and vacuum concentrated (Eppendorf, Hauppauge, NY, USA) to a final volume of 10 μL. A piece of blank gel, without spots, and a piece of albumin from the molecular mass markers were submitted to the same procedure, and used as negative and positive controls, respectively.

2.8. Protein identification by matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI-ToF/ToF): spots from the seminal plasma

MALDI-ToF/ToF-MS acquisition was performed by an ABI 4700 Proteomics Analyzer (Applied Biosystems, Carlsbad, CA, USA) using 3.5-dimethoxy-4-hydroxycinnamic acid as matrix, as previously reported [17]. MS spectra were acquired in positive ion reflector mode with 1600 laser shots per spot, processed with default calibration and the six most intense ions submitted to fragmentation. PSD spectra were acquired with 2400 laser shots and 1 keV collision energy with CID off (1×18^{-8} Torr). Resulting uninterpreted tandem mass spectra were searched against the non-redundant protein sequence databases from the National Center for Biotechnology Information (NCBI) and Swissprot using the Mascot MS/MS ion search tool (version 2.1; www.matrixscience.com). The search parameters used included no restrictions on protein molecular mass, one tryptic missed cleavage allowed, non-fixed modifications of methionine (oxidation) and cysteine (carbamidomethylation). Peptide mass tolerance in the searches was 1.0 Da for MS spectra and 0.6 Da for MS/MS spectra. Peptides were considered to be identified when the scoring value exceeded the identity or extensive homology threshold value calculated by Mascot, based on the MOWSE score. In cases of protein identification based on a single peptide, the minimum threshold of the probability based Mascot score was 40. Mass spectra with lower scores, but presenting a reasonable tandem mass spectrum, were manually verified. The occurrence of false positives was determined by running searches using the same parameters against a decoy database. Peptide sequence coverage and domain distributions of the

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major proteins and major trains identified by mass spectrometry were graphically localized in the primary sequences of the proteins using the Caititu software [18].

2.9. Protein identification by electrospray ionization-quadrupole-time of flight (ESI-Q-ToF) mass spectrometry: spots from the seminal plasma, cauda epididymal and seminal vesicle fluids

For LC-MS/MS [19], the digested samples were injected using the nanoAcquity UPLC sample manager and the chromatographic separation was performed using a UPLC C18 column (75 μm × 10 cm) with a flow of 0.6 μL/min. The mass spectra were acquired in a Synapt G2 HDMS instrument (Waters Co., Milford, MA, USA) using a data dependent acquisition (DDA), where the three top peaks were subjected to MS/MS. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The gradient conditions used were as follows: 0 min with 3% of B, increasing linearly to 30% B in 20 min, then it increased up to 70% B in 40 min where it remained until 50 min and in the next minute it was decreased to 3% of B. The data were processed using Mascot Distiller (Matrix Science Ltd) and subjected to database search using Mascot Server 2.3 [20]. The searches were made with the assumption that there was a maximum of one missed trypsin cleavage and that peptides were mono-isotopic and using partially oxidized methionine residues, and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to ±0.1 Da, respectively, for MS/MS ion searching. However, candidate peptide IDs were only accepted if the m/z values were observed within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate ID, as determined when manually reviewing MASCOT search results.

2.10. Gene ontology analysis

Protein data from the protein lists of seminal plasma, cauda epididymal and vesicular gland fluids obtained after MASCOT search were analyzed using the software for researching annotations of proteins (STRAP), an open-source application [21]. Gene ontology terms for biological process and molecular function were obtained from UniProtKB and EBI GOA databases.

2.11. Western blots

Seminal plasma, cauda epididymal and vesicular gland fluid proteins, representing a pool from 3 animals, were separated by one dimensional SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL, GE Lifesciences, USA) using a TE 70 transfer unit (GE Lifesciences, USA) at 208 mA for 90 min. A total of 20 μg of protein were used for each transfer. Membranes were blocked overnight at 4 °C with PBS-T (50 mL of PBS with 0.5% Tween 20) containing BSA (5% w/v), under mild agitation, followed by a for 2-hour incubation with primary antibodies against bovine Binder of Sperm Protein (BSP) 1 (1:1000) and bodhesin 2 (1:500). Membranes were then washed three times in PBS-T and incubated with donkey anti-rabbit IgG coupled with alkaline phosphatase (Abcam, Cambridge, MA, USA) for another 2 h, washed again three times in PBS-T and rinsed twice with Tris–HCl (50 mM). Immunoreaction was visualized by exposing the membranes to a solution containing BCIP® (5-bromo-4-chloro-3-indolyl phosphate, 0.15 mg/mL), NBT (nitro blue tetrazolium, 0.30 mg/mL), Tris (100 mM) and $MgCl_2$ (5 mM), pH 9.5. Reaction was stopped by washing the membranes several times with milli-Q water. Controls consisted of use of only the primary or secondary antibodies with the sample and the use of a blocked membrane without sample exposed to both antibodies (data not shown). Antibodies against BSP 1 [22] were kindly provided by Dr. Puttaswamy Manjunath (Department of Medicine, University of Montréal, Canada). Binder of Sperm Proteins is expressed in bulls and rams, among other species. The bovine BSP (BSP 1) and its ovine homolog (RSVP 14) share a high degree of homology in amino acid sequence [23]. Polyclonal antibodies against bodhesin, commercially developed (Rhea Biotech, Campinas, SP, Brazil), were raised in rabbits against a peptide (SSNQPVSPFDIFYYERPSA) corresponding to the C-terminus sequence of bodhesin-2 (Bdh-2; gi: 121484235).

2.12. Immunocytochemistry

For immunocytochemistry, both ejaculated and cauda epididymal sperm samples, each containing 5×10^6 cells, were fixed in paraformaldehyde (2%) for 10 min and washed twice in PBS (700 *g*, 15 min, 4 °C), as previously described [24]. To block nonspecific sites, spermatozoa were next incubated for 2 h, under gentle agitation and at 4 °C, in PBS-Tween 20 containing 5% of bovine serum albumin — BSA (w/v; Sigma, USA). Sperm cells were then incubated with the primary antibodies in the same blocking solution for 2 h (under gentle agitation and 4 °C), using the following antibody concentrations: 1:300 for anti-BSP 1 and 1:200 for anti-bodhesin 2. After this first incubation, sperm were washed three times with PBS-Tween 20 (500 *g*, 5 min, 4 °C) and incubated with the secondary antibody, an Alexa 488-conjugated anti-rabbit IgG (1:300, Alexa Fluor 488 Goat Anti-Rabbit SFX kit (Life Technologies, Grand Island, NY, USA) for 1 h in a solution with PBS-Tween 20 and 1% BSA (w/v). Three additional washes followed this incubation (as described above) and smears were immediately prepared in a dark room, using an anti-fade reagent (Prolong Gold, Life Technologies, Grand Island, NY, USA), for confocal analysis. Antibodies used in this protocol were the same as described for the Western blots.

Images of sperm incubated with Alexa 488-conjugated antibodies were acquired by a laser scanning confocal microscope using an emission wavelength of 510 nm, and Zen™ software (LSM 710 Confocal Microscope, Carl Zeiss Inc., Thornwood, NY, USA). We obtained images as a series of sequential planes taken every 0.125 μm, with a total depth of 5 μm [24]. The image stacks were deconvoluted to improve the resolving power and to eliminate noises and out-of-focus blur, using Autoquant X2 (Media Cybernetics Inc., Silver Springs, MD, USA).

2.13. Isolation of total RNA from epididymis and seminal vesicles

We collected samples from animals at different development states (pre-puberty and adulthood) to verify the sites of ovine

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BSP and spermadhesin gene expression in the reproductive tract of the Santa Ines rams. Immediately postmortem, testis samples measuring approximately 1 cm^3 taken from the innermost region of the organ, samples from the cauda epididymis (as described by Fouchécourt et al. [25]) and vesicular glands were snap frozen in liquid N_2 and transported to the laboratory. Total RNA was isolated using approximately 100 mg of the collected tissues. For this procedure, Trizol™ Reagent (Life Technologies, USA) was used following manufacturer's recommendations. Briefly, the frozen tissue was macerated in 1 mL of the Trizol reagent, and the organic portion was separated from the aqueous phase by adding 200 μL of chloroform and centrifuged at 12,000 *g* for 15 min, at 4 °C. After centrifugation of this mixture, we added to the supernatant the same volume of 70% ethanol. Total RNA was purified from this supernatant using the column system PureLink™ RNA Mini Kit (Life Technologies, USA), also according to manufacturer's instructions. RNA preparations were subjected to DNase I treatment (PureLink™ DNase; Life Technologies, USA), and the RNA was washed three times with the provided Wash Buffer Solution II and then re-suspended in diethylpyrocarbonate 0.1% (DEPC)-treated water. RNA quality and concentration were determined in a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). We considered that one unit of absorbance at 260 nm corresponded to 40 μg/mL RNA.

2.14. *Reverse transcription-PCR*

Complementary DNA (cDNA) was synthesized using 1 μg of total RNA with SuperScript™ III Reverse Transcriptase (Life Technologies, USA) and PCR reactions were conducted in two steps. In the first one, 1 μg of RNA, 50 ng/mL of random hexamer primers, 10 mM dNTP mix and DEPC-treated water (for a total volume of 13 μL) were heated to 65 °C for 5 min and immediately placed on ice for at least 1 min. The second step consisted of adding to the first step solution: 200 U of SuperScript™ III RT, 10× RT Buffer, 0.1 M DTT and 40 U RNase out. Reverse transcription was performed at 25 °C for 5 min, 50 °C for 40 min and 70 °C for 15 min. The resulting cDNA was stored at −20 °C until later use.

Based on preliminary results, proteins identified as the Ram Seminal Vesicle Proteins (RSVP) with 14 and 22 kDa and bodhesins represented the most abundant components of the seminal plasma of the Santa Ines rams. RSVPs are Binder of Sperm Proteins of the ovine species and bodhesins belong to the spermadhesin family [23,26]. Thus, candidate genes were chosen on the basis of RSVP 14 and 22 kDa and bodhesins 1 and 2. Gene sequences were obtained from the UniGene database (NCBI). The primers were designed according to the published *Ovis aries* (RSVPs 14 and 22) and *Capra hircus* (bodhesins 1 and 2) mRNA sequences available at the UniGene, using the online, free access program Primer 3 (for sequences of primers, see Table S1).

Quantitative real-time PCR (qPCR) was carried out on an iQ5 cycler (Bio-Rad, USA). Reactions were performed in 20 μL with 1 μg of each cDNA, 1× Power SYBR™ Green (Life Technologies, USA) PCR Master Mix, 0.4 μM of both sense and antisense primers and ultra-pure water completing the reaction volume. The qPCR protocol included a first denaturation at 95 °C for 10 min, then 40 PCR cycles (15 s 95 °C, 1 min 60 °C, 1 min 72 °C), followed by a final extension of 10 min at 72 °C. The specificity of each primer set was tested by generating a melting curve, carried out between 60 and 95 °C for all sequences, starting fluorescence acquisition at 60 °C and taking measurements at 10-second intervals until the temperature reached 95 °C. GAPDH was used as reference gene to normalize the sequences assayed. All samples were run in triplicates and qPCRs were repeated at least twice. As negative controls, samples with reverse transcriptase but without cDNA were used. Relative gene expression was estimated using the ΔΔCt-method [27]. Threshold and Ct (cycle threshold) values were automatically determined by Bio-Rad iQ5 software, using internal parameters, and Ct and Tm (melting temperature).

3. Results

3.1. *Seminal plasma proteins*

Semen samples of the Santa Ines rams used on the present study contained, on average, $3.6 \pm 0.4 \times 10^9$ spermatozoa/mL and $5.4 \pm 0.3 \times 10^9$ total sperm/ejaculate, with 90 ± 0.2% of motile cells and 75 ± 0.3% of sperm with progressive motility. On average, 302 ± 16 spots were detected by PDQuest software in the match set of ten seminal plasma gels constructed within the 4–7 pH range. Based on the master gel image (Fig. 1A), which included proteins of the reference gel (Fig. 1B) and spots from additional members of the match set, 143 spots consistently appeared in every gel and intensities of these spots corresponded to 65.5 ± 1.5% of the intensities of all spots depicted in the seminal plasma maps. Seventy three spots were identified by mass spectrometry, corresponding to 41 different proteins (Table 1). Combined, intensities of the identified spots accounted for 42.5 ± 0.9% of the intensities of all spots and 65.1% of the intensities of spots presented in every protein map.

Two spots were identified as Ram Seminal Vesicle Protein 14 kDa — RSVP 14 (spots #66 and #67) and eleven others as Ram Seminal Vesicle Protein 22 kDa — RSVP 22 (spots #17, 18, 51, 52, 53, 55, 56, 60, 61, 62 and 70; Fig. 1A; Table 1). RSVP spots corresponded to 20.3% of the total intensity of all spots detected in the gels. Six other spots showed homology to isoforms of either bodhesin 1 (spot #71) or bodhesin 2 (spots #57, 63, 64, 68 and 69) and the intensities of such components represented 11.4% of all spot intensities quantified in the ram seminal plasma gels. The 19 spots recognized as RSVPs and bodhesins numerically represented only 6% of all spots detected in the PDQuest match set, but their combined optical densities corresponded to 31.8% of the total spot intensities estimated in the protein maps. Some of the proteins identified in the 2-D gels were present as multiple isoforms, including matrix metalloproteinase 2 (train 1), albumin (train 2), lactotransferrin (train 3) and clusterin (train 4; Fig. 1B).

Conserved domains and other structural features of selected proteins described in Fig. 1 and Table 1 are graphically represented using the Caititu software (Fig. S1). The sequence of trypsin-digested peptides obtained from RSVP 14 spots matched to both fibronectin type II (FNII) domains of the full protein. Tryptic peptides from five RSVP 22 spots had one sequence matching to each of the FNII

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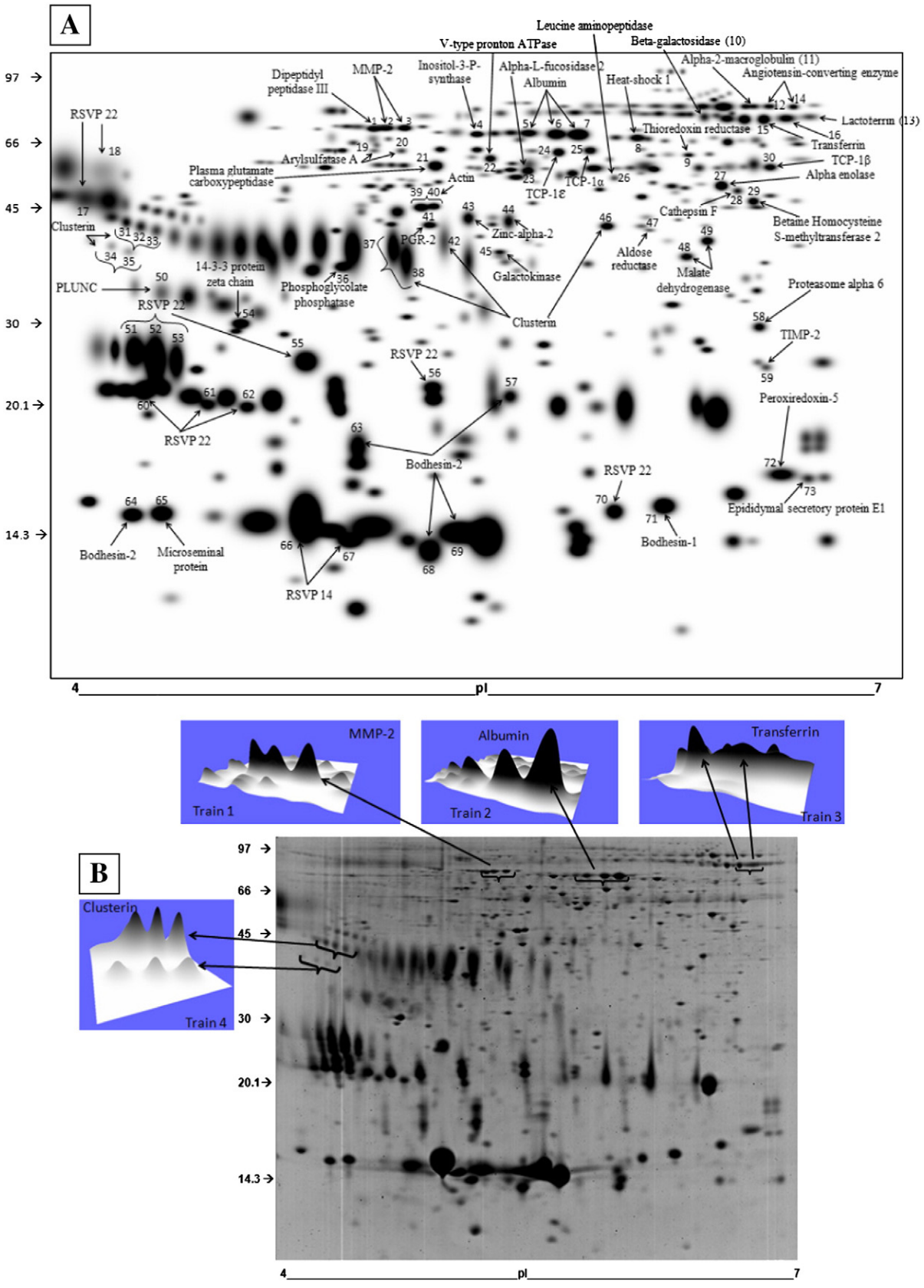

Fig. 1 – Two-dimensional map of seminal plasma proteins from Santa Ines rams. (A) represents the master gel generated by PDQuest software (Bio Rad, USA), based on a match set with all gels used in the study. (B) corresponds to the reference map (from ram #3) from which the master was mostly generated. Proteins were stained with Coomassie blue and identified by mass spectrometry (MALDI-ToF/ToF and ESI-Q-ToF). Spot numbers refer to those shown in Table 1.

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Table 1 – Proteins of the seminal plasma of Santa Ines rams identified by two-dimensional electrophoresis and mass spectrometry (MALDI-ToF/ToF and ESI-Q-ToF). Table includes spots detected in 13-cm gels, within the 4–7 pH range. Spot numbers refer to those in Fig. 1. Full table showing sequences of matched peptides, ion scores, m/z and z values is shown as Table S2.

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Dipeptidyl peptidase III				
Spot 01	73.0/5.2	296471603	202	19
Matrix metalloproteinase 2				
Spot 01	73.0/5.2	27807447	59	9
Spot 02	73.0/5.2	27807447	179	10
Spot 03	72.7/5.3	27807447	111	7
Inositol-3-P-synthase 1				
Spot 04	71.3/5.6	114051253	221	14
Serum albumin				
Spot 05	71.3/6.0	57164373	259	9
Spot 06	71.6/5.9	57164373	268	11
Spot 07	71.6/5.8	57164373	273	11
Heat shock 70 kDa protein 1-like				
Spot 08	70.7/6.2	268607682	327	12
Thioredoxin reductase				
Spot 09	65.6/6.4	27807129	207	14
Beta-galactosidase				
Spot 10	76.1/6.5	78042544	48	4
Alpha-2-macroglobulin				
Spot 11	80.0/6.6	157954061	76	1
14-3-3 protein zeta chain				
Spot 12	80.0/6.7	112687	62	5
Spot 54	29.3/4.7	253706	233	22
Lactoferrin				
Spot 13	76.6/6.9	32130549	62	1
Angiotensin-I converting enzyme				
Spot 14	79.9/6.8	66710717	166	10
Spot 74	79.9/6.9	66710717	92	4
Spot 75	79.9/7.0	331284213	52	2
Transferrin				
Spot 15	73.8/6.7	114053269	51	3
Spot 16	73.8/6.6	114053269	58	5
Ram Seminal Vesicle 22 kDa Protein				
Spot 17	54.9/4.1	219521812	67	15%
Spot 18	61.4/4.2	219521812	146	20%
Spot 51	29.4/4.3	219521812	68	15%
Spot 52	28.4/4.4	219521812	161	20%
Spot 53	28.2/4.5	219521812	176	20%
Spot 55	28.2/5.0	219521812	52	12%
Spot 60	25.0/4.3	219521812	145	20%
Spot 61	23.6/4.6	219521812	157	26%
Spot 62	23.4/4.8	219521812	167	20%
Spot 56	25.4/5.5	219521812	200	20%
Spot 70	15.3/6.1	219521812	93	20%
Arylsulfatase A				
Spot 19	66.7/5.2	47522624	78	4
Spot 20	66.7/5.3	115497982	36	9
Plasma glutamate carboxypeptidase precursor				
Spot 21	62.5/5.4	115495837	116	11
V-type proton ATPase subunit B				
Spot 22	62.5/5.7	297129	115	11
Spot 75	62.5/5.8	27806225	36	7
Alpha-L-fucosidase 2				
Spot 23	61.7/5.8	76626175	65	12
Chaperonin containing TCP-1 subunit ε				
Spot 24	67.7/6.0	74353962	66	8
Chaperonin containing TCP-1 subunit α				
Spot 25	67.6/5.9	57032236	122	5
Leucine aminopeptidase				
Spot 26	63.0/6.1	410689	183	12
Alpha enolase				
Spot 27	42.2/6.4	4927286	184	12
Cathepsin F				
Spot 28	56.6/6.5	115495381	104	10
Betaine-homocysteine S-methyltransferase 2				
Spot 29	40.0/6.4	60687508	47	4
Chaperonin containing TCP-1 subunit β				
Spot 30	60.5/6.6	468546	90	15
Clusterin				
Spot 31	49.7/4.3	27806907	114	7
Spot 32	48.9/4.4	27806907	247	12
Spot 33	48.1/4.5	27806907	144	7
Spot 34	45.2/4.2	27806907	51	5
Spot 35	44.2/4.3	27806907	319	19
Spot 37	43.4/5.2	27806907	450	16
Spot 38	42.5/5.3	27806907	37	2
Spot 42	41.3/5.4	27806907	64	3
Spot 46	46.5/6.1	27806907	67	6
Spot 76	48.7/6.1	27806907	225	11
Phosphoglycolate phosphatase				
Spot 36	41.5/5.1	84000329	167	13
Actin				
Spot 39	51.1/5.4	8809716	94	15
Spot 40	51.2/5.4	8809716	85	15
Prostaglandin reductase 2				
Spot 41	49.1/5.4	115497482	133	12
Zinc-alpha-2-glycoprotein				
Spot 43	47.8/5.5	77735615	95	8
Spot 77	49.9/5.7	77735615	52	8
Galactokinase 1				
Spot 45	41.3/5.7	95767475	78	4
Aldose reductase				
Spot 47	48.1/6.2	113594	56	13
Malate dehydrogenase				
Spot 48	40.0/6.4	77736203	86	6
Spot 49	41.2/6.4	77736203	129	24
Palate, lung and nasal epithelium clone				
Spot 50	32.9/4.4	27806071	102	7
Bodhesin-2				
Spot 57	24.4/5.7	121484235	217	18
Spot 63	19.2/5.2	121484235	87	18
Spot 64	15.1/4.3	121484235	34	18
Spot 68	13.2/5.5	121484235	162	27
Spot 69	14.1/5.6	121484235	57	18
Proteasome alpha 6 subunit				
Spot 58	28.8/6.6	6755198	78	11
Tissue inhibitor metallopeptidase (TIMP-2)				
Spot 59	25.4/6.6	75832065	190	13
Microseminoprotein				
Spot 65	15.2/4.5	76656898	55	9
Spot 77	15.2/4.5	76656898	155	26
Ram Seminal Vesicle 14 kDa Protein				
Spot 66	14.9/5.0	219521810	215	48
Spot 67	13.7/5.2	219521810	179	34
Bodhesin				
Spot 71	16.3/6.3	77864607	73	9

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Table 1 (*continued*)

Protein	Experimental[a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Peroxiredoxin 5				
Spot 72	17.8/6.7	74354725	158	22
Epididymal secretory protein E1				
Spot 73	17.5/6.9	27806881	150	31

[a] Experimental values were deduced from the respective 2D map by the PDQuest software.

domains; five other spots were found with one sequence belonging to FNII 1 domain and two sequences to the FNII 2 domain. One RSVP 22 spot had sequences matching only the FNII 2 domain. The sequence of trypsin-digested peptides from Bdh-1 spot matched to one single region of the CUB domain represented in the full protein. Peptides from three Bdh-2 spots also matched to only one region of the same domain of the protein, while one Bdh-2 spot had peptide sequences equivalent to two different regions of this domain. Angiotensin-converting enzyme sequences all matched to the main peptidase M2 domains, which comprise most of the protein. Sequences of the seven clusterin spots were found inserted in the beta chain and only one spot also in the alpha chain. Peptide sequences obtained after tryptic digestion of MMP-2 spots (#2 and 3) were part of the zinc-dependent metalloproteinase domain but only one of these spots (#3) had its sequence in the fibronectin type II and HX domains.

Electrofocusing seminal plasma proteins within pH ranges of 3.9–5.1, 4.7–5.9 and 5.5–6.7 allowed the detection of 104±8, 159±7 and 81±5 spots in the 2-D gels (Fig. 2B–D). In the 3.9–5.1 pH range, there were 45 spots identified by mass spectrometry, corresponding to 15 different proteins. From these, seven proteins were not detected in the original map constructed within the 4–7 pH range (glucose–fructose oxidoreductase domain-containing protein 2, proteasome subunit alpha 5, lysozyme, gelsolin, alpha-2-HS glycoprotein, ubiquitin carboxyl-terminal hydrolase isoenzyme L3 and alpha-1-antitrypsin). For the 4.7–5.9 pH interval, there were 76 spots identified, respective to 23 proteins, including seven that had not been detected before in the original seminal plasma gels within the 4–7 pH range:

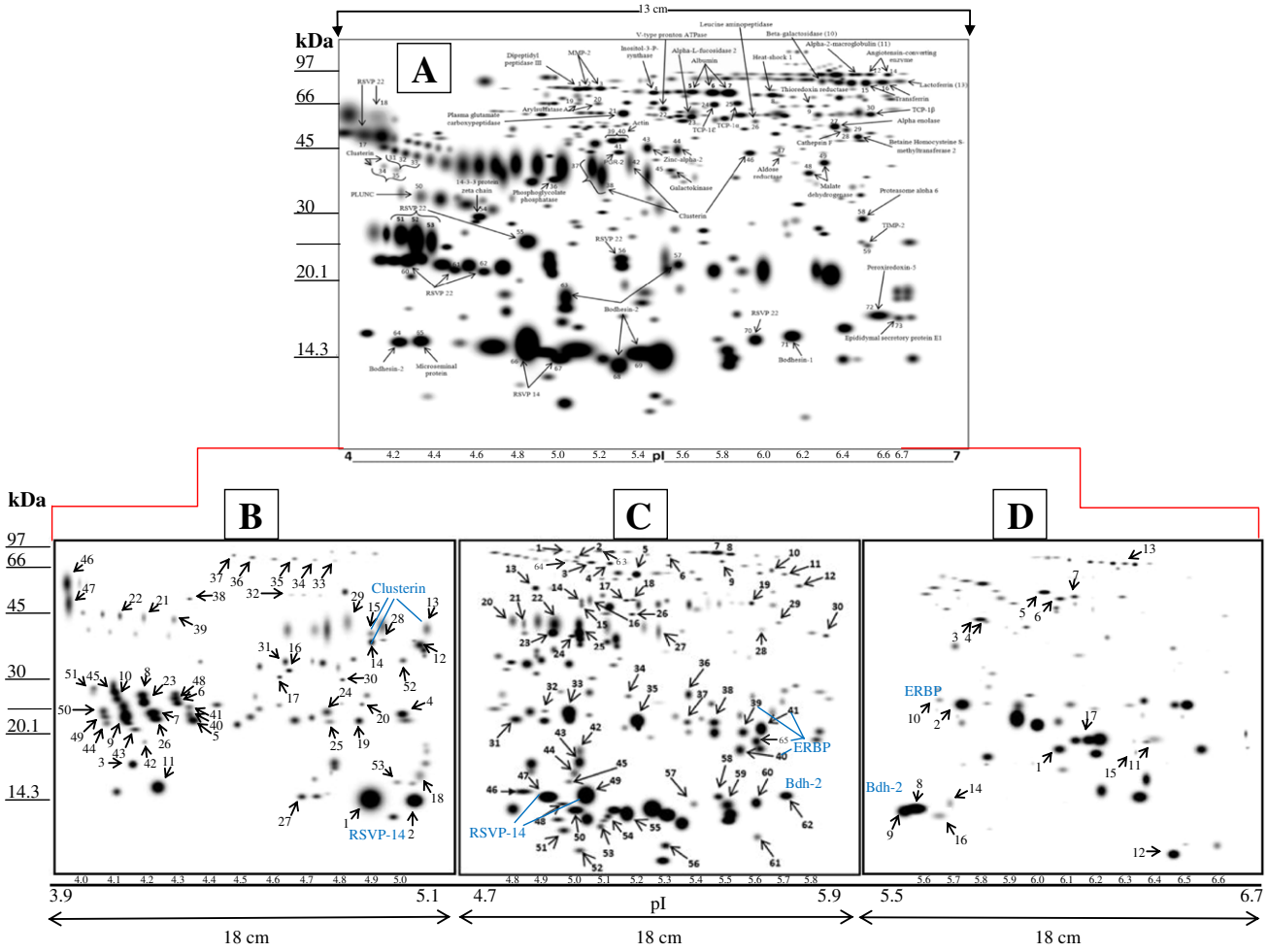

Fig. 2 – Two-dimensional map of seminal plasma proteins from Santa Ines rams. (A) represents the master gel generated by PDQuest software (Bio Rad, USA), based on a match set with all 13-cm gels, within the 4–7 pH interval. (B), (C) and (D) correspond to the master gels based on match sets constructed with 18-cm gels, within three different pH intervals (3.9–5.1, 4.7–5.9 and 5.5–6.7). Proteins were stained with Coomassie blue and identified by tandem mass spectrometry. Spots numbers refer to those shown in Table 2.

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alpha-N-acetylgalactosaminidase, aldose-1-epimarase, gelsolin, apolipoprotein A1, phosphoglycerate kinase 1, glutamate carboxypeptidase and prostaglandin-H2 D-isomerase. As regard to the 5.5–6.7 interval, 14 spots were identified, which corresponded to 14 proteins. From this list, six had not appeared in the first set of gels: superoxide dismutase, phosphoglycerate kinase 1, sperm acrosome membrane-associated protein 3, epididymis-specific ERBP-like protein, polyubiquitin and alpha-2-HS glycoprotein (Table 2). Spots were highlighted in the overlapping regions of the gels with narrow pH range. As shown, spots identified as clusterin and RSVP 14 in the 3.9–5.1 pH interval in the first gel (Fig. 2B) also appear in the 4.7–5.1 interval of the second gel (Fig. 2C). Similarly, ERBP and bodhesin-2 spots identified in the 5.5–5.8 pH interval of this gel (Fig. 2C) were also present in the third map (Fig. 2D), in the same pH range.

3.2. *Proteins of the cauda epididymal and vesicular gland fluid*

On average, we detected 113±7 spots per gel of cauda epididymal fluid (Fig. 3A) and 62 spots were consistently present on all maps. Combined, these spots represented 46.9±3.2% of the intensities of all spots detected in the 2-D maps. Twelve different proteins were identified and the most abundant proteins in these gels appeared as albumin and transferrin. These proteins were present as trains of multiple isoforms and together corresponded to 27.3% of all spot intensities. Other proteins identified include clusterin, alpha-1-anti-trypsin, prostaglandin D synthase, alpha-2-HS glycoprotein and actin (Table 3).

Concerning the vesicular gland fluid (VGF), 215±12 spots were detected per gel (Fig. 3B) and 104 spots consistently expressed in all maps. The combined intensity of these spots represented 38.0±2.2% of the intensities of all spots detected in the VGF maps. Nineteen different proteins were identified by tandem mass spectrometry in the VGF maps, being the most abundant the ones identified as RSVPs 14 and 22, bodhesin 2 and albumin. These proteins were present as trains of multiple isoforms and their combined intensities corresponded to 38.4% of all spot intensities. Additional proteins identified in the VGF include cystatin B, apolipoprotein A1, clusterin, disulfide isomerase, aldose reductase, alpha-enolase and ERP29 (Table 3).

Western blots confirmed that antibodies against BSP 1 reacted with a single band around 14 kDa in 1-D gels with seminal plasma, vesicular gland and cauda epididymal fluid. However, intensity of staining was much more pronounced in the first two samples as compared to the cauda epididymal secretion. In the case of Bdh-2, antibody reaction was evident for seminal plasma and vesicular fluid but weak in the cauda epididymal fluid (Fig. 4).

3.3. *Interactions of anti-BSP 1 and anti-Bdh 2 with ejaculated and cauda epididymal sperm*

Antibodies against BSP 1 were detected on the acrosome, equatorial segment and midpiece of ejaculated sperm with intact acrosome (Fig. 5A). On sperm with reacted acrosome, anti-BSP 1 binding was restricted to the equatorial and midpiece region. Epididymal sperm were completely absent of immunofluorescence for BSP 1 antibody. As regard to anti-Bdh 2, reaction

Table 2 – Proteins of the seminal plasma of Santa Ines rams identified by two-dimensional electrophoresis and mass spectrometry (MALDI-ToF/ToF and ESI-Q-ToF). Table includes spots detected in 18-cm gels, obtained within the 3.9–5.1, 4.7–5.9 and 5.5–6.7 pH intervals. Spot numbers refer to those in Fig. 2. Full table showing sequences of matched peptides, ion scores, m/z and z values is shown as Table S3.

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
pH range: 3.9–5.1				
Ram Seminal Vesicle 14 kDa Protein				
Spot 01	15.2/4.8	219521810	945	70
Spot 02	15.4/5.0	219521810	47	17
Glucose-fructose oxidoreductase domain-containing protein 2				
Spot 03	18.9/4.1	62460418	41	2
Ram Seminal Vesicle 22 kDa Protein				
Spot 04	24.9/4.9	219521812	143	36
Spot 05	22.8/4.4	219521812	131	20
Spot 06	26.7/4.3	219521812	188	20
Spot 07	25.0/4.2	219521812	131	20
Spot 08	28.3/4.2	219521812	176	20
Spot 09	25.1/4.1	219521812	131	20
Spot 10	28.0/4.1	219521812	118	20
Spot 23	27.0/4.2	219521812	172	24
Spot 26	22.9/4.4	219521812	115	20
Spot 45	30.2/4.1	219521812	183	24
Microseminoprotein				
Spot 11	15.3/4.4	296472032	62	50
Phosphoglycolate phosphatase				
Spot 12	41.6/5.1	84000329	254	17
Spot 28	42.2/4.9	84000329	71	8
Clusterin				
Spot 13	43.5/5.1	27806907	569	18
Spot 14	42.0/4.9	27806907	108	4
Spot 15	42.6/4.9	27806907	54	7
Spot 21	44.6/4.2	27806907	175	8
Spot 22	44.8/4.1	27806907	155	12
Spot 29	44.6/4.8	27806907	341	13
14-3-3 protein zeta chain				
Spot 16	29.5/4.7	253706	409	22
Proteasome subunit alpha 5				
Spot 17	28.4/4.6	296489341	187	34
Bodhesin 2				
Spot 18	17.6/5.0	121484235	101	18
Lysozyme				
Spot 19	23.0/4.8	112030802	94	12
Proteasome subunit alpha 6				
Spot 20	26.8/4.8	77735687	170	12
Gelsolin				
Spot 24	27.1/4.8	327346104	54	2
Spot 25	25.8/4.8	327346104	63	2
Alpha-2-HS-glycoprotein				
Spot 27	15.4/4.7	27806751	47	6
Spot 28	42.2/4.9	27806751	138	12
Spot 31	33.2/4.7	27806751	108	12
Spot 32	33.2/4.7	27806751	125	12
Spot 33	69.5/4.8	27806751	126	12
Spot 34	69.5/4.7	27806751	100	12
Spot 36	69.5/4.6	27806751	99	12
Spot 37	69.5/4.6	27806751	82	12
Spot 38	31.8/4.3	27806751	124	12
Spot 39	44.8/4.3	27806751	112	12
Spot 40	25.3/4.4	27806751	116	12
Spot 41	25.9/4.4	27806751	105	12

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Table 2 (*continued*)

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Alpha-2-HS-glycoprotein				
Spot 42	18.9/4.2	27806751	107	12
Spot 43	20.6/4.1	27806751	58	5
Spot 44	21.4/4.0	27806751	114	12
Spot 48	28.0/4.3	27806751	56	5
Spot 49	23.1/4.0	27806751	50	5
Spot 50	24.2/4.0	27806751	43	5
Spot 51	29.8/4.0	27806751	45	5
Spot 52	33.4/4.9	27806751	117	12
Spot 53	15.8/4.9	27806751	63	5
Ubiquitin carboxyl-terminal hydrolase isozyme L3				
Spot 30	28.5/4.8	94966875	199	24
Alpha-1-antitrypsin				
Spot 35	69.5/4.6	57526646	190	12
Transcobalamin-1-like				
Spot 46	65.4/4.0	345327379	56	4
Spot 47	52.6/4.0	345327379	55	4
pH range: 4.7–5.9				
Matrix metalloproteinase 2				
Spot 01	71.8/4.9	261244994	162	33
Spot 02	69.3/5.0	261244994	38	4
Serum albumin				
Spot 02	69.3/5.0	57164373	56	20
Spot 03	67.1/5.0	57164373	79	1
Spot 04	66.6/5.1	57164373	280	36
Spot 07	71.3/5.5	57164373	63	1
Spot 08	70.7/5.5	57164373	602	51
Spot 28	43.8/5.7	57164373	66	1
Spot 52	10.8/4.9	57164373	115	1
Spot 57	14.5/5.5	57164373	89	2
Spot 62	15.3/5.8	57164373	40	4
Cathepsin B				
Spot 06	70.2/5.4	27806671	51	9
Clusterin				
Spot 09	67.5/55	27806907	37	2
Spot 20	46.3/4.7	27806907	32	10
Spot 21	45.5/4.8	27806907	95	19
Spot 22	45.4/4.9	27806907	79	4
Spot 23	42.9/4.9	27806907	285	18
Spot 24	42.1/4.9	27806907	67	15
Spot 25	42.9/4.9	27806907	347	27
Spot 27	43.2/5.3	27806907	66	15
Spot 64	67.7/5.1	27806907	68	13
Lactoferrin				
Spot 10	63.9/5.7	254656113	59	1
Spot 11	62.3/5.7	254656113	194	16
Spot 12	57.8/5.8	254656113	65	1
Alpha-2-HS-glycoprotein				
Spot 13	57.0/4.8	57526674	88	5
Arylsulfatase A				
Spot 14	52.5/5.0	115497982	68	7
Spot 15	51.0/5.0	115497982	74	2
Plasma glutamate carboxypeptidase				
Spot 05	62.2/5.2	115495837	376	32
Spot 16	52.8/5.0	115495837	117	11
Spot 37	24.4/5.4	115495837	47	9
Spot 44	17.6/4.9	115495837	134	16
Actin				
Spot 17	52.8/5.2	8809716	152	15
Spot 18	52.9/5.2	8809716	175	15
Leucine aminopeptidase 3				
Spot 19	51.8/5.6	88682884	112	31

Table 2 (*continued*)

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Prostaglandin reductase 2				
Spot 26	48.3/5.2	115497482	69	32
Aldose-1-epimerase				
Spot 28	43.8/5.7	77736588	100	21
Alpha-N-acetylgalactosaminidase				
Spot 29	45.8/5.7	187607477	46	2
Enolase alpha				
Spot 30	42.2/5.9	4927286	50	20
Ram Seminal Vesicle 22 kDa Protein				
Spot 31	24.8/4.8	219521812	54	20
Spot 33	25.9/4.9	219521812	127	20
Spot 34	28.8/5.2	219521812	21	20
Spot 35	24.5/5.2	219521812	278	35
Spot 51	12.3/4.9	219521812	49	20
Spot 60	14.6/5.6	219521812	50	20
Gelsolin				
Spot 32	26.1/4.8	327346104	44	2
Prostaglandin D synthase				
Spot 36	29.1/5.4	57164293	96	14
Bodhesin 2				
Spot 37	24.4/5.4	121484235	55	35
Spot 38	24.3/5.5	121484235	214	35
Spot 39	22.9/5.6	121484235	25	18
Spot 42	20.3/4.9	121484235	90	35
Spot 43	18.9/4.9	121484235	108	18
Spot 44	17.6/4.9	121484235	100	18
Spot 45	16.7/4.9	121484235	64	18
Spot 47	15.2/4.8	121484235	124	27
Spot 48	14.5/4.9	121484235	147	35
Spot 50	14.0/4.9	121484235	90	18
Spot 53	12.6/5.1	121484235	36	27
Spot 54	13.4/5.1	121484235	86	18
Spot 59	14.4/5.5	121484235	79	18
Spot 60	14.6/5.6	121484235	45	18
Epididymis-specific ERBP-like protein				
Spot 39	22.9/5.6	296481974	57	4
Spot 40	20.4/5.6	296481974	51	4
Spot 41	23.4/5.6	296481974	58	4
Spot 65	21.7/5.6	296481974	98	4
Ram Seminal Vesicle 14 kDa Protein				
Spot 45	16.7/4.9	219521810	20	17
Spot 46	15.7/4.8	219521810	177	30
Spot 47	15.2/4.8	219521810	1246	70
Spot 49	15.4/5.0	219521810	214	49
Spot 55	13.7/5.2	219521810	149	49
Phosphoglycerate kinase 1				
Spot 44	17.6/4.9	215983082	102	22
Spot 56	11.1/5.4	215983082	67	8
Apolipoprotein A1				
Spot 44	17.6/4.9	162678	50	4
Spot 52	10.8/4.9	162678	490	8
Spot 55	13.7/5.2	162678	372	4
Spot 57	14.5/5.5	162678	175	4
Spot 58	20.4/5.6	162678	46	8
Spot 61	11.8/5.6	162678	409	8
Spot 63	72.0/5.1	162678	772	21
Bodhesin				
Spot 61	11.8/5.6	77864607	64	17
pH range: 5.5–6.7				
Superoxide dismutase [Cu–Zn]				
Spot 01	20.6/6.1	223633904	100	21

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Table 2 (*continued*)

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Ram Seminal Vesicle 14 kDa Protein				
Spot 02	20.6/5.7	219521810	61	17
Galactokinase 1				
Spot 03	42.5/5.8	150247075	41	2
Phosphoglycerate kinase 1				
Spot 04	41.8/5.8	215983082	183	12
Alpha enolase				
Spot 05	46.2/6.1	87196501	85	4
Cathepsin F				
Spot 06	45.4/6.2	115495381	211	12
Betaine-homocysteine S-methyltransferase 2				
Spot 07	45.9/6.2	60687508	119	6
Bodhesin 2				
Spot 08	14.6/5.6	121484235	191	35
Sperm acrosome membrane-associated protein 3				
Spot 09	14.4/5.6	157279923	60	6
Epididymis-specific ERBP-like protein				
Spot 10	21.1/5.7	297460193	62	4
Peroxiredoxin 5				
Spot 11	20.1/5.6	74354725	115	10
Spot 15	20.4/6.4	74354725	118	10
Polyubiquitin				
Spot 12	11.4/6.5	163575	74	15
Alpha-2-macroglobulin				
Spot 13	80.3/6.4	157954061	79	1
Alpha-2-HS-glycoprotein				
Spot 14	15.4/4.7	27806751	47	6
Spot 16	14.2/5.7	27806751	53	5
Spot 17	18.7/6.2	27806751	35	5

[a] Experimental values were deduced from the respective 2D maps by the PDQuest software.

was detected on ejaculated sperm and immunostaining was not evident on epididymal sperm (Fig. 5B).

3.4. *Quantitative expression of genes related to the major seminal plasma proteins: RSVPs 14 and 22 and bodhesins 1 and 2*

Expression of both RSVP 14 and RSVP 22 genes, as evaluated by relative abundance of their mRNA, was mainly detected in the vesicular glands (VES) of Santa Ines rams at both prepuberty and yearling age. RSVP 14 and 22 transcripts were also present in the cauda epididymis (CAU) but at much lower magnitude (Fig. 6). Transcripts related to bodhesin 1 and 2 genes were also detected in samples from the cauda epididymis and vesicular glands, but with much greater magnitude in the latter (Fig. 6).

3.5. *Gene ontology*

Gene ontology (GO) terms for biological process, molecular function and cellular component associated with proteins of the seminal plasma, cauda epididymal and vesicular gland fluid is presented in Fig. 7. The most prominent biological processes linked to seminal plasma, CEF and VGF were binding and catalytic activity. Enzyme regulator activity was also described for components of seminal plasma, CEF and VGF, while antioxidant activity was associated with proteins of the seminal plasma and VGF. Top three molecular functions of seminal plasma and CEF were characterized as cellular process, metabolic process and regulation. Based on the analysis of gene symbols, major molecular functions of VGF proteins were termed as regulation, cellular process and localization. Proteins of the seminal plasma, CEF and VGF were mainly defined as extracellular components, according cellular component localization. Cytoplasmic components were also annotated for all fluids and membrane proteins, for the seminal plasma and VGF.

4. Discussion

The present study characterizes a diverse cohort of seminal plasma proteins from reproductively normal Santa Ines rams as well as the main components of the cauda epididymal and vesicular gland fluids. The Santa Ines evolved from local herds in a semi-arid region of the Brazilian Northeast and has become the most common hair sheep raised in Brazil. Rams of such breed represent a unique genotype because of their adaptability to both the tropical environment and low external input production systems.

From seminal plasma maps obtained within the 4–7 pH range, we identified 73 spots and 41 different proteins, equivalent to 42.5% of all spot intensities detected in the gels. From the core of 143 spots present in all maps, we identified 49 spots, also representing the majority of the intensities of all spots. The number of spots in the gels with narrow pH intervals was greater (343 spots) than the number obtained with gels within the 4–7 pH range (302 spots). However, such comparisons need to consider that gels with the 4–7 pH and narrow pH intervals were constructed using different strip sizes (13 and 18 cm) and protein amounts (400 and 750 μg, respectively). Thus, compensations in protein loads and pH ranges obviously influenced the outcome of the 2-D-PAGE. Regardless of spot counts, the use of narrow gels allowed the identification of 20 proteins that had not been detected in the original gels.

Spots identified as Ram Seminal Vesicle Proteins 14 and 22 kDa (RSVPs) and bodhesins 1 and 2 represented the major components of the seminal plasma gels constructed within the 4–7 pH range. RSVPs were the single most abundant protein group, contributing with 20.4% of the intensity of all spots detected in those maps. RSVPs belong to the family of Binder of Sperm Proteins, which are characterized by two tandemly-arranged, fibronectin type II domains [28]. Graphic representation of the trypsin-digested RSVP showed that RSVP 14 and RSVP 22 spots had peptides in at least one of the FNII domains. RSVPs 14 and 22 also appeared as the most abundant proteins in the vesicular gland fluid 2-D maps. We confirmed this fact by showing that antibodies against BSP 1 reacted with components of the vesicular gland fluid but elicited only a very weak reaction with cauda epididymal fluid. Studies conducted in the bovine [29], goat [30] and bison [31] similarly showed that BSPs are the major seminal plasma proteins and of vesicular gland origin. Both RSVP 14 and 22 genes were also mainly expressed in the vesicular glands of the Santa Ines rams. Also, very low but consistent expression

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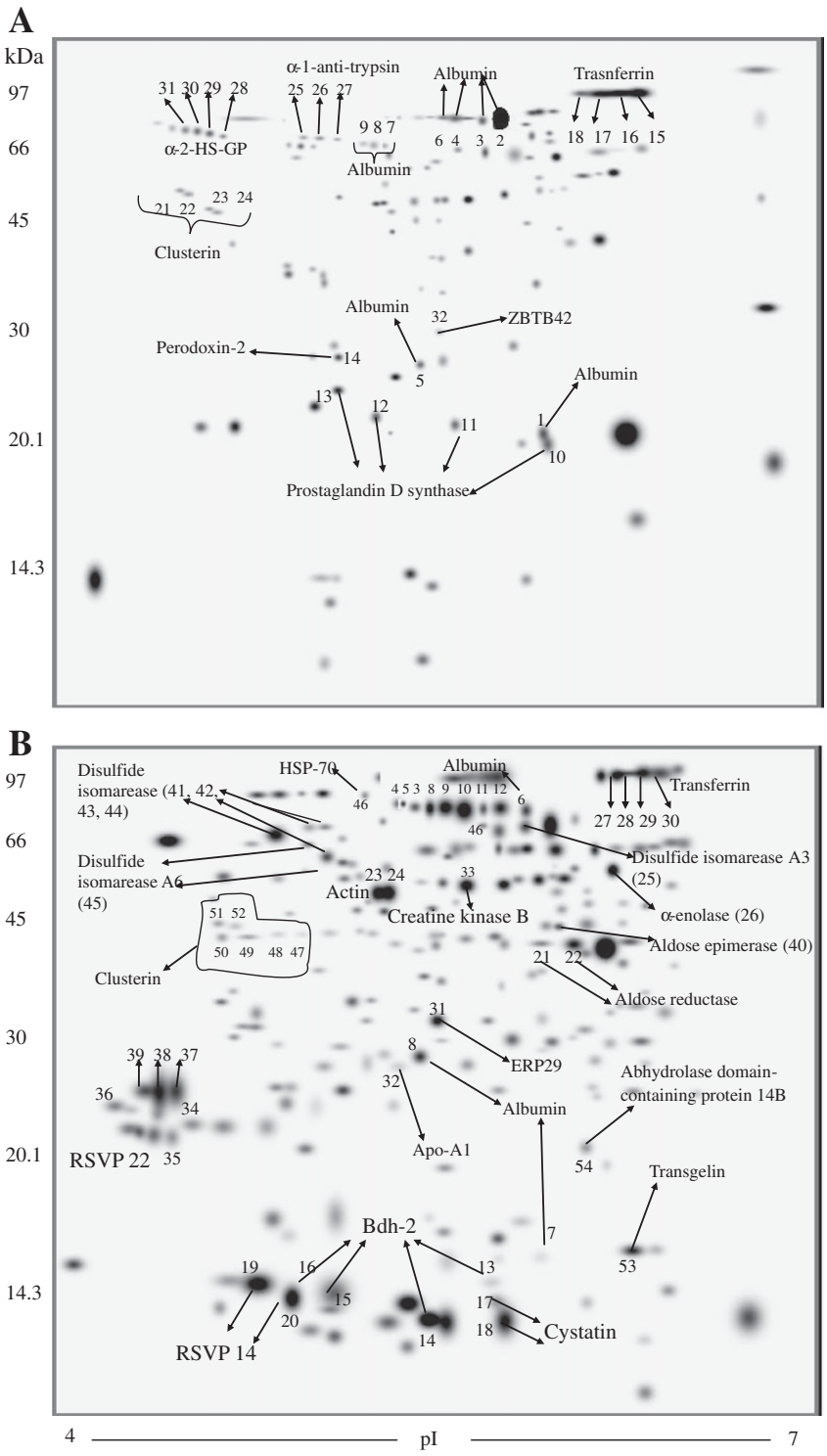

Fig. 3 – Two-dimensional map of cauda epididymis (A) and vesicular gland (B) fluid proteins from Santa Ines rams. In both cases, the figures represent the master gel generated by PDQuest software (Bio Rad, USA), based on a match set with all gels used in each set of samples. Proteins were stained with Coomassie blue and identified by tandem mass spectrometry. Spot numbers refer to those shown in Table 3.

of those genes was detected in the cauda epididymis, in accordance with the results from Western blots. Finding that RSVPs are present in the cauda epididymis confirms our previous data showing that RSVP 14 mRNA was detected in the epididymis of post-pubertal Morada Nova rams, also in lower magnitude as compared to the expression in the seminal vesicles [32]. Morada Nova is another breed of Brazilian hair sheep. Low amounts of BSP homologs have been described in the epididymis of humans and mice [33], similar to what we presently report for Santa Ines rams.

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Table 3 – Proteins of the cauda epididymal and seminal vesicle fluid from Santa Ines rams identified by two-dimensional electrophoresis and mass spectrometry (MALDI-ToF/ToF and ESI-Q-ToF). Spot numbers refer to those in Fig. 3. Full table showing sequences of matched peptides, ion scores, m/z and z values is shown as Table S4.

Protein	Experimental [a] kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)
Cauda epididymal fluid				
Serum albumin				
Spot 01	27.2/6.2	57164373	193	12
Spot 02	72.8/6.0	57164373	584	21
Spot 03	72.8/5.9	57164373	536	31
Spot 04	72.8/5.8	57164373	196	34
Spot 05	36.8/5.9	57164373	78	4
Spot 06	72.8/5.7	57164373	72	7
Spot 07	76.1/5.6	57164373	36	3
Spot 08	76.3/5.6	57164373	108	5
Spot 09	76.5/5.5	57164373	306	24
Prostaglandin D synthase				
Spot 10	26.2/6.2	57164293	48	1
Spot 11	26.5/5.8	57164293	49	1
Spot 12	27.3/5.5	57164293	57	1
Spot 13	28.9/5.2	57164293	48	1
Peroxiredoxin 2				
Spot 14	30.4/5.3	261244978	121	14
Transferrin				
Spot 15	80.2/6.7	114326282	79	7
Spot 16	80.2/6.6	114326282	81	7
Spot 17	80.2/6.6	114326282	89	7
Spot 18	80.2/6.5	114326282	74	7
Beta-actin				
Spot 19	52.9/5.4	194676388	74	11
Spot 20	52.9/5.4	194676388	140	19
Clusterin				
Spot 21	49.7/4.3	27806907	25	2
Spot 22	49.3/4.3	27806907	77	6
Spot 23	48.9/4.4	27806907	65	3
Spot 24	48.4/4.4	27806907	99	8
Alpha-1-anti-trypsin				
Spot 25	72.4/5.1	57526646	92	13
Spot 26	72.2/5.2	57526646	113	23
Spot 27	72.1/5.3	57526646	105	11
Alpha-2-HS-glycoprotein				
Spot 28	72.3/4.7	57526674	178	14
Spot 29	72.5/4.6	57526674	159	14
Spot 30	72.7/4.5	57526674	159	17
Spot 31	73.0/4.4	57526674	165	12
ZBTB 42 protein-like				
Spot 32	33.6/5.7	296475269	50	1
Vesicular gland fluid				
Sulfotransferase 1C2				
Spot 01	33.4/6.8	297459963	36	4
Serum albumin				
Spot 02	27.2/6.2	57164373	191	8
Spot 03	72.8/6.0	57164373	998	51
Spot 04	72.8/5.9	57164373	1597	49
Spot 05	72.8/5.9	57164373	440	19
Spot 06	72.8/6.5	57164373	95	9
Spot 07	17.1/5.9	57164373	104	6
Spot 08	72.8/6.2	57164373	56	5
Spot 09	72.9/6.2	57164373	66	2
Spot 10	72.9/6.3	57164373	849	27
Spot 11	73.0/6.3	57164373	1173	45
Spot 12	72.9/6.4	57164373	789	21
Bodhesin 2				
Spot 13	13.2/5.5	121484235	170	3
Spot 14	14.1/5.6	121484235	29	18
Spot 15	15.1/4.3	121484235	59	18
Spot 16	12.2/5.2	121484235	58	18
Cystatin B				
Spot 17	13.4/5.9	82697343	62	15
Spot 18	11.8/6.0	82697343	83	27
Ram Seminal Vesicle 14 kDa				
Spot 19	12.2/5.2	219521810	81	28
Spot 20	13.7/5.3	219521810	60	28
Aldose reductase				
Spot 21	39.8/6.0	60302887	353	31
Spot 22	39.8/6.2	60302887	153	36
Beta-actin				
Spot 23	52.9/5.4	194676388	1110	64
Spot 24	52.9/5.4	194676388	425	27
Disulfide isomerase A3				
Spot 25	72.8/6.4	251823897	479	47
Alpha-enolase				
Spot 26	57.4/6.5	87196501	621	36
Transferrin				
Spot 27	73.8/6.7	296490958	45	3
Spot 28	73.8/6.8	296490958	36	4
Spot 29	73.8/6.8	296490958	40	3
Spot 30	73.8/6.9	296490958	38	2
Endoplasmic reticulum resident protein 29				
Spot 31	33.2/5.8	187607549	104	8
Apolipoprotein A1				
Spot 32	26.4/5.9	162678	398	38
Creatine kinase B				
Spot 33	53.7/6.0	208427064	135	53
Ram Seminal Vesicle 22 kDa				
Spot 34	25.4/4.9	219521812	74	26
Spot 35	23.4/4.8	219521812	98	20
Spot 36	29.4/4.3	219521812	82	15
Spot 37	32.6/4.5	219521812	129	20
Spot 38	32.6/4.4	219521812	78	20
Spot 39	32.4/4.3	219521812	97	26
Aldose 1-epimerase				
Spot 40	43.4/6.1	77736588	126	19
Protein disulfide-isomerase				
Spot 41	68.5/5.2	27806501	893	40
Spot 42	60.4/5.5	148878430	372	49
Spot 43	70.2/4.9	148878430	730	39
Spot 44	69.0/4.8	148878430	549	45
Protein disulfide-isomerase A6				
Spot 45	63.2/5.4	151553573	299	17
Heat shock 70 kDa protein 1B				
Spot 46	73.2/5.5	73853769	74	3
Clusterin				
Spot 47	44.6/5.2	27806907	91	8
Spot 48	44.2/5.1	27806907	53	5
Spot 49	44.5/5.0	27806907	109	5
Spot 50	44.6/4.9	27806907	81	6
Spot 51	49.7/4.7	27806907	110	7
Spot 52	48.1/4.6	27806907	121	7
Transgelin				
Spot 53	18.2/6.7	296480261	99	12
Abhydrolase domain-containing protein 14B				
Spot 54	23.6/6.4	157428006	86	10

[a] Experimental values were deduced from the respective 2D maps by PDQuest Software, shown in Fig. 4.

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Fig. 4 – Western blots of seminal plasma, cauda epididymal and vesicular gland fluid proteins incubated with anti-BSP 1 (A) and anti-Bdh-2 (B). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

In the bovine, BSPs interact with sperm during ejaculation and after sperm cells come in contact with oviductal fluids [24]. Our results from indirect immunocytochemistry show that anti-BSP binds to ejaculated sperm before and after acrosome reaction, confirming that proteins of the BSP family interact with the ovine sperm as well. A recent study indicates that the bovine BSP forms oligomers and acts as a chaperone, binding to misfolded proteins and preventing their precipitation under conditions of stress [34]. This might also be the case for RSVP 22, that would explain the fact that we found high molecular weight spots (60 kDa) corresponding to this protein in our seminal plasma maps. Members of the BSP family are expressed in the reproductive fluids of several species, including bovine [16,22], equine [35], swine [36] and goats [30]. BSPs interact with sperm phospholipids and stimulate both cholesterol efflux and permeabilization of the membrane, events typically linked to sperm capacitation [37]. Also, ovine BSPs protect the sperm membrane against damages caused by cryopreservation [38] and, in the bull, BSPs mediate the interaction of sperm with the oviduct epithelium and

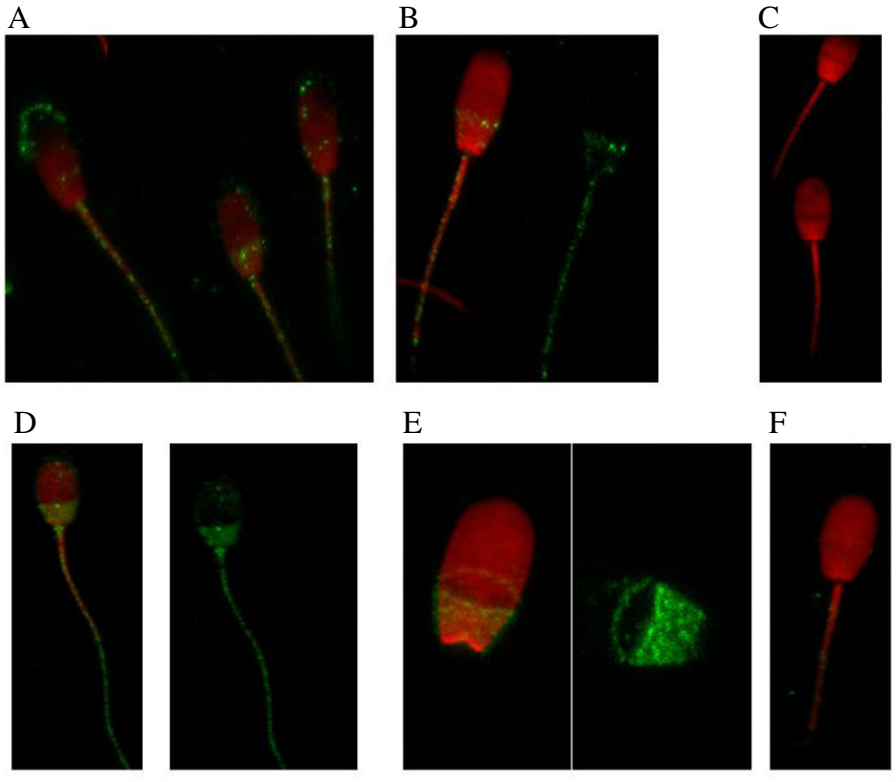

Fig. 5 – Patterns of BSP-1 and Bdh-2 binding to epididymal and ejaculated (intact and acrosome-reacted) sperm from Santa Ines rams. Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and AutoQuant X2 (Media Cybernetics Inc., USA). Anti-BSP-1: (A) acrosome intact, ejaculated sperm; (B) acrosome-reacted, ejaculated sperm; (C) cauda epididymal sperm. Anti-Bdh-2: (D) acrosome intact, ejaculated sperm; (E) acrosome-reacted, ejaculated sperm; (F) cauda epididymal sperm.

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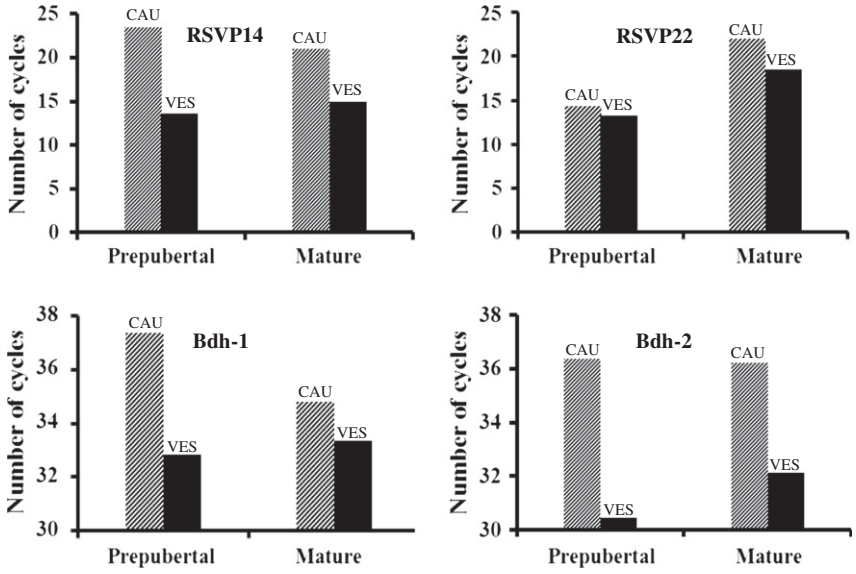

Fig. 6 – Gene expression of Ram Seminal Vesicle Protein 14 kDa (RSVP 14) and 22 kDa (RSVP 22), bodhesin 1 (Bdh-1) and bodhesin 2 (Bdh-2) in the reproductive tract of Santa Ines rams. Amplification plots of RSVP 14, RSVP 22, Bdh1 and Bdh2 genes are shown in prepubertal and sexually mature Santa Ines rams using cDNAs from vesicular glands (VES) and cauda epididymis (CAU).

contribute to sperm survival in that environment [39]. Moreover, BSPs regulate sperm volume, helping to maintain cell viability after the osmotic shock that occurs during ejaculation [40]. Because the bovine and ovine BSPs share a high degree of homology in their amino acid sequences [23], it is possible that these proteins share functional attributes as well. However, biochemical differences exist between these BSPs. For instance, results from our laboratory show that BSPs from the seminal plasma of Morada Nova rams apparently do not interact with heparin [41] and only show very weak affinity for gelatin, while bovine BSPs have strong affinity for those components [42].

The second most abundant proteins in the gels of Santa Ines seminal plasma were identified as bodhesins 1 and 2, belonging to the family of spermadhesins. Based on RT-PCR analysis, Bdh-1 and Bdh-2 were mainly expressed in the vesicular glands and in very low magnitude in the cauda epididymis of the rams, similar to what has been found for RSVPs 14 and 22. Western blots confirmed that Bdh-2 is primarily expressed in the vesicular gland fluid, but with some low expression in the cauda epididymal fluid as well. In goats, Bdh-2 mRNA was mainly found in the accessory sex glands, with epididymis displaying the smallest amount of transcripts [43], in agreement with the results reported in the present work.

Spermadhesins are components of the seminal plasma from bulls [10], pigs [44] and horses [45] and, in the goat, four transcripts (from Bdh-1 to Bdh-4) have been described as associated with those proteins [26]. In the present study, sequences of tryptic peptides from Bdh-1 and 2 spots matched to regions of the CUB domain, which mediates the interaction with carbohydrates [46], protease inhibitors, phospholipids [47] and glycoproteins of the zona pellucida [44]. CUB domains were named as such because of its presence in complement subcomponents (C1r/C1s), embryonic sea urchin protein (Uegf) and bone morphogenic protein 1 (Bmp1) [48]. Spermadhesins bound to swine [44] and horse sperm [45] have been shown to influence sperm capacitation, acrosome stabilization and sperm–oocyte interaction [49,50]. A 15.5-kDa spermadhesin has been described in the seminal plasma of Suffolk rams, with the N-terminal sequence sharing more than 70% identity with the amino acid sequence of swine spermadhesin (AQN-1; [51]). However, it remains to be determined if spermadhesins of the ram seminal plasma share the same functional attributes with those of other species. Our present results show, for the first time, that Bdh-2-like spermadhesin binds to ejaculated ovine sperm. Once sperm became acrosome reacted, binding to the acrosome was lost, but remained on the equatorial portion. Immunostaining associated with anti-Bdh-2 was visualized on the tail and midpiece of cauda epididymal sperm, although the biological significance of this finding is still unclear.

Major proteins of the ram seminal plasma, defined in the present study as RSVPs and spermadhesins, interact with and influence sperm function, as discussed above. However, the seminal fluid expresses other proteins that, although in lower amounts, potentially affect the male gametes as well. Thus, the following sections will discuss attributes of those proteins. Sperm release reactive oxygen species (ROS) as part of their normal physiology but, if produced in excess, these molecules are detrimental to the cells. Thus, a coordinated balance must exist between ROS production by the sperm and its scavenging by antioxidant systems in the reproductive tract fluids. Some of the proteins identified in the present work participate in such systems, such as albumin, peroxiredoxin, superoxide dismutase (SOD), transferrin and lactoferrin. Albumin protects sperm against oxidative stress possibly by trapping free radicals [52]. Peroxiredoxins (PRX) are peroxidases [53] that, together with glutathione peroxidase, catalase and SOD, comprise a reliable system for ROS scavenging [54]. Considering that spermatozoa have limited antioxidant capacity [55],

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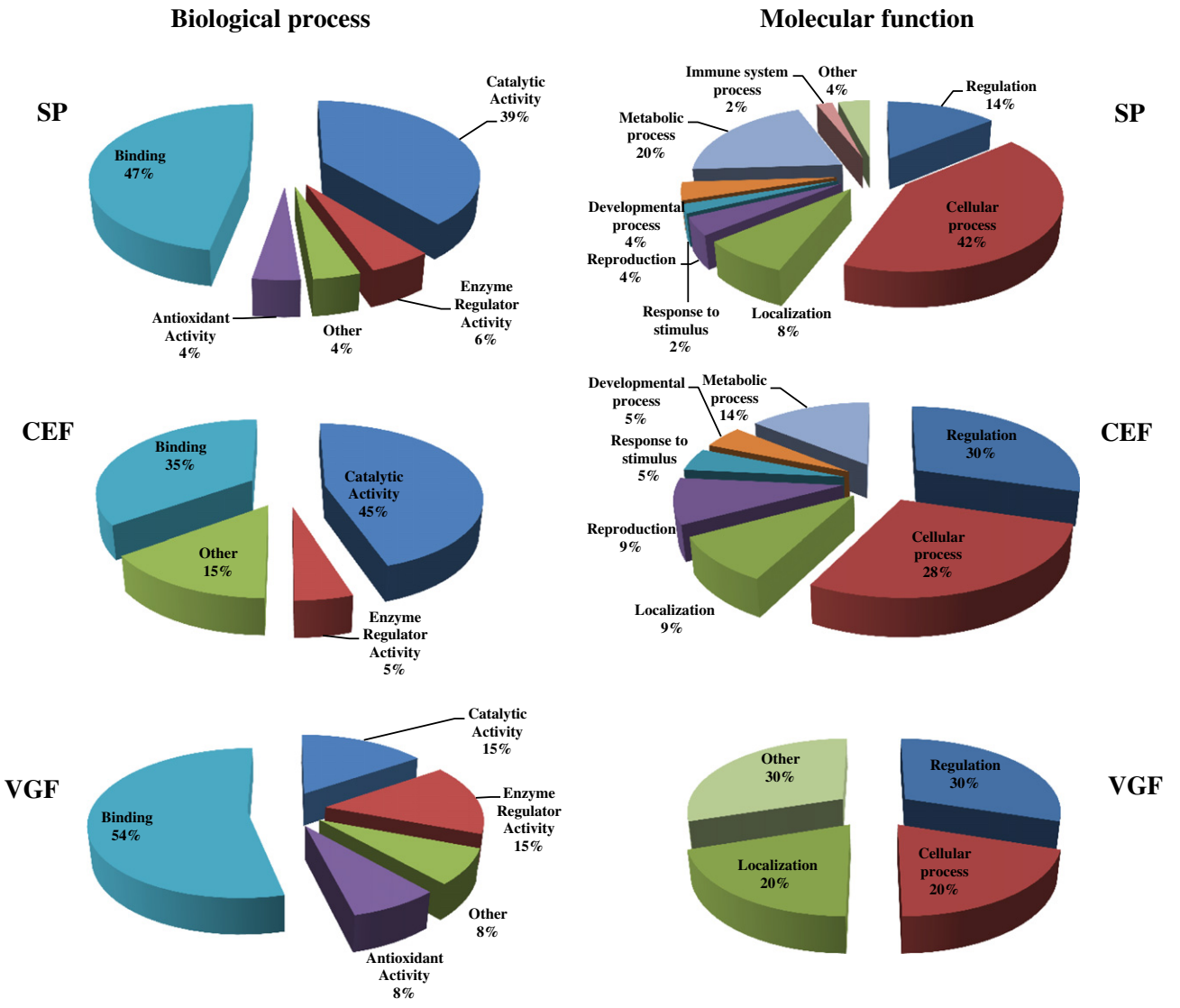

Fig. 7 – Pie charts showing gene ontology annotations of the Santa Ines ram seminal plasma (SP), cauda epididymal fluid (CEF) and vesicular gland fluid (VGF) proteomes based on their biological process, molecular function and cellular component. Protein data were analyzed using the software for researching annotations of proteins (STRAP [19]). Gene ontology terms were obtained from UniProtKB and EBI GOA databases.

these cells rely on the protection conferred by components of the reproductive tract fluids, especially during epididymal storage. Transferrin and lactoferrin are iron-binding proteins secreted by Sertoli cells, epididymal epithelium and accessory sex glands. Such proteins protect sperm by chelating free iron, thus preventing lipid peroxidation [56]. Moreover, through their ability to bind iron, an essential ion for bacterial growth, both transferrin and lactoferrin represent lines of defense against pathogenic microorganisms [57]. Palate, lung and nasal epithelium clone (PLUNC), detected in the Santa Ines maps, displays antibacterial properties as well, probably composing another barrier against infection [58].

Epididymal secretory protein E1 is a lipophilic component and among the most abundant proteins in the cauda epididymal fluid of bulls [15]. It binds to sperm [59] and may work to remodel sperm membrane during epididymal maturation. Apolipoprotein A1 (Apo-A1) is another lipophilic protein and it appears to be involved in sperm capacitation. Apo-A1 is a component of the High-Density Lipoprotein (HDL) that works as an acceptor for cholesterol and phospholipids during sperm capacitation induced by BSPs [60]. Alternatively, it has been suggested that Apo-A1 binds to an Apo-A1 binding protein located in the sperm acrosome and midpiece [61], causing an increase in sperm protein phosphorylation, characteristic of capacitation. Apo-A1 is present in the bovine cauda epididymal fluid [15] but, to the best of our knowledge, this is the first report of its occurrence in the seminal plasma of rams.

We reported several chaperones in the ram seminal plasma, including heat shock 70 kDa protein 1-like, T-complex containing chaperonins and clusterin, being the latter the most abundant and detected as nine different spots in the 4–7 pH gels. These proteins are secreted in response to cellular damage and protect cells by preventing accumulation of misfolded proteins and blocking apoptotic pathways [62]. Clusterin was

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reported as more abundant in the accessory gland fluid of high fertility bulls [10] compared to average and low fertility sires, but these authors did not report if this relationship was associated with chaperone activity.

Proteases participate in remodeling of the sperm membrane that occurs during epididymal transit and sperm capacitation [63,64]. In the present study, we detected several seminal plasma proteases, including leucine aminopeptidase, dipeptidyl peptidase 7, matrix metalloproteinase 2, angiotensin-converting enzyme, plasma glutamate carboxypeptidase, arylsulfatase A and cathepsins B and F. Cathepsins are cysteine proteinases that participate in the degradation of proteins and activation of enzyme precursors [65]. Cathepsin B is found mainly in the cauda epididymal environment [66] and pro-forms of this protein are secreted in the epididymal lumen [67]. Pro-cathepsin B is activated by other cathepsins [68] and it may regulate membrane remodeling or activate protein precursors secreted in the accessory sex gland fluid. Cystatins are inhibitors of cysteine proteinases and it could potentially exert a protective role against excessive activity of secreted cathepsins and other proteases in the reproductive tract and ejaculate [69]. Although the presence of cathepsin isoforms has been reported in the seminal plasma of other species, the present study is the first report of cathepsin B and F expression in the ovine reproductive tract fluids. The intensity of cathepsin D spots in 2-D gels of cauda epididymal fluid was positively associated with fertility rates of Holstein bulls [9]. This association may be related to cathepsin's ability to mediate sperm membrane remodeling during epididymal maturation [70]. The seminal plasma angiotensin-converting enzyme (ACE) results from its shedding from sperm during epididymal transit [71]. Epididymal ACE mediates the translocation of ADAM3 in the sperm membrane [72] and reduces sperm motility during epididymal storage [73]. Moreover, knockout animals for the ACE gene are sub-fertile [74], evidencing the requirement of this enzyme for normal fertility. Matrix metalloproteinase 2 (MMP2) is a gelatinase present in the seminal plasma and its concentration is associated with sperm counts in humans [75]. Although these enzymes play important roles in sperm function, their activity needs be modulated by protease inhibitors, like the tissue inhibitor of metalloproteinase 2 (TIMP-2) found in the Santa Ines seminal plasma. TIMP-2 is expressed in bovine cauda epididymal [76] and accessory sex gland fluids [16], and bulls containing sperm-bound TIMP-2 are more fertile than those without TIMP-2 [8]. Alpha-2-macroglobulin, on the other hand, is a nonspecific protease inhibitor that appears to act in the epididymal environment to modulate protease action [70], avoiding undesirable cleavages. Alpha-2-HS glycoprotein (AHSG), a broad-range protease inhibitor [77], appears as acidic spots in the vesicular gland and seminal plasma maps of the rams, as it has been shown in humans [78]. AHSG is present in other body fluids, including blood plasma [79], and participates in pathways associated with inflammation and vascular calcification [80], growth signaling [81] and insulin resistance [79]. Given that treatment of semen samples with antibodies to AHSG decreases the percentage of motile sperm [82], this protein certainly regulates post-mating sperm function, although by a mechanism that is still unknown.

Seminal peptidases are believed to affect sperm function by cleaving peptide residues of the sperm membrane but an alternative role has been postulated for these molecules. In this case, a sperm membrane aminopeptidase functions as a zona pellucida receptor, being implicated in oocyte recognition and induction of acrosome reaction [83]. Arylsulfatase A, found in our 2-D maps, has been shown to interact with the sperm during epididymal transit and to participate in sperm–egg binding [84]. Other proteins of the Santa Ines seminal plasma that potentially affect gamete binding include the carbohydrate metabolizing enzymes beta galactosidase [85] and alpha fucosidase [86]. A report showing that the amount of epididymal fucosidase is associated with bull fertility [9] supports the notion that this enzyme plays an important role in sperm function. These seminal enzymes come from cauda epididymal fluid [15], in addition to alpha enolase and aldose reductase. The present study is the first to describe the expression of alpha enolase, aldose epimerase and aldose reductase in the vesicular gland fluid of rams. Aldose reductase is involved in the production of seminal fructose and it is believed that this enzyme causes accumulation of sorbitol in the cauda epididymis, contributing to sperm immobilization prior to ejaculation [87].

Albumin and transferrin were detected as the major proteins of the ovine cauda epididymal fluid, similar to studies conducted in the bovine [15]. Albumin and transferrin predominantly work to protect sperm from reactive oxygen species, as discussed above. Interestingly, transferrin was found in both vesicular and cauda epididymal fluids of the Santa Ines rams, with practically identical isoforms. Transferrin has been previously detected in the prostate fluid from boars [88] and dogs [89] but the present study is the first to report its expression in the vesicular gland fluid of ruminants. Although several proteins appeared common to both cauda epididymal and vesicular fluid, such as transferrin, albumin and clusterin, others became visible only in CEF gels of the present study, such as α-enolase and prostaglandin D synthase (PGDS). PGDS binds to the acrosomal cap of sperm from both the cauda epididymis and ejaculates [90] and its expression in the seminal plasma is related to bull fertility [7]. As a hypothesis, PGDS would act as a carrier of lipophilic components (such as retinol and testosterone) in the epididymis or across the epididymal-blood barrier [91], but knowledge of the exact mechanism by which PGDS functions in male reproduction is incomplete.

Actin was detected in both CEF and seminal plasma, whereas gelsolin was found in the seminal plasma. While gelsolin can be found in a soluble, besides the intracytoplasmic form [92], actin is a putative intracellular protein. Actin is also present in the cauda epididymal fluid of bulls [15] and it is possible that this protein leaked from damaged sperm. It is likely that soluble gelsolin in the epididymal fluid and seminal plasma protects sperm against deleterious effects of actin [93]. The 14-3-3 family of proteins detected in the Santa Ines seminal plasma is multifunctional and reportedly involved in cell signaling, cellular trafficking and cell adhesion [94]. Those are intracellular proteins also found in bovine epididymal sperm [95], where it regulates sperm motility. Although 14-3-3 isoforms have been localized to the testis [96], modulating Sertoli cell adhesion, evidence exists that 14-3-3 zeta can also be secreted [97], being present in tears. However, no function has been postulated for this protein when secreted into the reproductive fluids of the male.

The ubiquitin–proteasome system (UPS) represents a mechanism of protein processing. Proteasomes are large proteases

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composed of seven different subunits and present in almost every eukaryotic cell [98]. Although they are predominantly intracellular complexes, there are also secreted proteasomes involved in the processing of extracellular proteins and dying cells [99]. However, before proteins are recognized by these proteasomes, they have to be marked with a poly-ubiquitin chain [100]. Ubiquitin is a chaperone found in seminal plasma [101,102] and cauda epididymal fluid of humans [103], secreted by means of the epididymosomes [104]. In the epididymis, the membrane of damaged sperm is ubiquitinated and then degraded and recycled by extracellular proteasomes [105]. During proteasome cleavage of ubiquitinated proteins, poly-ubiquitin residues are removed by ubiquitin C-terminal hydrolase isoforms (UCH) [106]. UCHs have also been localized to the sperm head of boars and regulate acrosome reaction and gamete binding, preventing polyspermy [107]. Proteasomes are also detected on the sperm head [108] and their activities have been related to sperm capacitation and acrosome reaction [109], zona pellucida-binding and fertilization [110]. Recently, D'Amours et al. [2] showed that the proteasome subunit alpha 6 is more prevalent in the sperm membrane of low fertility than in high fertility bulls. In the present work, we found components of the UPS system in the seminal plasma, including two proteasome subunits (alphas 5 and 6), ubiquitin and UCH-L3. Thus, as it has been shown for other mammals [111], it is plausible to point out as a hypothesis that the ubiquitin–proteasome system also modulates sperm functions in the ram.

In summary, we detected several categories of proteins in the reproductive tract fluids of hairy rams using a classical proteomic approach based on 2-D SDS-PAGE and mass spectrometry. Gene ontology analysis confirms the functional diversity of seminal plasma proteins. This is the first study reporting such proteins from reproductive tract secretions of tropically-adapted rams. Also, it is noticeable that the superimposition of gels of cauda epididymal and seminal vesicle fluid does not exactly replicate the seminal plasma map. It is likely thus that when CEF and VGF mix, interactions occur among proteins from both secretions and from sperm as well. Based on our previous work and those published by others, we discuss the concept that seminal plasma proteins participate in sperm protection against oxidative stress, microorganism infection, protein misfolding and immune response, sperm capacitation, acrosome reaction, membrane remodeling and sperm–egg interaction.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.05.039.

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