Theriogenology 147 (2020) 135–145

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Proteomic identification of boar seminal plasma proteins related to sperm resistance to cooling at 17 °C

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A R T I C L E I N F O

Article history:
Received 2 August 2019
Received in revised form
18 November 2019
Accepted 19 November 2019
Available online 19 November 2019

Keywords:
Boar
Semen
Cooling
Proteomics
Seminal plasma

A B S T R A C T

The modern pig industry relies on extensive use of artificial insemination with cooled semen. It is important that semen doses maintain their quality during processing, transport and storage before insemination to guarantee maximum fertility rates. However, ejaculates may respond differently to liquid preservation at 17 °C, despite the optimal quality assessed before cooling. Thus, the aim of this study was to identify differences in seminal plasma proteome of ejaculates with a higher or lower seminal resistance to storage at 17 °C. A total of 148 ejaculates from 65 sexually mature healthy boars were classified as: High Resistance to cooling (HR, total motility > 60% at 144h) and Low resistance to cooling (LR, total motility <60 at 72h). To identify differentially expressed seminal plasma proteins between HR and LR ejaculates, ten ejaculates of each group were analyzed by 2D SDS-PAGE and ESI-Q-TOF mass spectrometry. The proteins associated with HR ejaculates were cathepsin B (spot 2803 and 6601, $p < 0.01$); spermadhesin PSP-I (spots 3101 and 3103, $p < 0.05$); epididymal secretory protein E1 precursor (spot 2101, $p < 0.05$) and IgGFc binding protein (spot 1603, $p < 0.01$). The protein associated with LR group was the Major seminal plasma PSPI (spot 9103, $p < 0.01$). To our knowledge, this is the first report of the association of boar seminal plasma proteins to semen resistance to cold storage at 17 °C. These results suggest the use of these proteins as biomarkers for semen resistance to preservation at 17 °C.

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1. Introduction

The modern pig industry relies on extensive use of artificial insemination (AI) with cooled semen [1]. This reproductive biotechnology is important for genetic improvement and an increase in herd prolificacy in a high efficiency manner [2,3]. To obtain successful AI results, semen of reliable quality must be used and, to this day, the evaluation of semen has progressively become of utmost importance [4–6]. Although the characterization of an infertile male is possible with exams and tests that are currently available, the identification of sires with superior fertility is difficult and more discriminating techniques need to be identified [4,7–9]. Many studies have been done to determine the consistency and efficacy of the use of semen quality parameters for selecting the best performing boars in routine analysis [10,11], but the conclusions are often controversial [12].

Proteomic analysis is increasing in importance in gamete and embryo biology as an independent tool of discovery and as a means of follow-up to transcriptional profiling [13]. Seminal plasma (SP) contains unique proteins necessary for sperm function and survival [14–18], and have been shown to influence sperm kinematics,

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https://doi.org/10.1016/j.theriogenology.2019.11.023
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protect spermatozoa, regulate sperm capacitation and acrosome reaction, and participate in sperm-egg fusion [19–22]. Seminal plasma proteins also modulate an immune response in male and female reproductive tracts, allowing the most competent spermatozoa to meet the oocyte during fertilization [23,24]. Consequently, seminal plasma proteins can serve as important biomarkers for male fertility [25–27].

The composition of boar seminal plasma reveals several roles of the proteins secreted in the seminiferous tubuli, epididymis and accessory glands. Gonzalez-Cadavid et al. (2014) identified a total of 39 proteins in the boar seminal plasma. The most abundant proteins were the spermadhesin porcine seminal protein (PSP)-I and PSP-II, as well as spermadhesins AQN-1, AQN-3 and AWN-1, accounting for 45.2% of the total intensity of all spots. Recently, a larger number of porcine SP proteins were described [28], expanding the complexity of SP composition. In addition, the association of SP proteins with seminal traits was demonstrated, with several proteins being suggested as markers for semen quality and freezability [29–32].

However, little is known about the association between swine SP proteins and sperm resistance to liquid preservation. In fact, one of the hurdles encountered when using cooled semen in AI in swine, is the occurrence of ejaculates that show either a higher or lower resistance to preservation at 17 °C, and can be observed as a reduction in sperm motility. The main consequence of reduced sperm motility is a lower fertility rate associated to doses with lower resistance, which were preserved for 6 days or more. Therefore, the goal of this study was to identify differences in seminal plasma protein profiles of ejaculates with a higher or lower seminal resistance to cool storage at 17 °C.

2. Material and methods

2.1. Animals, semen collection and handling

A total of 148 ejaculates from 65 sexually mature healthy boars (Large White, Duroc and Landrace breeds), routinely used as semen donors for artificial insemination were assigned for the study. The animals were housed in a boar stud and were routinely fed with a commercial corn-soybean meal diet and *ad libitum* water according to the nutritional requirement guidelines for adult boars. All procedures with animals during this study were approved by the local Animal Ethical Committee (CEUA/Univates n. 001/2015).

Ejaculate samples were collected using the gloved-hand method, into a gauze-covered container, followed by semen evaluation for the following parameters: sperm concentration (SpermaCue, Minitüb, GmBH), agglutination, sperm morphology, sperm motility and sperm vigor. Total (TM) and progressive (PM) motilities were estimated immediately by computer-assisted sperm analysis (CASA) (SpermVision, Minitube, Germany). The parameters were based on the following thresholds: total motility - VCL (curvilinear velocity) with a cut off value of 24 μm/s and ALH (amplitude of lateral head displacement) with a cut off value of 1 μm; progressive motility – VCL with a cut off value of 48 μm/s and VSL (straight line velocity), with a cut off value of 10 μm/s. Only ejaculates with progressive sperm motility greater than 70% immediately after collection and a minimum of 80% of spermatozoa showing normal morphology were used. Sperm morphology of each semen sample was analyzed using a phase contrast microscope at the laboratory. Spermatozoa defects were considered as the following: head defects (acrosome defect, abnormal head, detached head), proximal and distal protoplasmic droplet, midpiece defect, bent tail, coiled tail and teratologic format. Sperm classification was performed as previously described [33]. Samples considered normospermic were weighed (assuming 1 g of semen equals 1 mL) and diluted with AndroStar Plus (Minitube, GmBH) into semen doses (50 mL) with 1.5×10^9 spermatozoa per dose. Semen doses were cold-stored at 17 °C for up to 6 days.

Semen analyses were performed 72h and 144h after cooling to determine the seminal resistance to cold preservation at 17 °C. Ejaculates were assigned to experimental groups High Resistance (HR) and Low Resistance (LR) to cooling using the following criteria: HR, composed by ejaculate samples with total motility higher than 60% at 144h of preservation; and LR, when the ejaculates presented total motility lower than 60% after 72h of cooling [34]. Samples (n = 10) with highest total and progressive motilities at 144h were allocated to the High Resistance (HR) group, whereas samples (n = 10) with lowest total and progressive motilities at 72h where assigned to the Low Resistance (LR) group. The criteria for group classification were validated by hierarchical cluster analysis including a complete linkage (furthest neighbor) clustering algorithm, consisting of calculating the chi-squared frequencies from total motility at 72h and 144h. In addition, principal component analysis (PCA) was performed to correlate the seminal traits evaluated and the group composition using Past3 [35].

2.2. Seminal plasma protein samples preparation

Aliquots (3 mL) of the sperm rich fraction of the ejaculates were centrifuged at 800 x g for 10 min at room temperature, followed by a second centrifugation at 12,000 x g for 60 min at 4 °C. To the supernatant (seminal plasma free of spermatozoa and cell debris) 1% (v/v) protease inhibitors (Protease Inhibitor Cocktail, Sigma, EUA) were added and samples were stored at −80 °C until analysis. The BCA protein Assay Kit (Pierce) was used to quantify total protein in the samples.

2.3. Two-dimensional electrophoresis of seminal plasma proteins

Seminal plasma proteins of each sample were analyzed by 2D SDS-PAGE [36,37], using 400 μg of protein per run. Briefly, isoelectric focusing (IEF) was carried out using 7-cm IPG Strips (pH 3–10, Bio-Rad). Samples were mixed well with of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue) adjusting the volume for 125 μL and 400 μg of total protein. First, IPG strips were passively rehydrated for 60 min, followed by active rehydration for 16h. Isoelectric focusing was performed according to the following settings: linear gradient, 250V, 50 μAmp at 20 min; linear gradient, 4,000 V, 50 μAmp at 120 min; and fast gradient 4,000 V, 50 μAmp, to with a total of 10,000 Vh. After focusing, IPG strips were incubated (15 min) in equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 1.5 M Tris-HCl, pH 8.8 and 1% DTT) and re-equilibrated for additional 15 min in equilibration buffer II (similar to equilibration buffer I, but containing 4% de iodoacetamide instead of DTT). After equilibration, strips were placed on the top of homogeneous SDS-PAGE gels (15%), sealed with agarose (5% in SDS-PAGE running buffer), and ran at 80 with 20 mA per gel. Gels were n colloidal Coomassie Blue R-250 (Sigma) 0,1% in methanol (50%) and acetic acid (7%) for 16 h and bleached in methanol solution (50%) and acetic acid (7%) for 2 h. For each sample, a minimum of three gels were ran.

2.4. Analysis of gel images

Images of gels Two-dimensional gel were scanned in TIF file at 300 dpi using Image Scanner III (GE Life Sciences) and analyzed

using PDQuest software, version 8.1 (Bio-Rad Laboratories, Hercules, CA, USA). A total of 40 gels (20 per group) were analyzed. A master gel was generated, which included spots of a reference gel and spots consistently present in the other 2D maps. As internal reference controls, landmark spots present in key regions of the gels, were matched in every member of the match set. Quantitation of protein spots was given as parts per million (ppm) based on the total integrated optical density of spots [38].

2.5. Protein identification by electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry

Differentially expressed seminal plasma protein spots in seminal were excised from gels and digested with trypsin as previously described [39]. Tryptic peptides then were separated in BEH300 C18 columns (100 μm × 100 mm) using the nanoAcquity™ (Waters Corp., Milford, USA) system and eluted (600 μl/min) with acetonitrile gradient (5%–85%) with 0.1% formic acid. The liquid chromatography system was connected to a nanoelectrospray mass spectrometer source (SYNAPT HDMS system, Waters Corp.), which was operated in positive mode using a source temperature of 90 °C and capillary voltage of 3.5 kV. Instrument calibration was done with fragments of the double protonated ion [Glu1]-fibrinopeptide B (*m/z* 785.84), and the Lock mass used during the acquisition was the intact ion. The LC–MS/MS procedure was performed according to the data-dependent acquisition (DDA) method, selecting MS/MS doubly or triply charged precursor ions. Ions were fragmented by collision-induced dissociation using argon as the collision gas and ramp collision energy that varied according to the charge state of the selected precursor ion. LC–MS/MS records were acquired at an *m/z* range of 300–2,100 for the MS survey (1 scan/s) and at an *m/z* range of 50–2,500 for MS/MS. Then data were collected with MassLynx 4.1 software and processed using the Protein Lynx Global Server 2.4 (Waters Corp.) and were converted to peak list text files for database searching.

2.6. Bioinformatics

All identified proteins were categorized by Gene Ontology (GO) annotation [40] according to biological process and molecular function using the Blast2GO 5 tool [41]. Protein-protein interactions analysis were evaluated using STRING v.10.5 (http://string-db.org/) database, indicating physical and functional associations, generating an interaction network [42].

2.7. Statistical analysis

Spot intensities and semen parameters were first checked for normality using D'Agostino-Pearson omnibus test. All seminal derived variables were analyzed as two-way repeated measures ANOVA followed by Tukey's test. Spots differential expression was analyzed by Fisher's Exact test and Student's *t*-test. Also, to verify the association between spots densities and seminal parameters, Pearson's correlation test and linear regression analysis were performed. Data are presented as mean ± SD, and statistical significance was assumed when $P < 0.05$. Statistical analyses were carried out using GraphPad Prism 6 (La Jolla, USA).

3. Results

All 148 boar ejaculates evaluated for this study had total and progressive motilities at collection of 88.3 ± 4.6% and 76.4 ± 3.2%, respectively. Based on the sperm total and progressive motilities evaluated after 72h and 144h of storage at 17 °C, 86 ejaculates from 48 boars were classified as having high resistance to cooling and 40 semen samples from 23 boars as having low resistance to cooling. Twenty-two ejaculates were not classified because they presented a total motility >60% at 72h and <60% at 144h. As for boar distribution within groups, 26 and 7 males had all ejaculates classified as having high and low resistance, respectively. The overall distribution of boars based on their ejaculate's resistance to cooling at 17 °C is shown in Fig. 1A. The 20 ejaculates included in the HR and LR groups were collected from 19 different boars, with one individual (#4524) with one ejaculate in each experimental group (Fig. 1B). One boar (#1035) had two ejaculate samples included in the LR group. It was confirmed that the two categories (HR and LR) were maintained in a dendogram built to include the total sperm motility values at 72h and 144h (Fig. 2A). PCA analysis also confirmed the classification of ejaculates (Fig. 2B).

Regarding the differences in sperm parameters between the

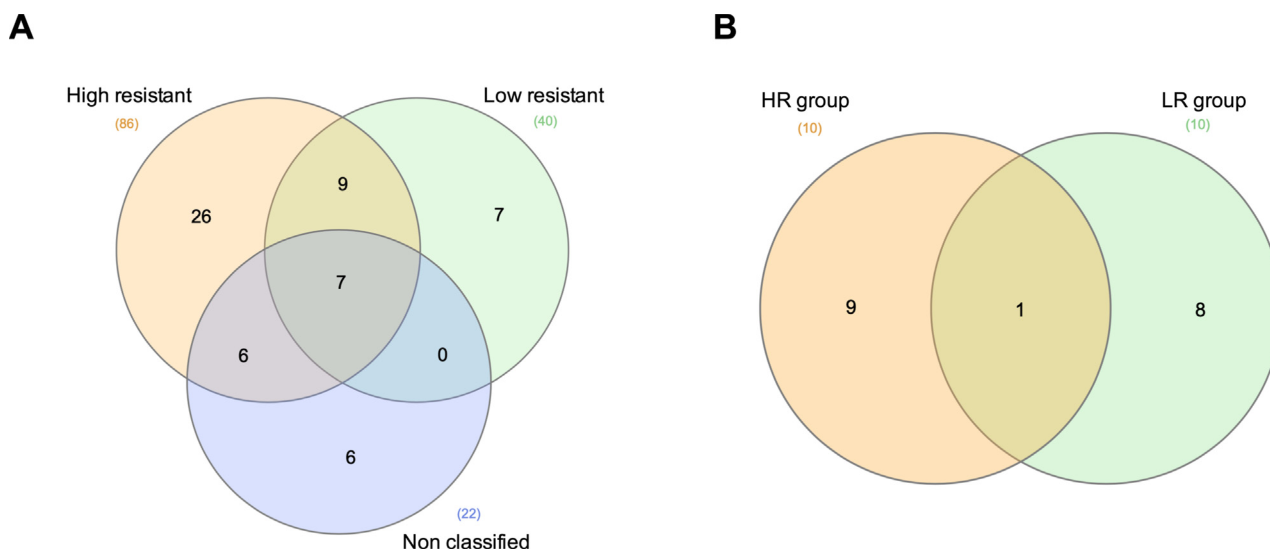

Fig. 1. Venn diagrams depicting the classifications of ejaculates per boar according to sperm resistance to cooling at 17 °C for 144h. A - Distribution of all boars (65) according to the classification of their ejaculates (total of 148) collected during the experiment. B – Boar distribution within the experimental groups High Resistance (HR) and Low Resistance (LR).

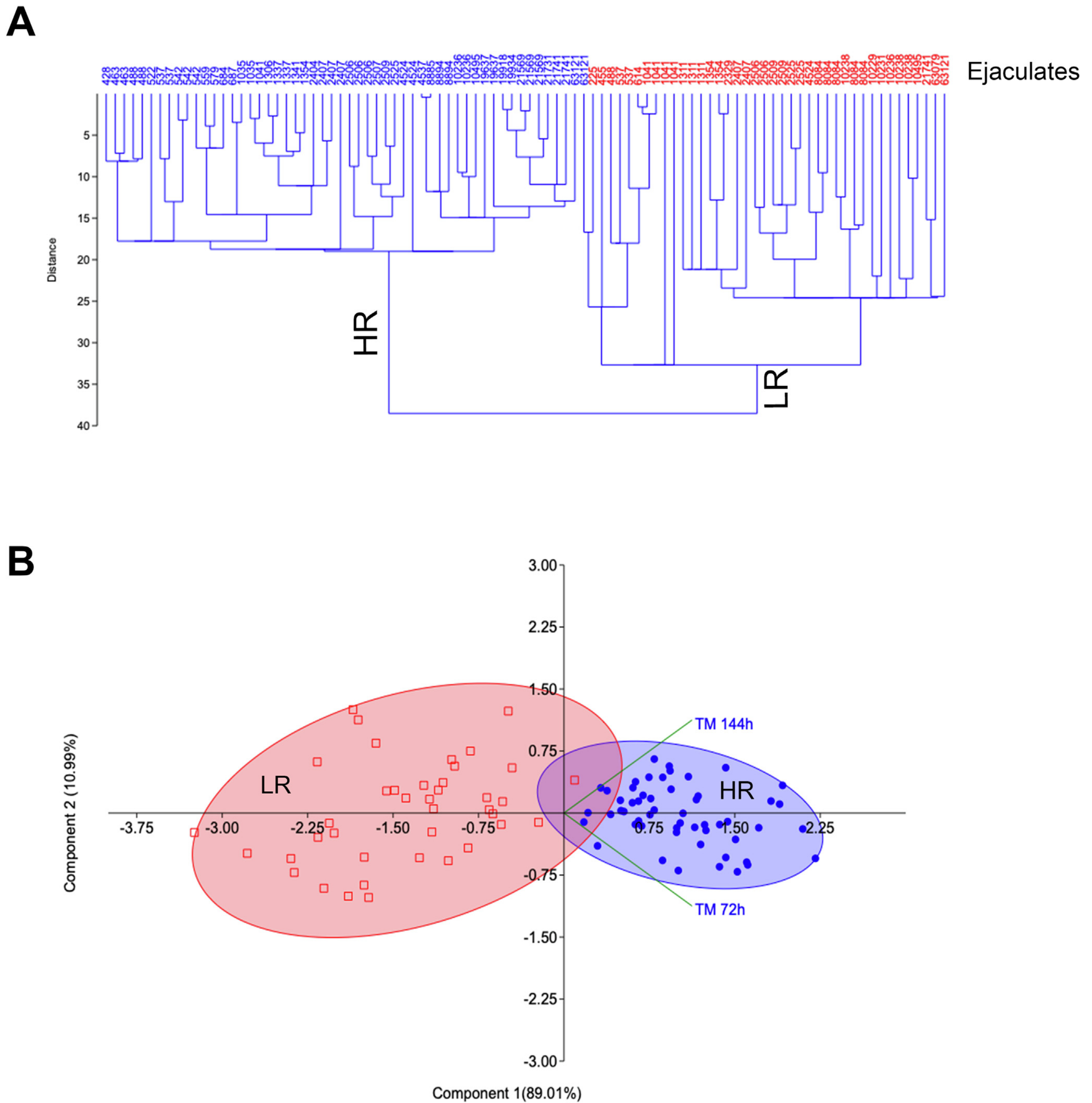

Fig. 2. Hierarchical cluster analysis (dendogram) (A) and Principal Component Analysis score plot (B) of the ejaculates classified as having high resistance (HR) or low resistance (LR) to cooling at 17 °C based on the total sperm motility at 72h and 144h. Red numbers and squares indicate LR ejaculates, whereas blue numbers and dots indicate HR samples.

experimental groups, no differences were observed at 0h. Total and progressive sperm motilities were affected by the time of preservation in both groups, with a marked reduction in the first 72h ($P < 0.05$), without a significant change at 144h. However, the LR group showed a more drastic reduction in total motility in the first 3 days of cold preservation (59.1%), whereas the HR group had 11.4% of reduction in total motility. Although, major and minor sperm defects did not differ between groups and timepoints, when only tail defects were considered, an increase ($P < 0.05$) was observed after 72h of preservation in samples of the LR group. All parameters evaluated in fresh and cold preserved boar ejaculates included in the experimental groups of HR and LR are presented in Table 1.

On average, the seminal plasma protein contents were 37.05 ± 11.75 mg/mL and 27.58 ± 10.58 mg/mL in the HR and LR groups respectively. We identified a total of 250 spots on the protein maps of both groups with no significant difference between groups (HR: 70 ± 15 spots; LR: 64 ± 16 spots). The comparison of the density of spots revealed six highly expressed spots highly in the HR group and one in the LR group (Fig. 3). The identities of the differently expressed spots are shown in Fig. 4 and Table 2. The proteins associated with ejaculates of a higher resistance to cooling at 17 °C were cathepsin B (spot 2803 and

Table 1
Sperm parameters of ejaculate samples of boar assigned to High Resistance (RH) and Low Resistance (LR) groups and submitted proteomic analysis of the seminal plasma. Data presented as mean ± SD.

Parameters	HR Group (n = 10)			LR Group (n = 10)		
	0h	72h	144h	0h	72h	144h
Total motility (%)	89.4 ± 3.3 [a,x]	79.2 ± 5.4 [b,x]	84.56 ± 2.55 [b,x]	86.80 ± 6.51 [a,x]	35.5 ± 5.5 [b,y]	38.8 ± 11.5 [b,y]
Progressive motility (%)	77.2 ± 3.9 [a,x]	74.4 ± 8.6 [b,x]	72.8 ± 8.22 [bx]	75.7 ± 2.7 [a,x]	14.37 ± 5,98 [b,y]	18.6 ± 10.2 [b,y]
Normal morphology (%)	79.0 ± 13.5	83.0 ± 11.7	77.0 ± 35.0	79.0 ± 14.32	64.0 ± 17.3	74.0 ± 14.3
Major defects (%)	15.3 ± 13,2	9.6 ± 7.69	10.8 ± 9.6	13.4 ± 10.9	18.2 ± 16.0	10.5 ± 6.3
Minor defects (%)	6.6 ± 6,2	6.2 ± 5.94	3.4 ± 3.7	9.1 ± 10.1	7.8 ± 6.1	9.3 ± 7.1
Morphology – tail defects	5.5 ± 3.2 [a,x]	3.0 ± 6.2 [a,x]	3 ± 2,74[a,x]	3.5 ± 4.7 [a,x]	8.0 ± 3.1 [b,y]	10.0 ± 5.7 [b,y]

[a,b] indicate difference between time points ($P < 0.05$).
[x,y] indicate difference between experimental groups ($P < 0.01$). Two-way repeated measures ANOVA followed by Tukey test.

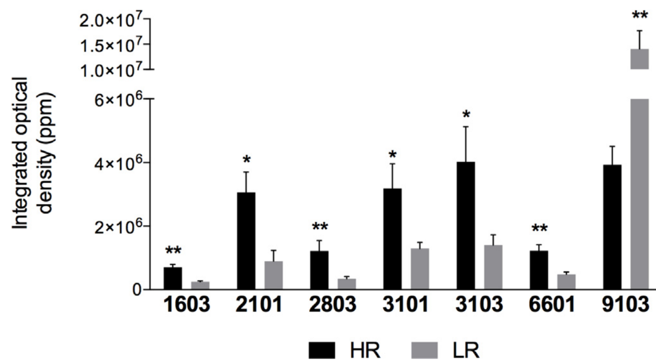

Fig. 3. Intensities (mean ± SD) of significantly more expressed spots in the two-dimensional maps of boar seminal plasma from ejaculates with higher (HR) or lower (LR) resistance to preservation at 17 °C. *$P < 0.05$; **$P < 0.01$.

6601, $P < 0.01$); spermadhesin PSP-I (spots 3101 and 3103, $P < 0.05$); epididymal secretory protein E1 precursor (spot 2101, $P < 0.05$) and IgGFc binding protein (spot 1603, $P < 0.01$). The protein associated with LR group was the Major seminal plasma PSPI (spot 9103, $P < 0.01$).

Associations between spot densities and seminal parameters were identified (Fig. 5). A positive correlation was observed between total sperm motility at 72h with epididymal secretory protein E1 precursor ($R^2 = 0.63$; $P = 0.0099$), spermadhesin PSP-I (spot 3101 $R^2 = 0.23$; $P = 0.0388$ and spot 3103 $R^2 = 0.33$; $P = 0.0315$), and cathepsin B (spot 6601: $R^2 = 0.55$; P = 0.0022). Regarding total sperm motility at 144h, a positive correlation was found for cathepsin B ($R^2 = 0.30$; $P = 0.0433$), spermadhesin PSP-I (spot 3101: $R^2 = 0.22$; $P = 0.0441$ and spot 3103: $R^2 = 0.34$; $P = 0.0294$) and cathepsin B (spot 6601: $R^2 = 0.48$; $P = 0.0062$). Major seminal plasma PSPI, which presented an increased expression in LR group, had a negative correlation ($R^2 = 0.37$; $P = 0.0016$) with total sperm motility at 144h. Also, this protein was the only spot associated with a sperm morphology parameter (sperm cauda defects at 144h; $R^2 = 0.33$; $P = 0.0035$).

The merge of protein-protein interactions networks provided by String analysis showed important biological processes with key roles in sperm survival and function (Fig. 6). Cathepsin B is directly involved with other proteases as well epididymal secretory protein E1 precursor. Protein-protein interaction network of PSP-I includes the spermadhesin family members, as well acrosome and sperm membrane-associated proteins, indicating its function in membrane stability and interaction with other cells.

4. Discussion

Proteomics is a valuable approach to identify protein markers for seminal traits and male fertility [43–45]. In the swine, several proteins have been identified and implicated in preserving cell integrity and motility, as well in sperm protection during cryopreservation [14,30,44,46,47]. The present work is the first to relate boar seminal plasma proteins with ejaculate resistance to preservation at 17 °C. We identified the proteins spermadhesin PSP-I, cathepsin B, epididymal secretory protein E1 precursor and IgG Fc binding protein associated with ejaculates that presented at least 60% of motility after six days of storage at 17 °C. Only one spot was associated with low resistance, identified as Major seminal plasma PSPI.

In the swine, the spermadhesin protein family comprises five members: AQN-1, AQN-3, AWN, PSP-I, and PSP-II [48–50]. Sequence variation, glycosylation and the aggregation state of spermadhesins contribute to the diversity of their biological activities [48–50]. In the present work, PSP-I was the only spermadhesin associated with sperm cooling resistance, showing positive correlations with sperm motility after 72 and 144h of storage at 17 °C. The spermatozoa exposure to the heterodimer PSP-I/PSP-II is associated to in vitro membrane integrity, motility, and mitochondrial activity. This protective effect appears to be related to its adhesion to the acrosomal region, suggesting a stabilizing effect on the fluidity of the sperm plasma membrane [51,52]. Also, it was demonstrated that the addition of the PSP-I/PSP-II heterodimer to sperm suspensions increased the number of viable spermatozoa with stable plasma membrane. A non-permanent decapacitating effect of PSP-I/PSP-II was observed on highly extended boar spermatozoa, defined by the delay in increase of [Ca^{2+}] levels [53]. This effect could explain a higher cooling resistance of boar ejaculates with higher PSP-I, since premature acrosome reactions could result in more fragile plasma membranes, leading to cell rupture and viability loss. In addition, *in silico* analysis showed interactions of PSP-I with other proteins, which could contribute to sperm cooling resistance. The interaction with other spermadhesins as well to binder of sperm protein 1 (BSP1), seminal plasma sperm motility inhibitor and sperm-associated acrosin inhibitor strengthens the hypothesis of a role of PSP-I on sperm protection during storage at 17 °C.

Cathepsins are papain-like cysteine proteases presenting unique reactive-site properties and an uneven tissue-specific expression pattern [54]. In living organisms, their activity is a delicate balance of expression, targeting, zymogen activation, inhibition by protein inhibitors and degradation [55]. Cathepsins have been already found in the reproductive tissues and fluids of several species [38,56–58], including the boar seminal plasma [59]. In men, Cathepsin B has been associated with normal sperm count despite

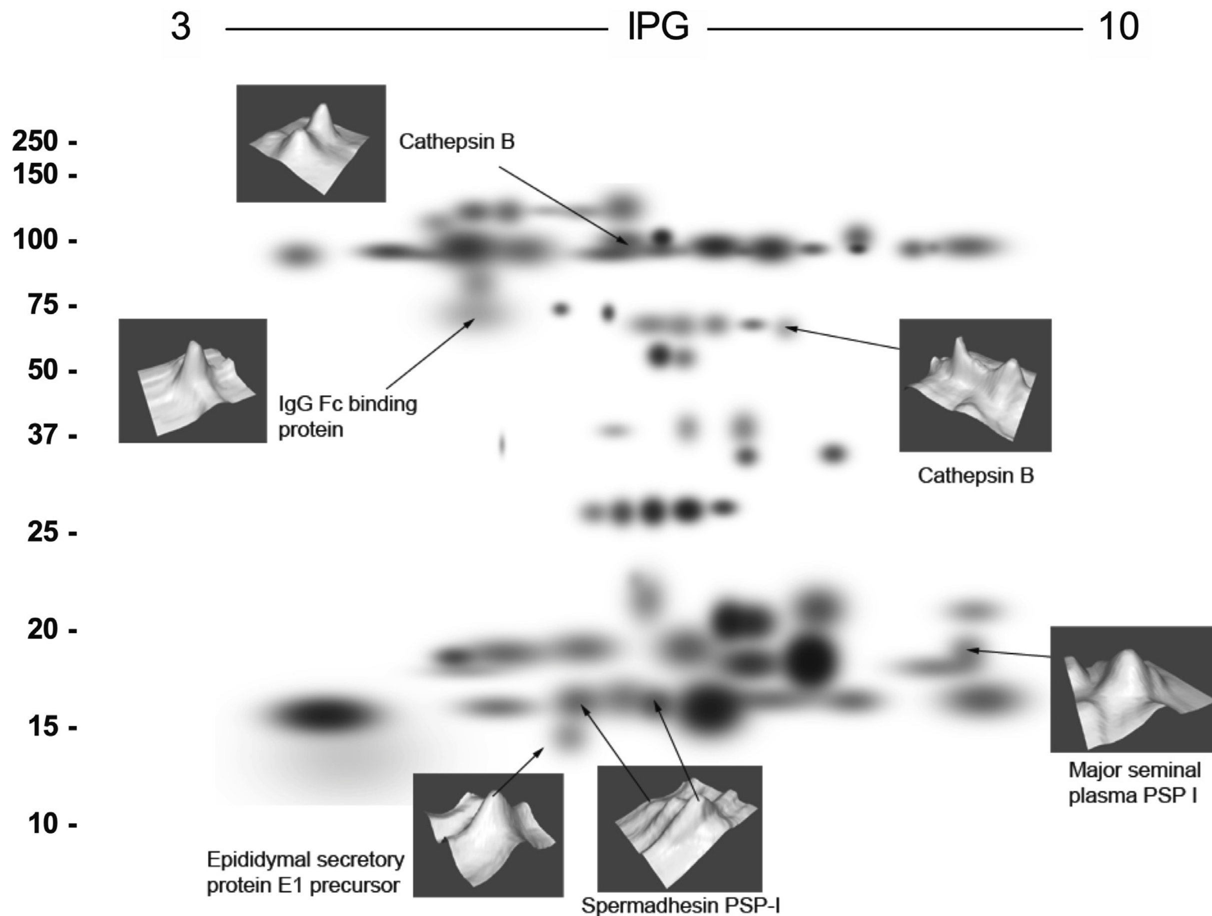

Fig. 4. Master gel of the boar seminal plasma proteome generated by PDQuest software (Bio-Rad, USA), based on a match set with all 2D gels used in the study. Greater detail is provided in the 3D representation of the differentially expressed spots.

sperm morphology values [58]. Although the mechanism by which cathepsin B could contribute to increased sperm resistance to cold storage is not clear, its interaction with other proteins could shed a light on this matter. The network provided by String analysis showed that cathepsin B potentially participates in the modulation of immune response through host immune response against pathogens [60] and in the induction of cytokine secretion and inflammation [61,62]. Also, cathepsins have been implicated in the degradation of apoptosis-regulating proteins of the B-cell lymphoma-2 (Bcl-2) family [63]. Apoptotic markers including caspase activation and phosphatidyl-serine exteriorization have been described in the mature spermatozoa of infertile men [64–66]. Thus, the interaction of cathepsins with Bcl-2 indicates a possible protection role by inhibiting sperm apoptosis via the intrinsic pathway.

As for cathepsin B, the association of the amount of IgG Fc binding protein in SP of ejaculates with higher resistance to cold preservation also suggests that the modulation of immune response during spermatogenesis and epididymal maturation is important for the production of more resistant gametes. Seminal plasma has immune modulating properties that are mediated by prostaglandins, complement inhibitors, cytokines, and proteins capable of binding IgG via the Fc fragment [23,67,68]. IgG binding proteins are Fc receptor-like proteins and can be regarded as antibody-binding proteins. The presence of antibody-binding proteins in human seminal plasma could contribute to the protection of sperm from immune-mediated damage [69].

Secreted in the epididymal lumen, epididymal secretory protein E1 (NPC2) was previously described in proteomic studies of the epididymal fluid and seminal plasma [38,59,70–72]. The characterization of the epididymal secretory protein E1 structure suggested that it binds to cholesterol from internal lysosomal membranes, enabling a physical interaction with NPC1 and allowing post lysosomal export of cholesterol [73]. Therefore, NPC2 have a key role in regulating the transport of lipoprotein-derived cholesterol to other cellular compartments and maintaining intracellular cholesterol homeostasis [74]. The cholesterol content in sperm membrane is first regulated during epididymal maturation and then during capacitation in the female genital tract. These two processes are necessary for the spermatozoa to acquire their fertilizing potential. Interestingly, Busso and co-workers demonstrated that NPC2$^{-/-}$ mice had a reduced cholesterol content compared with that of wild-type (WT) spermatozoa, despite the normal motility and morphology parameters. Moreover, capacitated NPC2$^{-/-}$ spermatozoa exhibited defective tyrosine phosphorylation patterns and a decreased ability to fertilize cumulus–oocyte complexes compared with WT spermatozoa, supporting the relevance of mouse epididymal NPC2 for male fertility [75]. Finally, the interaction between cathepsin B with NPC1, as demonstrated *in silico*, corroborates the evidence of a protective role of NPC2 of the spermatozoa integrity and functionality.

Table 2
Proteins differently expressed in boar seminal plasma according to semen resistance to cooling at 17 °C. Proteins were identified by 2D SDS-PAGE and mass spectrometry. Spot numbers refer to those shown in Fig. 3.

Protein	Experimental kDa/pI	NCBI reference sequence	Sequence covered	Matched peptides	Mass (Da)
Cathepsin B					
Spot 2803	127.3/5.3	NP_001090927	5%	$^{(58)}$LCGTFLGGPK$^{(67)}$	992.5233
				$^{(88)}$EQWPNCPTIK$^{(97)}$	1215.5826
Spot 6601	99.2/6.1	A0A287BF94	34%	$^{(133)}$DQGSCGSCWAFGAVEAISDR$^{(152)}$	2172.9073
				$^{(90)}$LCGTFLGGPK$^{(99)}$	1049.5448
				$^{(50)}$ESLHFQPLSDELVNFINK$^{(68)}$	2130.0865
				(51) SLHFQPLSDELVNFINK (68)	2001.0440
				$^{(49)}$RESLHFQPLSDELVNFINK$^{(68)}$	2286.1877
				$^{(326)}$WLVGNSWNTDWGDNGFFK$^{(343)}$	2142.9668
				$^{(347)}$GQDHCGIESEIVAGIPCTPHF$^{(367)}$	2324.0434
				$^{(198)}$GLVSGGLYDSHVGCRPY$^{(214)}$	1836.8697
				$^{(278)}$NGPVEGAFTVYSDFLQYK$^{(295)}$	2034.9807
Spermadhesin PSP-I					
Spot 3101	15.9/5.4	S23942	33%	$^{(1)}$LDYHACGGR$^{(9)}$	991.4414
				$^{(53)}$EYVEVLEGAPGSK$^{(65)}$	1377.6896
				$^{(53)}$EYVEVLEGAPGSK$^{(65)}$	1377.6896
				$^{(1)}$LDYHACGGR$^{(9)}$	991.4414
Spot 3103	14.8/5.7	S23942	65%	$^{(10)}$LTDDYGTIFTYK$^{(21)}$	1436.6944
				$^{(10)}$LTDDYGTIFTYK$^{(21)}$	1436.6944
				$^{(25)}$TECVWTLQVDPK$^{(36)}$	1418.6984
				$^{(53)}$EYVEVLEGAPGSK$^{(65)}$	1377.6896
				$^{(53)}$EYVEVLEGAPGSK$^{(65)}$	1377.6896
				$^{(70)}$FCEGLSILNR$^{(79)}$	1151.5877
				$^{(91)}$DSGHPASPYEIIFLR$^{(105)}$	1701.8594
				$^{(91)}$DSGHPASPYEIIFLR$^{(105)}$	1701.8594
				$^{(1)}$LDYHACGGR$^{(9)}$	991.4414
				$^{(1)}$LDYHACGGR$^{(9)}$	991.4414
				$^{(91)}$DSGHPASPYEIIFLR$^{(105)}$	1701.8594
Major seminal plasma PSPI					
Spot 9103	14.2/10.0	NP_99902.1	46%	$^{(34)}$LTDDYGTIFTYK$^{(45)}$	1436.6944
				$^{(34)}$LTDDYGTIFTYK$^{(45)}$	1436.6944
				$^{(49)}$TECVWTLQVDPK$^{(60)}$	1418.6984
				$^{(77)}$EYVEILEGAPGSK$^{(89)}$	1391.7052
				$^{(77)}$EYVEILEGAPGSK$^{(89)}$	1391.7052
				$^{(94)}$FCEGLSILNR$^{(103)}$	1151.5877
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
Epididymal secretory protein E1 precursor					
Spot 2101	14.0/5.1	NP_999371.1	28%	$^{(77)}$EYVEILEGAPGSK$^{(89)}$	1391.7052
				$^{(94)}$FCEGLSILNR$^{(103)}$	1151.5877
				$^{(115)}$DSGHPASPYEIIFLR$^{(129)}$	1701.8594
IgG Fc binding protein					
Spot 1603	98.6/4.6	XP_013844022.1	1%	$^{(148)}$DCHNTLDPQGVVR$^{(160)}$	1453.6852
				$^{(62)}$VPGSYSGALCGLCGNFNGDPADDLALR$^{(88)}$	2682.2286
				$^{(148)}$DCHNTLDPQGVVR$^{(160)}$	1453.6852

5. Conclusion

To our knowledge, this is the first report of an association of boar seminal plasma proteins to semen resistance to storage at 17 °C. Spermadhesin PSP-I, cathepsin B, epididymal secretory protein E1 precursor and IgG Fc binding protein were more abundant in ejaculates with higher resistance to cooling. Another PSP-I isoform (Major seminal plasma PSPI), was the only protein associated to ejaculated that presented total sperm motility below 60% after 72 h. These results suggest the use of these proteins as biomarkers for semen resistance to preservation at 17 °C. The identification of ejaculates with higher or lower resistance could improve the logistics of boar semen doses commercialization, reducing costs and increasing fertility rates with AI.

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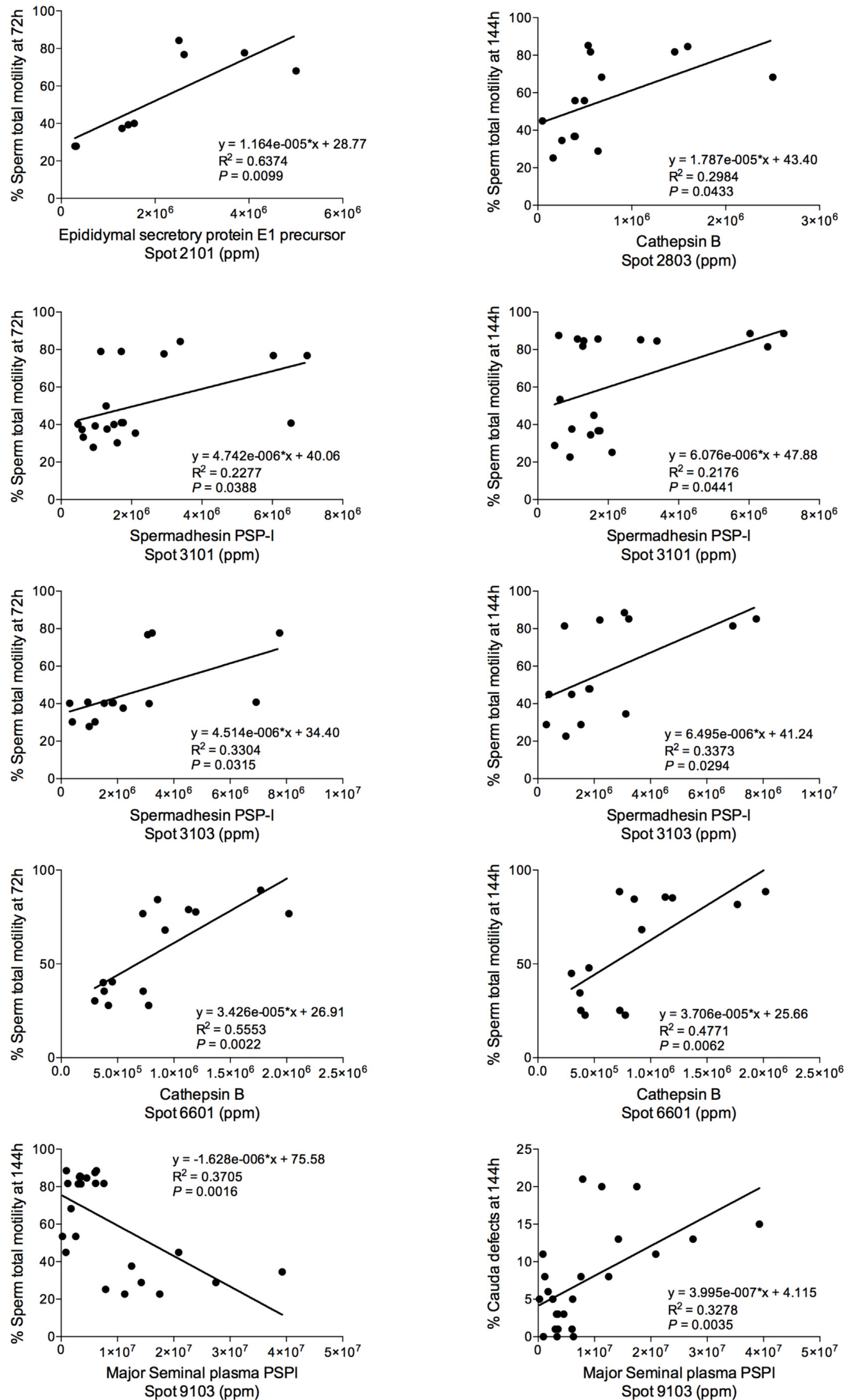

Fig. 5. Regression models showing the correlation between boar sperm parameters and seminal plasma proteins. Dependent variables (y): percentages of motile sperm and sperm with midpiece and tail defects. Independent variables (x) refer to the intensities of spots identified as each protein.

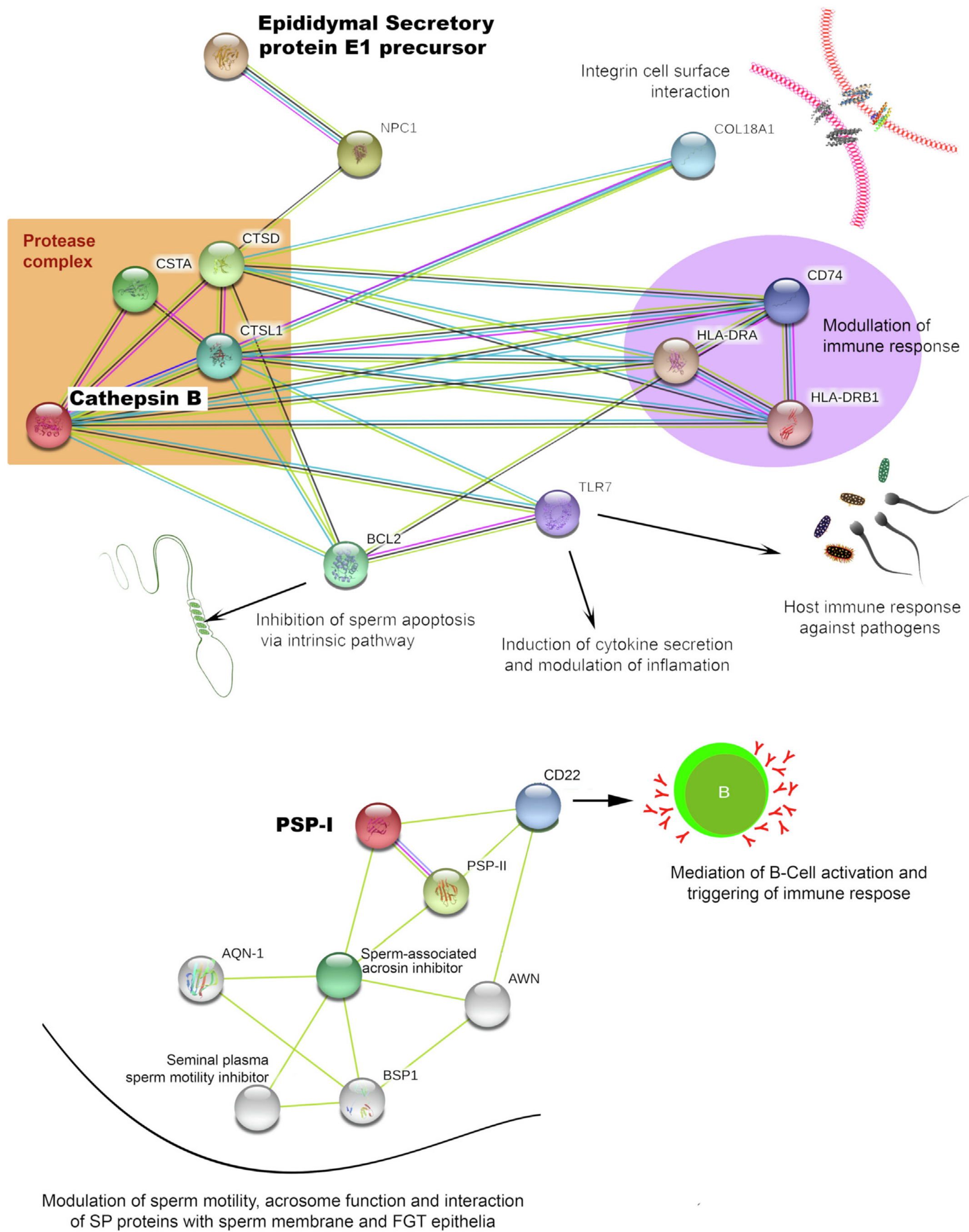

Fig. 6. Protein-protein interaction networks of the differentially expressed seminal plasma proteins (in bold) from ejaculates with high or low resistance to cooling at 17 °C. Lines indicate interaction; arrows indicate the biological process involved.

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Declaration of competing interest

The authors declare that there are no conflicts of interest that may have influenced that discussion presented herein.

Acknowledgements

The present work was financially supported by The Brazilian Council of Scientific and Technological Development (Grant CNPq/ Universal 447251/2014-7), Foundation for the Scientific and Technological Development of the State of Rio Grande do Sul (FAPERGS; grant 16/2551-3360000223-1), Foundation for the Scientific and Technological Development of the State of Ceará (FUNCAP) and FUVATES. Franciele Lucca De Lazari was a recipient of a CAPES scholarship. Authors would like to thank Prof. Dr. Camille Eichelberger Granada for statistics support, Ms. Allison Crawford for review the manuscript and to Associação de Criadores de Suínos do Rio Grande do Sul (ACSURS) for providing the semen samples and for allowing the use of the facilities and consumables.

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