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Original Research Article

Metabolome and oxidative stress markers in the seminal plasma of Holstein bulls and their relationship with the characteristics of fresh and frozen/thawed sperm

E. Giaretta [a,*], A. Damato [b], L. Zennaro [c], V. Bonfatti [a], B. Mislei [d], V. Vigolo [e], M.E. Falomo [f], F. Bertuzzo [g], G. Gabai [a], D. Bucci [h]

[a] Department of Comparative Biomedicine and Food Science, University of Padova, Via Dell'Università 6, 35020, Legnaro, PD, Italy
[b] Institute of Biomolecular Chemistry, CNR, Padova Unit, Department of Chemistry University of Padova, Via Marzolo 1, 35131, Padova, Italy
[c] Department of Molecular Medicine, University of Padova, Padova, Italy
[d] INFA-AUB, University of Bologna, Via Gandolfi 16, Cadriano, BO, Italy
[e] Department for Small Animals and Horses, Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Vienna, Austria
[f] Department of Animal Medicine, Production and Health (MAPS), Università di Padova, Viale Dell'Università 16, 35020, Legnaro, PD, Italy
[g] Intermizoo S.p.A, Via Dossetto 1, 30021, Caorle, VE, Italy
[h] Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano Dell'Emilia, BO, Italy

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ABSTRACT

Seminal plasma composition has important role in sperm functionality and its freezability. The objective of this study was to test the hypothesis that seminal plasma (SP) oxidative status and metabolome are associated with fresh semen characteristics and freezability of bull sperm. To accomplish this objective, oxidative status markers and metabolome of SP of ejaculates obtained from 20 Holstein bulls (3 for each bull) were analyzed using spectrophotometry and nuclear magnetic resonance (^{1}H NMR). The ejaculates were classified into higher motility fresh semen (HMF) and lower motility fresh semen (LMF), according to total motility (TM) and progressive motility (PM) values of fresh semen. Then the ejaculates was cryopreserved and assigned to higher motility thawed group (HMT) or lower motility thawed group (LMT) according to TM and PM at 0 h post-thawing. Multivariate analyses were performed to identify the association between the functional characteristics of fresh and thawed semen and the SP parameters, in terms of the oxidative status and the metabolomic composition. According to our results, the advanced oxidative protein products (AOPP) and thiol concentrations in SP are significantly related to some physiological characteristics of the thawed sperm, such as higher viability, TM, PM and LIN and lower mitochondrial and cytoplasmic superoxide production in viable thawed cells. In contrast, a higher amount of C in the SP was negatively related to TM and PM of thawed semen and was associated with higher mitochondrial and cytoplasmic superoxide production.

In addition, partial least squares-discriminant analysis (PLS-DA) performed on the ^{1}H NMR spectra indicated a discrete separation between HMF and LMF groups, and good discrimination between HMT and LMT groups. Higher levels of formic acid, lactate, glycerol and phosphocholine, were found in the SP of the HMF group than in the LMF group. On the other hand, alanine, phenylalanine, and tyrosine were higher in the SP of the LMF group than in the HMF group. GABA, glutamate, histidine and glycerol were found in higher concentrations in the HMT group than in the LMT group, while fructose decreased in the HMT group. Our results showed that the oxidative and metabolomic status of SP is related to the physiological properties of semen and its freezability and open new fields in research of SP biomarkers of bull semen preservation and fertility.

1. Introduction

Seminal plasma (SP) is endowed with an array of molecules whose actions are not fully understood. Nowadays the omic sciences are used for a comprehensive understanding of biological systems. In relation to bull SP, proteomic, transcriptomic, lipidomic and metabolomic allowed

* Corresponding author. Department of Comparative Biomedicine and Food Science, University of Padova, Via dell'Università 6, 35020, Legnaro, PD, Italy.
E-mail address: elisa.giaretta@unipd.it (E. Giaretta).

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the identification of different biochemical components important for sperm functions, such as sperm interaction with the oviduct, capacitation, acrosome reaction and fertilization [1–3]. To date, a few studies (reviewed in Ref. [4] have investigated the relationship between the sperm metabolome and fertility. These studies performed the metabolomics by different techniques such as GC-MS, LC-MS and nuclear magnetic resonance (^{1}H NMR) and evaluated the relation between SP metabolome and fertility score, based on the empirical evaluation of actual conception rates of cows inseminated with semen from the tested animals [5,6]. In addition, previous studies have already demonstrated that SP components contribute to the redox homeostasis of ejaculated sperm and improve its freezability [7–9]. Bull semen cryopreservation is known to increase the cellular reactive oxygen species (ROS) and contribute to a reduction in sperm motility and fertilizing ability [10,11]. The content of thiols, advanced oxidative protein products (AOPPs) and carbonylated proteins has been used in previous studies to characterize the oxidative status (OS) in biological matrices, such as blood plasma and milk [12–14]. Thiols concentration reflects the antioxidant power of SP, and it is determined by free-SH groups present in glutathione, ergothioneine, cysteine, and albumin. They can act as reducing agents that cause the release of a hydrogen atom, reducing the most important free radicals, such as hydroxyl radicals (HO•) and superoxide (O2 •−). A positive correlation was found between SP thiols levels and concentration and PM of human semen [15]. The AOPPs result mainly from the action of ROS on proteins, leading to the formation of di-tyrosine and modified albumin such as advanced glycation end products (AGE-HAS) [16,17]. In blood plasma, AOPP are mainly derived from oxidation-modified albumin aggregates or fragments, and since albumin is the most abundant SP protein, they can be considered as an indicator of oxidative damage of SP proteins. Although the concentration and function of AOPP has already been studied in various biological matrices [16], only our preliminary study [18] investigated the AOPP content in seminal plasma and its relationship with physiological semen parameters. In that study we investigated the relationship between biomarkers of oxidative stress in SP and the kinetic characteristics of fresh semen and observed that thiols and AOPP are positively associated to fresh semen with higher motility. Carbonylation of proteins is the other mechanism of protein modification and a marker of oxidative damage to proteins. It is an irreversible transformation consisting of the formation of reactive ketones and aldehydes by different mechanisms: the direct oxidation of amino acid residues by ROS, or the addition of reactive aldehydes to proteins, formed from oxidized unsaturated fatty acids and oxidized glucides [16,19]. A higher content of carbonylated proteins in semen was observed in infertile human patients and after bull cryopreservation [10,20,21].

As the understanding of the relationship between and the SP metabolome, its ability to protect against oxidative stress, and the functional parameters of bull sperm is still incomplete, this study aims at investigating the association between sperm cells' functional parameters both in freshly ejaculated and cryopreserved bull semen and the metabolome and the oxidative status of SP. In particular, this work explored the motility of fresh semen and other functional parameters in frozen-thawed bull sperm in relation to the metabolome and indicators of protein oxidation in SP.

2. Materials and methods

Semen samples, partially analyzed in the previous study [18] were used to investigate motility and viability, mitochondrial integrity, mitochondrial membrane potential, the intracellular superoxide and hydroperoxide at 0 h and 3 h after thawing and the metabolomic profile of SP. Briefly, 3 ejaculates were obtained from each of 20 young Holstein bulls aged between 10 and 12 months and housed at the Intermizoo A.I. station (Caorle, Italy). Semen was collected using an artificial vagina and was diluted 1:1 with the pre-warmed (+37 °C) commercial extender. An aliquot of seminal plasma, obtained after centrifugation was stored at −80 °C for metabolomic and oxidative stress analyses, as described below. After dilution and collection of the SP aliquot, semen samples were immediately cryopreserved, as follows. The diluted semen samples were packed into 0.5 mL labelled plastic straws (50–80 × 10^6 sperm/mL). This procedure was performed at 4 °C. Afterward, the straws were transferred to a programmable freezer. The freezing program consisted of the following rates: −4 °C/min from 4 °C to 0 °C, −1 °C/min from 0 °C to −4 °C, −12 °C/min from −4 °C to −40 °C, −30 °C/min from −40 °C to −140 °C. The straws were finally plunged into liquid N_2 (at −196 °C) for further storage.

2.1. Seminal plasma oxidative status

The oxidative status measured in the study by Vigolo et al. [18] were used in this study to investigate their relationship with flow cytometry parameters of thawed semen. The concentration of proteins (mg/nmol), AOPP (nmol/mL), thiols (nmol/mL) and protein carbonyls (nmol/mL), were evaluated as reported in the previous study [18].

Protein concentrations in SP were measured by the BCA method (BCA Protein as-say kit; Pierce Biotechnology, Rockford, IL, USA), following the manufacturer's instructions. The AOPP concentration was measured spectrophotometrically adding the acetic acid to the samples and measuring the absorbance after the reaction at 340 nm in a microplate reader. Protein carbonyls were measured following derivatization with DNPH (2,4 dinitrophenylhydrazine; Sigma-Aldrich Co., St, Louis, MO, USA). The carbonyl content was determined by reading the absorbance at 380 nm with a spectrophotometer. Thiol concentration in SP (nmol/mL) was determined according to the thiol/disulfide reaction using Ellman's reagent. Thiol concentration was measured by reading the absorbance at 412 nm after the addition of 3.5 μL of Ellman's reagent (Sigma), which was expressed as nmol/mL SP.

2.2. Seminal plasma metabolomics (^{1}H NMR analysis)

Seminal plasma samples were thawed at room temperature immediately before ^{1}H NMR analysis and centrifuged at 12,000×*g* for 30 min at 4 °C. Then, 450 μL of each supernatant were diluted to the final volume of 600 μL with 90 μL of potassium phosphate buffer (1.5 M, pH 6.80), containing 5 mM sodium azide (NaN_3) as antibacterial agent, and 60 μL of D_2O + TSP-d4 solution (deuterium oxide 99.99 % with 40 mM 2,2,3,3-d4-3-(trimethylsilyl)-propionic acid sodium salt). The potassium phosphate buffer was added to minimize pH differences between samples (final pH of diluted samples = 6.82 ± 0.03, mean ± sd), while the TSP-d4 was used as chemical shift reference for ^{1}H NMR spectra. The samples (600 μL) were transferred into 5 mm ^{1}H NMR tubes to be analyzed using a Bruker Advance III spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 300.13 MHz (proton Larmor frequency) and equipped with a ^{1}H–^{13}C probe (5 mm diameter) with Z gradient facility. The ^{1}H NMR spectra were acquired using two different one-dimensional (1D) pulse sequences: the 1D Noesy sequence (noesygppr1d) and 1D Carr–Purcell–Meiboom–Gill (CPMG) spin-echo sequence (gppr1d). While the Noesy sequence does not use any digital filters, the CPMG sequence uses them to attenuate the broad signals from macromolecules, which can cover the signals of low molecular weight species (metabolites). Before acquiring the ^{1}H NMR spectra with the above sequences, a pre-saturating pulse sequence (zgpr) was used to find for each sample the conditions to suppress the wide and intense peak of water. TopSpin 3.6.4 software (Bruker Bio-spin) was used for spectra acquisition and processing. The acquisition parameters were 64 scans for Noesy, 64 and 512 scans for CPMG, 64 K data points, spectral width of 20 ppm (6010 Hz), 4 dummy scan, 4 s as relaxation delay, and 300 ± 0.1 K as constant temperature. A line broadening of 0.3 Hz was applied after the Fourier transform and all the acquired 1D spectra were aligned by setting the TSP peak at 0.0 ppm. To allow the sample temperature to stabilize, 5 min were waited between inserting each ^{1}H NMR tube into the probe and starting spectra acquisition. A preliminary identification

of metabolites in seminal plasma samples was performed by comparing the measured chemical shifts and split patterns of proton signals with information available in online databases (BMDB - Bovine Metabolome Database; HMDB - Human Metabolome Database; LMDB - Livestock Metabolome Database; ChemicalBook) and literature [6,22,23]. To confirm the metabolic profile obtained, standard solutions at known concentrations of the pure metabolites (Sigma-Aldrich) were prepared in order to acquire their ^{1}H NMR spectra and compare their signals with those present in the spectra of seminal plasma samples. The same standard solutions were also used to conduct spike-in experiments: by adding an appropriate volume of a metabolite's standard solution to a seminal plasma sample and repeating the acquisition of the sample's ^{1}H NMR spectrum, the presence of the metabolite under investigation can be confirmed by appreciating the increase in intensity of the corresponding spectral signals.

2.3. Sperm functional evaluation

Freshly ejaculated semen was analyzed for motility (the results has been already reported in the study by Vigolo et al. [18], whereas thawed semen (3 ejaculates for each bull) was evaluated for motility and flow-cytometric parameters at 0 h and 3 h after thawing.

Sperm samples were assessed for motility by a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12), using the standard bull parameters as reported by Vigolo et al. [18].

Flow cytometric analyses were performed on thawed semen (3 ejaculates for each bull) immediately and 3 h after thawing process, according to recommendations of the International Society for Advancement of Cytometry [24] with a BD FACS Calibur cytometer (Becton Dickinson, Milan Italy) with an argon ion laser 488 nm emission and a 635 nm emission red diod laser. Debris (non-sperm events) were gated out on the basis of forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest; a total amount of 10,000 cells gated in were recorded. Emission of each fluorescent probe was detected using different filters: 530/30 band-pass (green/FL1), 585/42 band-pass (orange/FL2), >670 long pass (far-red/FL3) and 661/16 band-pass (orange for red laser/FL4). Sperm samples were diluted using Tyrode's modified medium at a final concentration of 1×10^{6} spz/mL. In CM-H_2DCFDA/PI, DHE and MitoSOX assessments, percentages of non-DNA containing particles (debris) were determined to avoid an overestimation of sperm particles as described by Petrunkina et al. [25].

Sperm viability was assessed by checking the membrane integrity using two separate fluorochromes SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). SYBR-14 is a membrane-permeable dye, which stains the head of viable spermatozoa in green, while PI is a membrane-impermeable dye that only penetrates through disrupted plasma membrane, staining the sperm heads of non-viable cells in red. Sperm samples were stained with 5 μL SYBR-14 working solution (final concentration: 100 nM) and with 2.5 μL of PI (final concentration: 12 μM) for 10 min at 37 °C in darkness. Viable sperm exhibited a positive staining for SYBR-14 and negative staining for PI (SYBR-14+/PI-). Single-stained samples were used for setting the voltage gain for FL1 and FL3 photomultipliers.

CM-H2DCFDA is a non-fluorescent agent that accumulates in the cell cytoplasm due to deacetylation and emits green fluorescence upon oxidation by H2O2, detected by FL1 photomultiplier, being converted to the highly fluorescent 20,70-dichlorofluorescein (DCF). This staining was coupled with PI that stains sperm with disrupted plasmalemma (dead sperm) emitting red/orange fluorescence detected by the FL3 photomultiplier. Sperm samples were diluted in 500 mL of Tyrode's medium and stained with 2.5 mL CM-H2DCFDA (in DMSO, 50 mM final concentration), 2.5 mL PI (in water, 2.4 mM final concentration). Samples were incubated at 37 °C for 30 min in the dark. H_2O_2 production was assessed by H_2CDFDA oxidation in viable cells.

Dihydroethidium (DHE) is a cell-permeable dye emitting blue wavelength when not oxidized and in orange/red (610 nm) when oxidized mainly by superoxide radicals ($O_2^{\bullet-}$), detected by the FL2 photomultiplier; the staining was coupled with YOPRO1, that stains in green apoptotic cells, by the FL1 photomultiplier. In brief, sperm samples were diluted in 500 mL of Tyrode's medium and stained with 2.5 mL of 50 mM DHE (in DMSO, 5uM final concentration) and 2.5 mL of YO PRO 1 (in DMSO, 100 nM final concentration). Samples were incubated at 37 °C for 30 min in the dark and O_2^- production was assessed on non-apoptotic cells.

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was used to evaluate mitochondrial membrane potential. After the compound enters mitochondria with high membrane potential, JC-1 forms multimers (known as J-aggregates) and emits orange fluorescence at 590 nm, which is detected by FL-2 photomultiplier. In contrast, when mitochondria have low membrane potential, JC-1 maintains its monomeric form (M-band) and emits green fluorescence at 530 nm, which is detected by FL-1 photomultiplier. Sperm samples were stained with 2.5 μL JC1 (at a final concentration of 1 μg/mL); samples were successively incubated at 37 °C for 20 min in the dark. Cells were analyzed in a FL-1/FL-2 plot. High mitochondrial membrane potential cells (HMMP) stained orange (higher FL-2) and low mitochondrial membrane potential cells (LMMP) stained green (higher FL-1).

MitoSOX Red is a lipid-soluble, cell-permeable cation that selectively targets the mitochondrial matrix and can detect superoxide radicals (O2•-) generation in this organelle. MitoSOX Red (MX) emits red fluorescence upon oxidation, detected by the FL 3 photomultiplier. It was coupled with YO PRO 1 as a counterstaining to distinguish sperm with early changes in membrane permeability subpopulation from the intact ones. YO PRO 1 stains sperm with early changes in membrane permeability and emits green fluorescence upon binding to DNA, detected by the FL-1 photomultiplier. The MitoTracker deep red probe was included to assess mitochondrial integrity simultaneously. MitoTracker is excited by the red diode laser, and the FL-4 photomultiplier detects its fluorescence.

Sperm samples were diluted with Tyrode's and stained with 2.5 μL YO PRO 1 (in DMSO, 100 nM final concentration), 1 μL MitoSOX (in DMSO, 1 μM final concentration), and 2.5 μL M T. (in DMSO, 100 nM final concentration). Samples were incubated at 37 °C for 30 min in the dark.

The mitochondrial production of ROS by non-apoptotic/live cells with intact mitochondria was recorded in this analysis. In this study, we used the population of live sperm (ignoring YO PRO 1+ events), distinguishing cells with high and low O2•- generation in the subpopulations with intact mitochondria: MX + MT+, MX–MT+, MX + MT–, MX–MT–. The population with mitochondrial superoxide production (considering both cells with active and non-active mitochondria) was named MXpos.

2.4. Statistical analyses

Statistical analyses were performed using R (version 4.3.0). Values are expressed as mean ± standard deviation (SD), unless otherwise specified, and level of significance was set at $P < 0.05$.

First, all traits were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests. When necessary, data were normalized using a box-cox transformation [25]. The ability of SP oxidative status to predict semen traits assessed by CASA and flow cytometry was investigated by a set of generalized linear models using the function glm in the package *lme4* of the R software, in which SP traits (AOPP, thiols and C) were used as predictors and semen quality traits as response variables; time after thawing (2 levels: 0 h; 3 h) was used as fixed-effect factor. Despite having repeated observations for each bull, the random effect of the bull (i.e., the common effect that a given bull exert on each of their ejaculates) was omitted from the model: including this effect in the statistical models under study was not appropriate

because the aim was testing the ability of such models to serve as prediction tools of semen quality for future samples, and the nature of bull effects is random (for future semen samples, the quantitative effects of the bull on the depended variables are unknown when the prediction is made). Fresh semen was classified into Higher Motility Fresh semen (HMF) and Lower Motility Fresh semen (LMF), according to TM and PM values as in the study by Ref. [18], whereas thawed semen was assigned to Higher Motility Thawed group (HMT) or Lower motility Thawed group (LMT) according to TM and PM at 0 h post-thawing. Briefly, sperm motility parameters (VAP, VSL, VCL, ALH, BCF, TM, and PM), measured in both FR and TH samples, underwent principal component analyses (PCA). Then, the first three principal components (PCs), which explained more than 90 % of the variance, were used to perform k-means cluster analyses on both FR and TH samples. As a result, two clusters were obtained for the FR samples, HMF (n = 38) and LMF (n = 22) groups, and two clusters were obtained for the TH samples, HMT (n = 45) and LMT (n = 15) groups.

Mixed linear models were performed to investigate the difference in flow cytometry traits between the thawed motility semen groups, using bull as the random effect factor, and post-thawing storage time (2 levels: 0 h and 3 h) and motility thawed groups (2 levels: HMT and LMT) as fixed effects factors.

2.4.1. ^{1}H NMR spectra editing

In order to reduce the data dimensionality and correct for peak shifting [26], ^{1}H NMR spectra variables were divided into evenly spaced windows (0.05 ppm) named bins. The intensities inside each bin were summed, so that the area under each spectral region was used instead of individual intensities. Therefore, a new smaller set of variables (each one being the result of the sum of intensities) was created and the width of the buckets was set to cover the chemical shift variability around the peaks, reducing their misalignment. After binning and elimination of 54 bins which signal intensity was close to 0 with very low variation, the total number of ^{1}H NMR spectra variables was 146. Outlier inspection was performed by PCA. The standardized Mahalanobis distance (GH) was used to determine the distance of each sample from the centroid and based on this criterion, one sample with GH > 3 was removed as an outlier. Hence, 51 samples were used for further analyses.

Each of the ^{1}H NMR spectra variables was adjusted for the random effect of the bull using a linear mixed model and the residuals of the model were used for the prediction of SP oxidative status, flow-cytometric traits and motility groups (before and after thawing). Among ^{1}H NMR variables, the effect of the bull explained on average 56 % of the variance, and it explained at least 16 % of the variance in the 90 % of the variables.

2.4.2. Prediction of SP oxidative status and flow-cytometric traits by ^{1}H NMR spectra

Values of SP oxidative status and flow-cytometric traits were predicted by the 146 ^{1}H NMR spectra variables, after their adjustment for the random effect of the bull. In preliminary analyses, three multivariate prediction algorithms were tested: partial least squares regression (PLS), sparse partial least squares regression (s-PLS), and orthogonal projections to latent structures (o-PLS). Data were mean-centered and divided by the standard deviation of each variable before the analysis. The number of variables tested in s-PLS ranged from 20 to 180, with a 20-variable increase at each iteration. The optimal number of components was identified based on the decrease in the prediction error rate provided by each additional component. PLS, s-PLS, and o-PLS analyses were performed in the R software (R Core Team, 2023), using the packages "*PLS*", "*MixOmics*", and "*ropls*", respectively, and in-house scripts. The prediction accuracy (measured by the R^2) of each method was tested in a 4-fold random cross-validation. In preliminary analyses, the PLS algorithm provided the highest accuracy and was then selected as the method of choice. The variable importance in projection (VIP) scores were estimated to identify the spectral variables that mostly contributed to the prediction. They reflect both the loading weights for each component and the variability of the response explained by this component [27,28]. Commonly, a variable with a VIP score greater than 1 is considered important in a given model [29].

2.4.3. Prediction of motility groups (before and after thawing) by ^{1}H NMR spectra

Differences in the ^{1}H NMR spectra between motility groups were preliminarily inspected by an unsupervised method (PCA) to highlight possible clusters (data not reported). In preliminary analyses, PLS discriminant analysis (PLS-DA), s-PLS-DA and o-PLS-DA were performed to maximize the separation between groups. PLS-DA, s-PLS-DA, and o-PLS-DA analyses were performed in the R software (R Core Team, 2023), using the packages "*mdatools*", "*MixOmics*", and "*ropls*", respectively. The motility group was modeled as the sole response variable and ^{1}H NMR spectra variables adjusted for the random effect of the bull as predictors. Data were mean-centered and divided by the standard deviation of each variable before the analysis. s-PLS-DA algorithm provided the highest accuracy and was then selected as the method of choice. The optimal parameters (number of components and number of variables) in the model were identified using a 4-fold random cross-validation repeated for 50 times, based on the maximization of the Mahalanobis distance between groups and minimization of the misclassification error rate. The maximum number of components tested was 20, whereas the number of variables tested ranged from 20 to 140, with a 20 variable increase at each iteration. After model tuning, the optimal number of components used were 9 and 1, for fresh and thawed semen, respectively. The error rate across number of components and the number of selected variables for each component, are reported in the Supplementary material (Supplementary Fig. 1a and b). The final s-PLS-DA models used all the tuned parameters. The overall error rate (measured as the proportion of correctly identified samples to the total number of samples) and the balanced error rate (measured as the average proportion of wrongly classified samples in each class, weighted by the number of samples in each class) of final models were tested in a 4-fold random cross-validation as used as measures of model accuracy. The variables with the 12 highest VIP scores, representing the spectral variables contributing the most to the variance between groups, were identified and discussed.

3. Results

3.1. Relationship between flow cytometry parameters and motility groups of thawed semen

As shown in Table 1 the differences between HMT and LMT groups, at 0 h and 3 h after thawing, in terms of flow-cytometry parameters. Overall, incubation time affected all flow cytometry parameters, decreasing the quality of thawed semen. The percentage of live cells negative for cytoplasmic superoxide and peroxide production was higher in HMT groups and decreased after 3 h storage in HMT group. In live cells, no differences on mitochondrial integrity (Live MT+) and superoxide production (Live MX+) were observed between the HMT and LMT groups. A significantly higher percentage of sperm with higher mitochondrial membrane potential (HMMP population) was observed in the HMT groups than in the LMT.

3.2. Relationship between oxidative status of SP and thawed semen parameters

Significant positive correlations were found between thiols and AOPP concentrations ($r = 0.82$, $p < 0.05$), while protein carbonyls negatively correlates both with thiols ($r = -0.60$, $p < 0.05$) and AOPP ($r = -0.65$, $p < 0.05$). In addition, TM and PM significantly ($p < 0.05$) positively correlate with low superoxide production (LIVE_DHE-, $r = 0.69$) in live sperm and higher mitochondrial membrane potential sperm

Table 1
Motility and flow-cytometry parameters of frozen-thawed semen in high and low motility groups (HMT and LMT, respectively) at 0 h and 3 h storage time.

	0 h		3 h	
	HMT	LMT	HMT	LMT
TM	56.14 ± 7.77[a]	24.27 ± 9.75[b]*	38.01 ± 12.06[a]	15.53 ± 8.42[b]
PM	32.59 ± 6.55[a]*	11.47 ± 5.25[b]*	17.06 ± 7.76	5.7 ± 4.18
LIVE	53.77 ± 8.14[a]*	41.92 ± 16.06[b]*	44.49 ± 11.12[a]	32.07 ± 12.44[b]
LIVE DHE neg	50.42 ± 9.39[a]*	29.99 ± 15.52[b]*	35.48 ± 11.17	20.47 ± 19.53
LIVE DHE pos	1.74 ± 1.89*	3.73 ± 3.9	4.41 ± 6.17	2.66 ± 1.59
LIVE H_2CDFDA neg	56.1 ± 8.65[a]*	39.51 ± 14.40[b]	46.54 ± 11.17[a]	30.97 ± 19.99[b]
LIVE H_2CDFDA pos	0.27 ± 0.41*	0.21 ± 0.38	0.72 ± 0.29	0.16 ± 0.24
HMMP	45.91 ± 12.19[a]	36.59 ± 17.89[b]	39.29 ± 12.09[a]	25.34 ± 16.12[b]
Live Mtpos	86.27 ± 14.97*	82.66 ± 17.16*	78.83 ± 17.63	72.27 ± 16.74
Live MXpos	1.97 ± 2.34*	2.98 ± 2.48	8.75 ± 11.40	7.13 ± 5.67

The lower-case letters indicate significantly different means ($p < 0.05$) for each variable between groups (HMT vs LMT) within the same storage time. Asterisks indicate significantly different means ($p < 0.05$) for each variable between storage time (0 h and 3 h) within the same group.

subpopulation (HMMP, $r = 0.45$). On the other hand, TM, PM and LIN negatively correlate with low mitochondrial activity in living cells (MTnegMXneg, $r = -0.48$ for TM and PM; $r = -0.42$ for LIN).

As shown in Fig. 1, AOPP concentration affected semen quality parameters. Seminal plasma with increasing concentration of AOPP was associated to higher TM, PM, and LIN of thawed semen. In addition, live sperm obtained from SP with higher concentration of AOPP were characterized by lower production of mitochondrial and cytoplasmic superoxide after thawing. Higher amounts of AOPP in SP was positively associated with the percentage of viable cells.

As shown in Fig. 2. SP with higher thiols concentration (nmol/mL) were related to an increase of TM, PM and a decrease of mitochondrial superoxide production on thawed semen. In addition, SP with a higher concentration of thiols was associated with lower cytoplasmic hydroperoxide production in live cells (low H_2DCFDA fluorescence).

In contrast, viable sperm with a higher mitochondrial superoxide production (Mxpos) and a lower TM and PM presented higher concentration of C in SP, whereas a lower cytoplasmic H_20_2 production (Live H_2DCFDAneg) was associated with a lower C concentration in SP (Fig. 4). In addition, an increase in apoptotic cells with higher cytoplasmic superoxide production (Apoptotic DHEpos) was observed with increasing concentration of C in SP (Fig. 3).

After 3 h storage time a decrease of all kinematics and an increase of intracytoplasmic ROS and mitochondrial superoxide production was observed.

3.3. Characterization of the metabolome of SP

A total of 29 metabolites were identified in the ^{1}H NMR spectra of seminal plasma samples: acetate, acetone, alanine, arginine, aspartate, carnitine, glycerophosphocholine, citrate, isopropanol, glycerol, creatinine, ethanol, formate, fructose, fumarate, GABA, glucose, glutamate, histidine, isoleucine, lactate, leucine, lysine, phenylalanine, proline, propynoic acid, tyrosine, uridine, and valine.

3.4. Relationship between ^{1}H NMR spectra of SP and motility groups of fresh and thawed semen

Among ^{1}H NMR variables, the effect of the bull explained on average 56 % of the variance, and it explained at least 16 % of the variance in the 90 % of the variables. Differences between motility groups were first investigated using the unsupervised multivariate approach of PCA. The first three principal components accounted for 32.6, 23.3, and 11.1 % of the total spectral variance, respectively. The effect of the group was not visible on the distribution of the PCA scores (data not reported). Scores of the s-PLS-DA for the classification of fresh semen obtained after model tuning are displayed in Fig. 4 a. The score plot shows a good separation between the two motility groups, dependent largely on components 2 and 3, with some overlapping. The accuracy of s-PLS-DA to discriminate between motility groups, as measured by the overall and balanced error rate, is reported in Table 2. Despite the score plot showing a good degree of separation between the two groups, the overall error rate of the model was 0.402, indicating a relatively limited accuracy of the model to classify samples not belonging to the training set. Nevertheless, the accuracy of the model was significantly different from a random allocation of the samples to each of the motility groups (error rate = 0.5). For thawed semen, the s-PLS-DA score plot after model tuning (Fig. 4 b) was obtained by plotting the scores of the first component, as the number of selected components was 1. The score plot showed a good separation between classes of motility. Compared to fresh semen, the accuracy of the model for the classification of thawed semen motility was more accurate. The overall error rate was 0.297, with a very similar value of balanced error rate. Moreover, both motility groups showed similar misclassification rates, with the one of the LMT group being slightly higher (0.36) than that observed in the HMT. The model performed significantly better than a random allocation of sample to the two categories.

The 12 ^{1}H NMR variables that contributed the most to the classification in high and low motility group of the fresh semen samples are reported in Fig. 5. Some of these spectral ranges were not identified because they are missing in the online libraries and are listed as "Unknown". In addition, in some spectral regions there is an overlap of several metabolites. Therefore, Fig. 5 identifies the 12 most representative metabolites in SP. The compounds identified in the ^{1}H NMR spectral bins with the highest VIP score for the prediction of fresh semen groups are formate, alanine, glycerophosphorylcholine, phenylalanine, lactate, and tyrosine. Some metabolites such as alanine, lactate and phenylalanine present a higher VIP score in several spectral ranges, confirming their importance in the prediction of fresh semen groups. The distribution of the ^{1}H NMR signal in each VIP, across motility groups in fresh semen, is reported in Fig. 6. Higher levels of formic acid, lactate, glycerol and phosphocholine, were found in the SP of the HMF group than in the LMF group. On the other hand, alanine, phenylalanine, and tyrosine were higher in the SP of the LMF group than in the HMF group.

The 12 ^{1}H NMR variables that contributed the most to the classification in high and low motility group of the thawed semen samples are reported in Fig. 7. GABA, glutamate and histidine and glycerol were found in higher concentrations in the HMT group than in the LMT group, while fructose decreased in the HMT group. The distribution of the ^{1}H NMR signal in each VIP, across motility groups in thawed semen, is reported in Fig. 8. As reported in the heat maps (Fig. 9 a and 9 b) significant positive correlations were found between the ^{1}H NMR ranges identified with the same compound such as alanine (ppm: 1.525; 1.475), lactate (ppm: 1.325; 1375) and phenylalanine (ppm: 7.325; 7.375).

4. Discussion

The relationship between SP components and bull semen quality has been investigated in previous studies, but few studies, to date, have examined the oxidative status and metabolome of SP plasma and their relationships with fresh and thawed bull sperm quality.

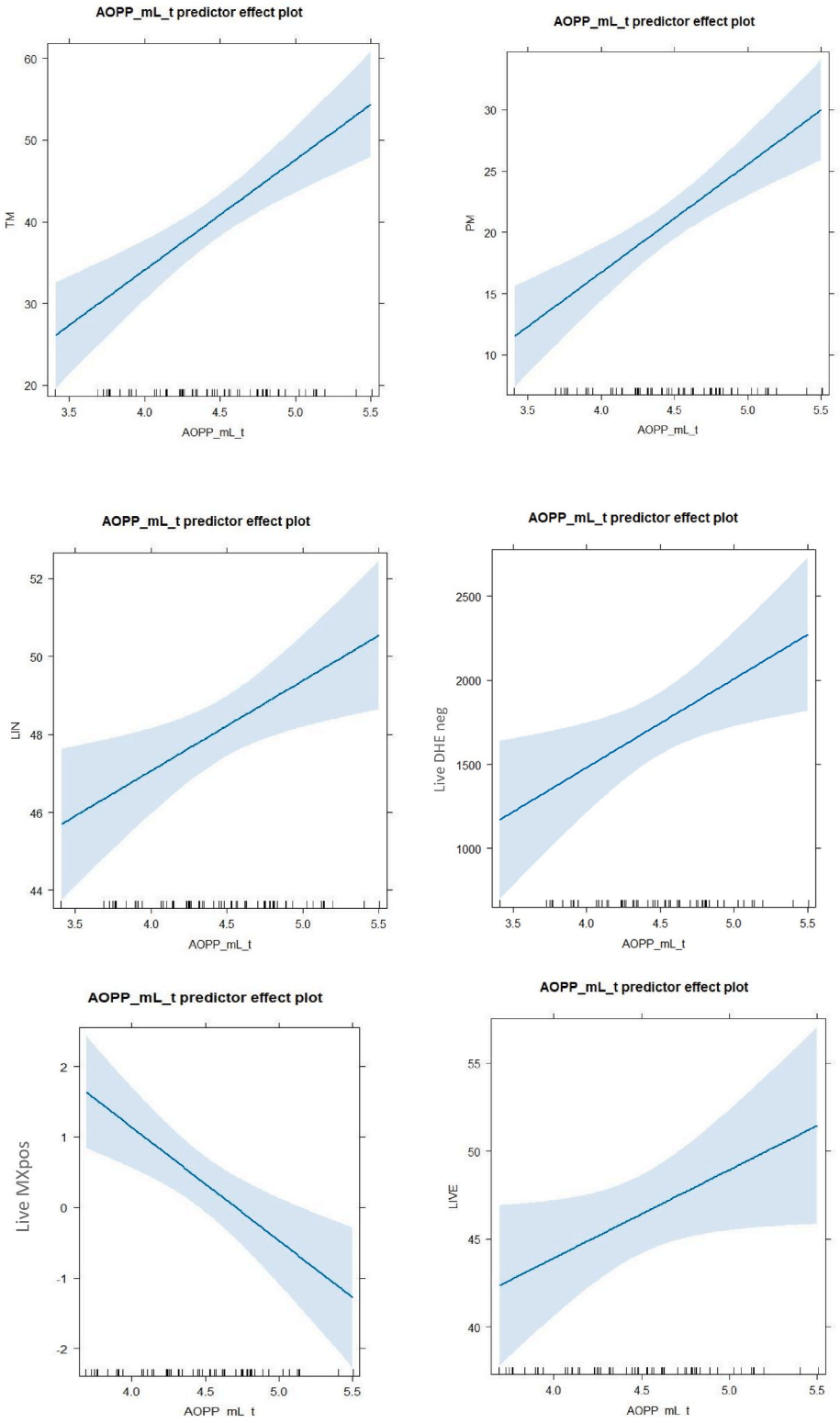

Fig. 1. Relationship between AOPP concentration in SP and quality parameters of thawed semen. Plots represent the fitted values on the vertical axis versus the focal predictor on the horizontal axis (AOPP (nmol/mL). The shaded area is a pointwise confidence band for the fitted values, based on standard errors computed from the covariance matrix of the fitted regression coefficients. The rug plot at the bottom of the graph shows the location of the AOPP (nmol/mL) values.

4.1. Different patterns of protein oxidation in SP alter semen quality parameters in a different manner

The results of this study add new information to our previous study [18], in which AOPP and thiol concentrations in SP were positively related with a few kinetic characteristics, as LIN and BCF, in freshly sperm characterized by high motility. A minimal part of SP is produced in the testes and epididymis during spermatogenesis, while at ejaculation secretions of the ampulla, seminal vesicle, prostate, and bulbourethral glands complete the SP composition. SP proteins play a

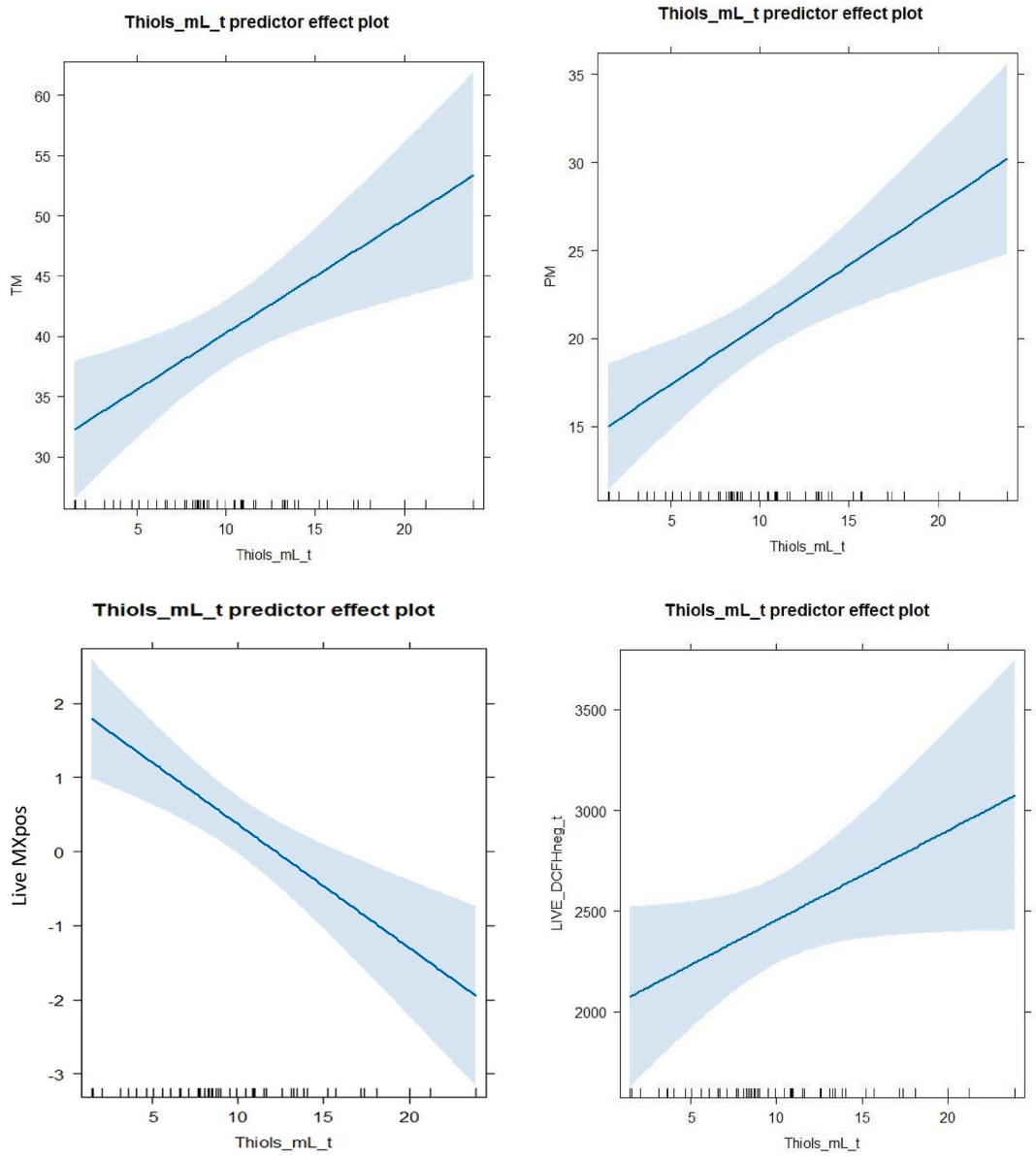

Fig. 2. Relationship between thiol concentration in SP and quality parameters of thawed semen. Plots represent the fitted values on the vertical axis versus the focal predictor on the horizontal axis (AOPP (nmol/mL). The shaded area is a pointwise confidence band for the fitted values, based on standard errors computed from the covariance matrix of the fitted regression coefficients. The rug plot at the bottom of the graph shows the location of the AOPP (nmol/mL) values.

fundamental role in the maintenance of numerous physiological processes such as plasma membrane stability, motility, capacitation, sperm-egg interaction and fertilization [3,30,31]. Many proteins, called sperm binding proteins, a major component of ruminant SP, bind to sperm, influencing membrane structure and sperm function, protecting sperm from cryopreservation damage, and affecting bull sperm fertilizing ability [32]. In the study by Gomez et al. [32] 445 proteins were identified in SP and it was demonstrated that they are differentially expressed in bulls with higher and lower semen freezability; additionally, specific potential biomarkers of sperm freezability were recognized within these proteins.

Contrasting results on the role of AOPP in SP are reported in human studies, in which higher levels of AOPP in SP were associated with male infertility by Kratz et al. [33,34], or with improved motility and morphology by Matschke et al. [35]. According to our results, the alteration of proteins through carbonylation or the formation of AOPP may have a different meaning. Our results determine a relationship between SP features and sperm cells' ones, in particular regarding the predictivity of some functional parameters such as motility or ROS production by sperm cells, given some specific information (AOPP, thiols, carbonyls levels in SP). Our data, anyway, do not permit to find the biological physiological relationship between these parameters, as it falls out of the scope of the present research. In addition, the positive relationship between AOPP and sperm viability and motility should be considered with prudence. Proteins can scavenge up to 75 % of free radicals and be oxidized in more than 35 ways [16]. In particular, AOPP in blood plasma increase because of neutrophil or monocyte activation [36], and represent useful markers of the enzyme myeloperoxidase, which catalyses the reaction between hydrogen peroxide and chlorine ions to produce hypochlorous acid, a potent oxidant. However, AOPP composition is heterogeneous, as they may contain chromophores induced by chlorinated oxidants, dityrosine and disulphide bridges, and carbonyl groups [16]. Therefore, spectrophotometric assays for the determination of AOPPs are unspecific, potentially detecting a wide range of optically absorbing species [37]. Indeed, testing the potential interference of different substances is the utmost importance when

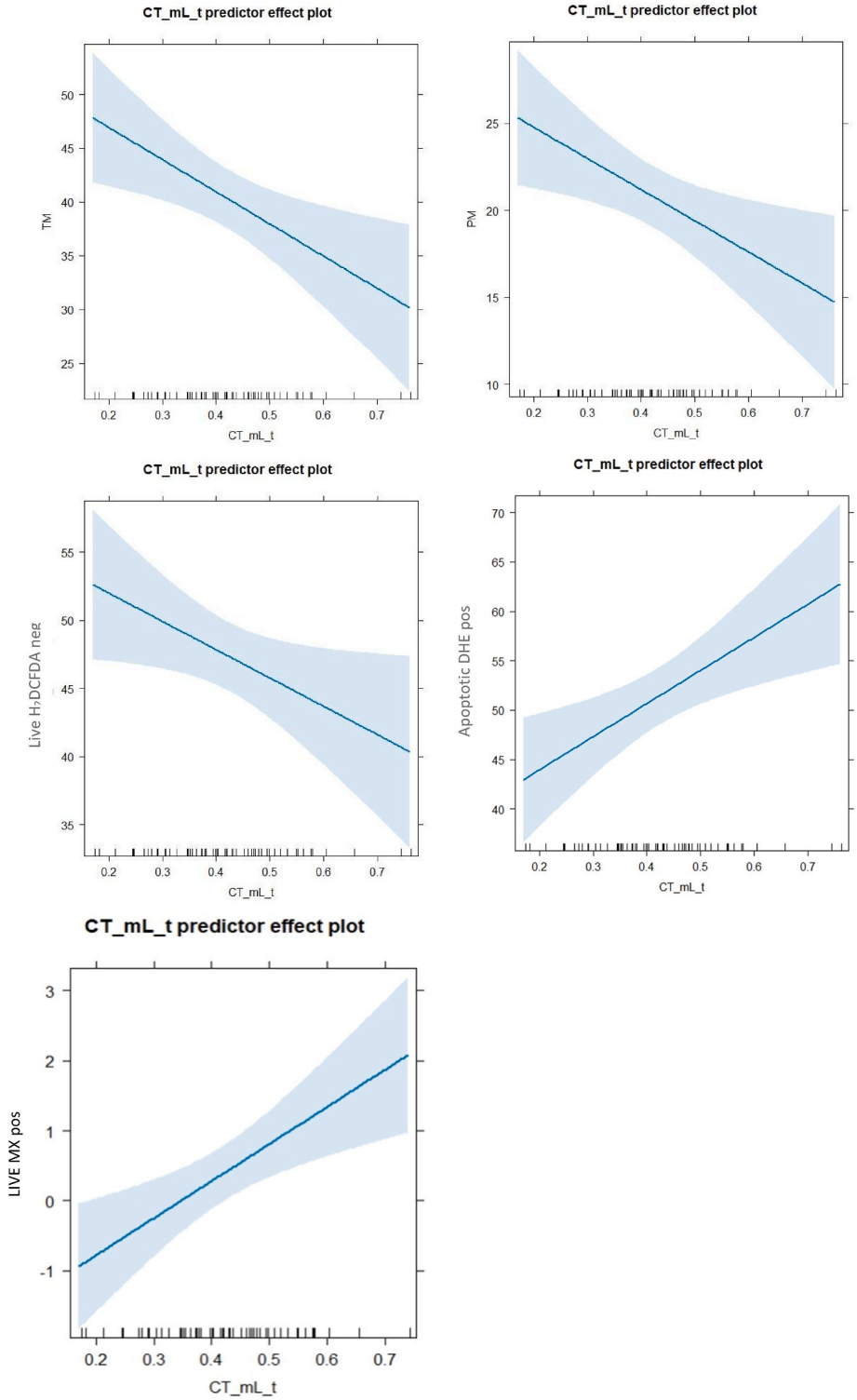

Fig. 3. Relationship between CT concentration in SP and quality parameters of thawed semen. Plots represent the fitted values on the vertical axis versus the focal predictor on the horizontal axis (CT, nmol/mL). The shaded area is a pointwise confidence band for the fitted values, based on standard errors computed from the covariance matrix of the fitted regression coefficients. The rug plot at the bottom of the graph shows the location of the CT (nmol/mL) values.

applying the AOPP assay to a new animal species or to a different biological matrix and, therefore, variations in AOPP content in SP need a thorough investigation.

According to our results, the total ROS production in the mitochondria and cytoplasm decreased when the concentrations of AOPP and thiols in SP increased. These findings corroborate the hypothesis that SP proteins could minimize or balance the intracellular ROS production by acting as ROS scavengers. The redox state regulation starts from the testis and epididymis and continues after semen ejaculation [38]. We can hypothesize that during the different phases of sperm maturation, the thiols and proteins of extracellular fluids could counteract intracellular ROS productions and together with the intracellular and extracellular antioxidant enzymes contribute to the maintenance of cellular redox homeostasis. This could help maintain the

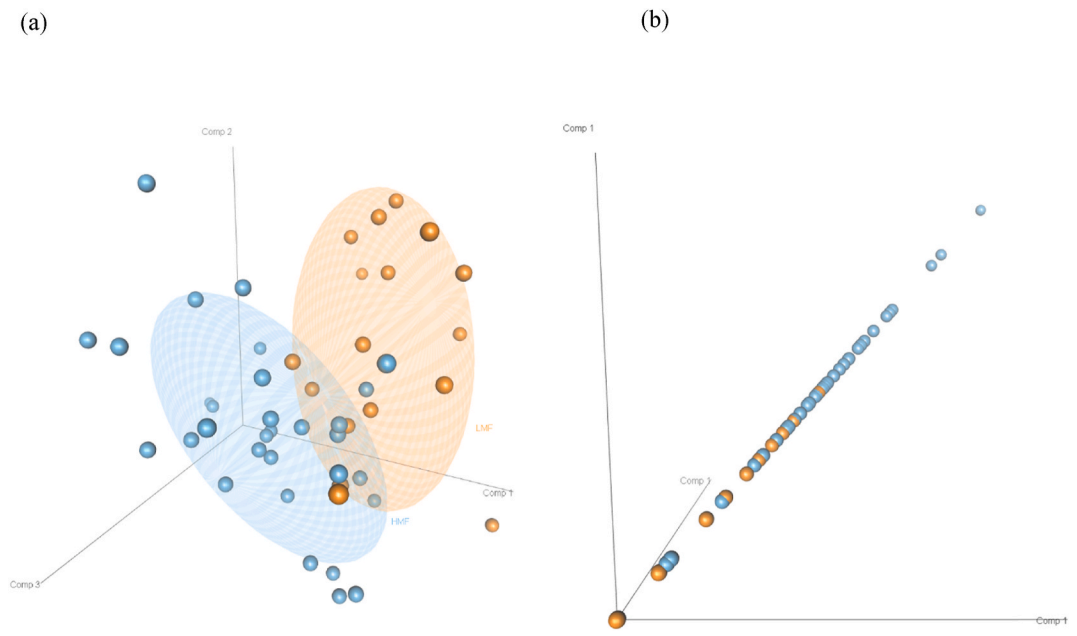

Fig. 4. ^{1}H NMR spectra sPLS-DA score plots for component 1, 2 and 3, after model tuning. Each point represents a SP sample. Samples were classified in two groups according to the motility of fresh semen (a) (HMF and LMF) and the motility of frozen-thawed semen (b) (HMT and LMT).

Table 2
Overall error rate (±SD), balanced error rate (±SD), and error rate for the high and low motility groups in fresh and thawed semen, across cross-validation folds of the s-PLS-DA model performed on the ^{1}H NMR spectra.

Error term	Fresh semen	Thawed semen
Overall error rate	0.402 ± 0.052	0.297 ± 0.030
Balanced error rate	0.455 ± 0.056	0.319 ± 0.040
Error rate in the high motility group	0.283	0.279
Error rate in the low motility group	0.628	0.360

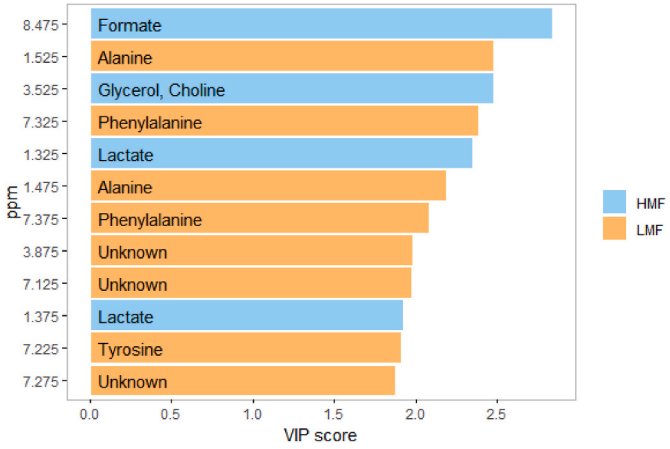

Fig. 5. Variable importance in projection (VIP) scores of the ^{1}H NMR bins with the 12 highest VIP score obtained by sparse partial least square discriminant analysis for the classification of fresh semen motility. Light blue and orange colors indicate a higher amount of the corresponding metabolite in the high (HMF) or low (LMF) motility group, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

functionality of cells in fresh sperm and reduce the deleterious effects of cryopreservation. It is known that, although bull glycolysis and oxidative phosphorylation can be used alternatively for sperm cells' energetic needs, motility can be maintained for some time by glycolysis, but should eventually be supported by mitochondrial activity at an optimal level [39]. This is confirmed by the higher percentage of HMMP sperm observed in the HMT group. As claimed in a recent study by Blanco-Prieto et al. [39], the freezing-thawing process leads to mitochondrial damage that uncouples the mitochondria of these cells. These results are also supported by our study, in which a moderate percentage of thawed cells with high mitochondrial superoxide production (Live--MXpos population) was found, and a low percentage of intracellular superoxide and peroxide production was observed in the same samples (Table 1). Although our results do not allow us to confirm the mechanism of action, other studies suggest that an induced protein response due to sublethal stress may be responsible for improved cryosurvival in gametes [40–42] The positive relationship between high sperm motility and thiol concentrations support the hypothesis that more energy-efficient sperm do not produce high superoxide radicals and, thus, less thiols are consumed in the SP. It is also possible, although speculative, that high quality semen is characterized by both energy-efficient sperm and thiol rich SP. Conversely, less energy-efficient sperm cells produce more superoxide radicals that can leak out from the plasma membranes and increases SP protein oxidation, resulting in higher C concentrations. In this sense, carbonylation of SP protein could be considered as a consequence of irreversible semen oxidative damage, as reported in the recent study by Renato de Oliveira Menegassi et al. [8], who found that thermal stress affects semen redox status and sperm quality parameters of breeding bulls by decreasing motility and increasing intracellular C and peroxide levels.

Further studies are needed to clarify the level of protein oxidation and thiols in sperm cells, in comparison to the protein oxidation of SP.

4.2. ^{1}H NMR metabolome of the bull SP

Although spectra obtained by a 300 MHz ^{1}H NMR spectrometer are characterized by a lower resolution compared with those generating higher magnetic fields, all spectrometers have the capability to acquire a wide range of molecular metabolite data, which subsequently undergo multivariate statistical evaluation and, thus, can provide a view of the biological processes at a specific time-point [43]. Although there are few studies investigating the metabolome of SP in relation to conception rate

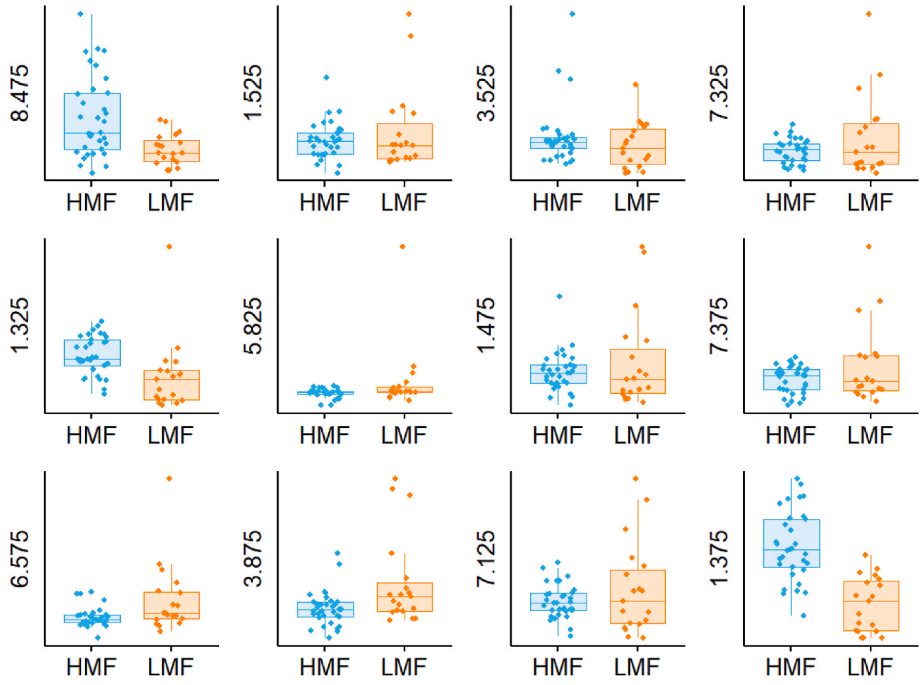

Fig. 6. Distribution of the ^{1}H NMR signal for the bins with the 12 highest values of variable importance in projection scores in the high (HMF) or low (LMF) fresh semen motility group.

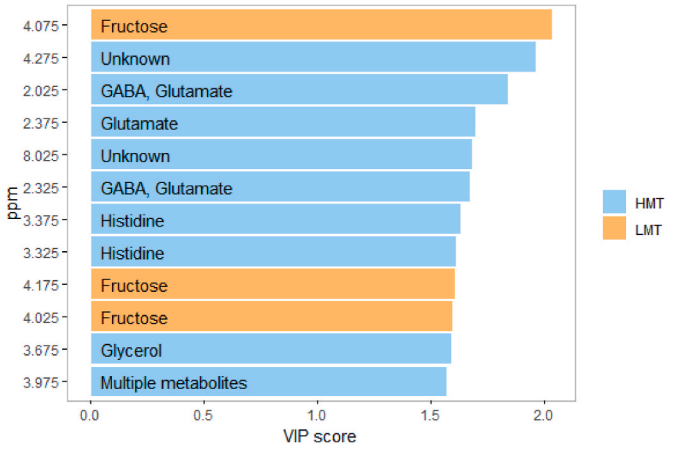

Fig. 7. Variable importance in projection (VIP) scores of the ^{1}H NMR bins with the 12 highest VIP score obtained by sparse partial least square discriminant analysis for the classification of fresh semen for the classification of thawed semen motility. Light blue and orange colors indicate a higher amount of the corresponding metabolite in the high (HMT) or low (LMT) motility group, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[5,44], to our knowledge this is the first study that investigates the relationship between the ^{1}H NMR metabolome of SP and the physiological characteristics of fresh and thawed bull semen. However, the study was conducted on 30 highly selected breeding bulls, with a narrow sperm motility range, so the differences between lower and higher quality semen may not be sufficiently emphasized.

The sPLS-DA analysis showed that the SP metabolome was different between the experimental groups, as it allowed a good separation between them. In the discussion, the first twelve bins (VIP score >1.5) responsible for the differences between high and low motile sperm from both fresh and thawed semen were considered. However, only metabolites clearly identifiable in one or more bins are discussed, as the 300 MHz spectra resolution and the bucketing process may lead to overlapping of signals from different metabolites within the same bin, which can affect the data analysis [26,43].

When considering the kinetic characteristics of the fresh sperm, the levels of formic acid, lactate and glycerol phosphocholine were higher in the SP of the HMF than in the LMF group. On the other hand, alanine, phenylalanine and tyrosine were lower in the SP of the HMF than in the LMF group. When considering the kinetic characteristics of thawed sperm, GABA, glutamate and histidine levels were higher in the HMT group than in the LMT group, while fructose levels were lower in the HMT group.

4.2.1. *Metabolites related to sperm energy metabolism*

Besides citric acid and fructose, lactate is one of the most abundant metabolites in bull SP [5]. It is the product of the glycolytic pathway which, together with mitochondrial activity, plays a central role in ATP production in bull sperm cell. A recent study found a higher concentration of lactate in sperm of high fertility bull indicating a more efficient utilization of the anaerobic glycolytic pathway in highly fertile bull sperm. Bull semen is equally dependent on glycolysis and oxidative phosphorylation to maintain intracellular energy status, in contrast to other species (e.g. swine, human and equine) that rely on mitochondrial ATP production to maintain motility [39,45]. The higher amount of lactate in SP of HMF semen is in line with a higher energy production through the glycolytic pathway and a lower utilization of this metabolite as a substrate for mitochondrial ATP production by the Krebs cycle [46].

Several ^{1}H NMR spectra ranges related to fructose were negatively associated with sperm freezability (Figs. 7 and 8). Although, these ranges associated with fructose (ppm = 4.025, 4.075 and 4.175) negatively correlated with those of glutamic acid and GABA (Fig. 7), as reported by Velho et al. [5], in our study it was observed a negative effect of fructose on the motility of thawed semen, which is not consistent with the positive effect of fructose on bull fertility based on the actual conception rates, observed by the same author [5]. Although fructose and other metabolites have been identified as biomarkers for bull fertility in previous studies, it is known that fertility and freezing ability are not always linked. The amount of fructose in the SP may depend on the ability of sperm to utilize it, and it has been observed that higher concentrations of fructose in SP correlate with lower sperm TM [47,48].

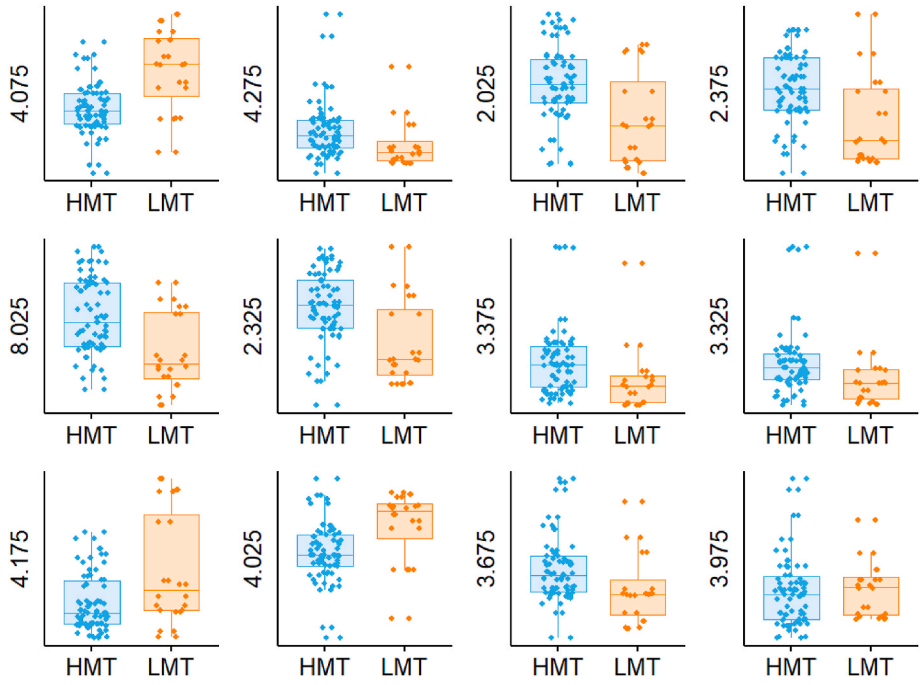

Fig. 8. Distribution of the ^{1}H NMR signal for the bins with the 12 highest values of variable importance in projection scores in the high (HMT) or low (LMT) thawed semen motility group.

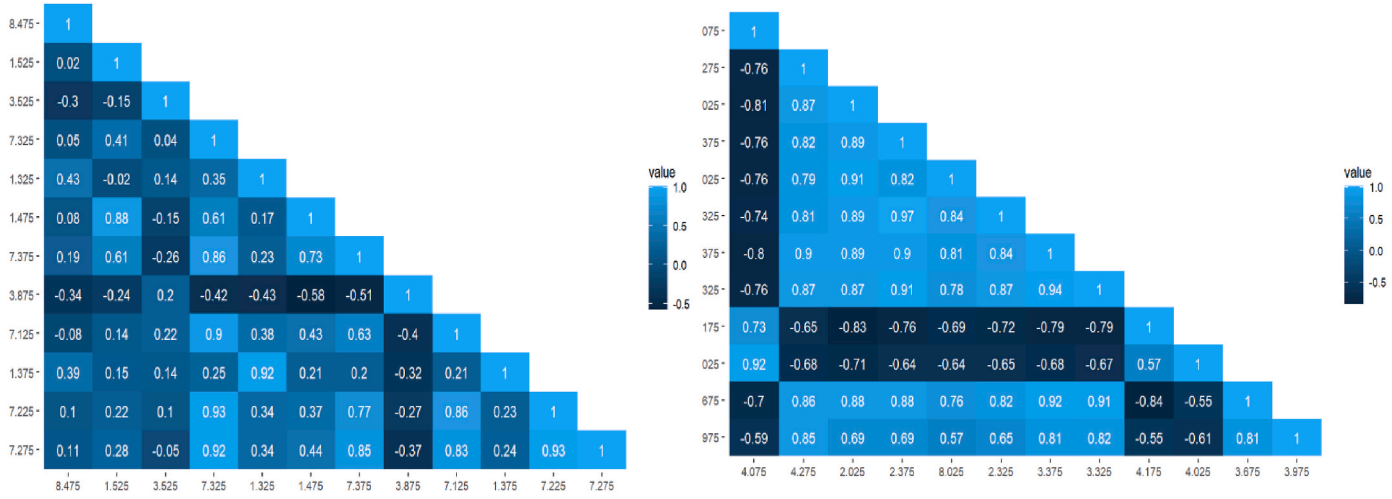

Fig. 9. Heatmaps of Pearson's correlations among the top 12 ^{1}H NMR bins for variable importance in projection score identified in bull seminal plasma for the prediction of fresh semen groups (A) and thawed semen groups (B).

Fructose is the major saccharide in the SP of ruminant, and it is synthesized from blood glucose by accessory sex glands stimulated by testosterone [30] and is used as a metabolic substrate in sperm to produce ATP. The greater amount of fructose in SP could therefore be associated with a reduced fructose utilization by the sperm or increased fructose production in seminal vesicles. The uptake of fructose monosaccharide occurs through specific protein carriers, the so-called GLUTs transporters [49]. As previous studies have shown, the activity and localization of GLUTs may change under different physiological conditions, such as during semen capacitation or storage protocols as cryopreservation (reviewed in Ref. [49]). These changes could modulate sperm metabolism and its freezability, but at present this is only a hypothetical explanation of this phenomenon, as our data do not allow us to certainly define this process. Once inside the cell, fructose is phosphorylated by ketohexokinase (KHK), and its metabolism can be faster than that of glucose under hypoxic conditions. In this sense, the fructolytic activity of sperm can overcome the mitochondrial damage that occurs after the cryopreservation process and improve the sperm motility of thawed sperm [50]. Therefore, we can hypothesize that an increased amount of fructose in SP may reflect a lower utilization of this substrate by sperm for ATP production, possibly due to differential expression and functionality of fructose transporters.

4.2.2. *Amino acid composition of SP is related to semen quality and freezability*

The content of alanine, phenylalanine and tyrosine in SP had a significant negative effect on the motility of the fresh sperm, while histidine, proline and glutamate were associated to a better semen freezability. Higher phenylalanine content (identified in the ranges of 7.325 and 7.375) in SP was associated with the LMF group (Fig. 5), while

in the study by Ugur et al. [51], phenylalanine was present in greater quantity in SP of bull with higher semen viability after thawing. This finding is supported by our results in thawed semen, where phenylalanine was one of the first 50 metabolites in SP with a VIP score >1.5 associated with the HMT group (data not shown). Phenylalanine is actively involved in the oxidoreductive process of cells [51] and, although its negative association with HMF semen is not clear, its beneficial effect on thawed semen could be due to the higher oxidative damage after the cryopreservation process, which could be counteracted by a higher concentration of phenylalanine in SP, leading to a higher motility of thawed semen.

Alanine, histidine and glutamic acid, which are associated to better freezability in our study, have already been used as cryoprotectants in various species due to their antioxidant activity and protection of the sperm surface [52]. The VIP values obtained in this study confirm the hypothesis put forward by Ugur et al. [51] of a possible cryoprotective effect of glutamic acid, the most abundant amino acid in bull SP (more than 53 % of total amino acids), followed by alanine, glycine, and aspartic acid. Curi et al. [53] reported the important role of glutamine, a derivative of glutamic acid, in various cellular functions such as maintenance of redox potential, energy metabolism and cellular integrity. In agreement with previous studies demonstrating the effect of GABA on sperm motility and acrosome reaction, through GABA A and B receptors [53,54], our results confirm that higher GABA concentrations in SP could improve sperm motility of thawed semen.

5. Conclusion

To summarize, this study has shown that the oxidative and metabolomic status of SP is related to the physiological properties of semen and its freezability. Considering that metabolomics and oxidative stress markers can precisely describe the physiological characteristics of a biological matrix, the investigation of novel biomarkers in different semen physiological phases, e.g. during testicular and epididymal maturation and after ejaculation, could be utilized in further studies to gain insights into the biochemical reaction networks during sperm maturation, seminal plasma production and semen ejaculation. Furthermore, given the significant impact of genetics and environmental conditions (e.g., different housing or diet of bulls) on reproductive performance, further investigation is warranted to explore how these aspects may influence the metabolome and oxidative status of seminal plasma, as well as the physiological parameters of both fresh and thawed semen.

CRediT authorship contribution statement

E. Giaretta: Writing – original draft, Methodology, Data curation. **A. Damato:** Methodology. **L. Zennaro:** Methodology, Investigation. **V. Bonfatti:** Validation, Formal analysis, Data curation. **B. Mislei:** Methodology, Investigation. **V. Vigolo:** Data curation. **M.E. Falomo:** Conceptualization. **F. Bertuzzo:** Resources, Investigat. **G. Gabai:** Writing – review & editing, Supervision. **D. Bucci:** Writing – review & editing, Investigation.

Ethical issues

According to the Italian law for the protection of experimental animals (Law Decree n. 26 issued on March 4, 2014, art. 2), the approval by an ethical committee is not required under the circumstances that this trial was carried out. The owner's informed consent has been correctly collected.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.01.015.

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