Differences in the proteome of stallion spermatozoa explain stallion-to-stallion variability in sperm quality post-thaw†

Differences in the proteome of stallion spermatozoa explain stallion-to-stallion variability in sperm quality post thaw

[1] Gemma Gaitskell-Phillips, [1]Francisco E. Martín-Cano, [1] José M Ortiz-Rodríguez, [2] Antonio Silva-Rodríguez, [1]Maria C. Gil, [1]Cristina Ortega-Ferrusola, [1]* Fernando J. Peña

[1] *Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain.*
[2] *Facility of Innovation and Analysis in Animal Source Foodstuffs, University of Extremadura, Cáceres, Spain.*

*Correspondence to Dr. FJ Peña, Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine University of Extremadura Avd. de la Universidad s/n 10003 Cáceres Spain. E-mail fjuanpvega@unex.es
phone + 34 927-257167
fax +34 927257102

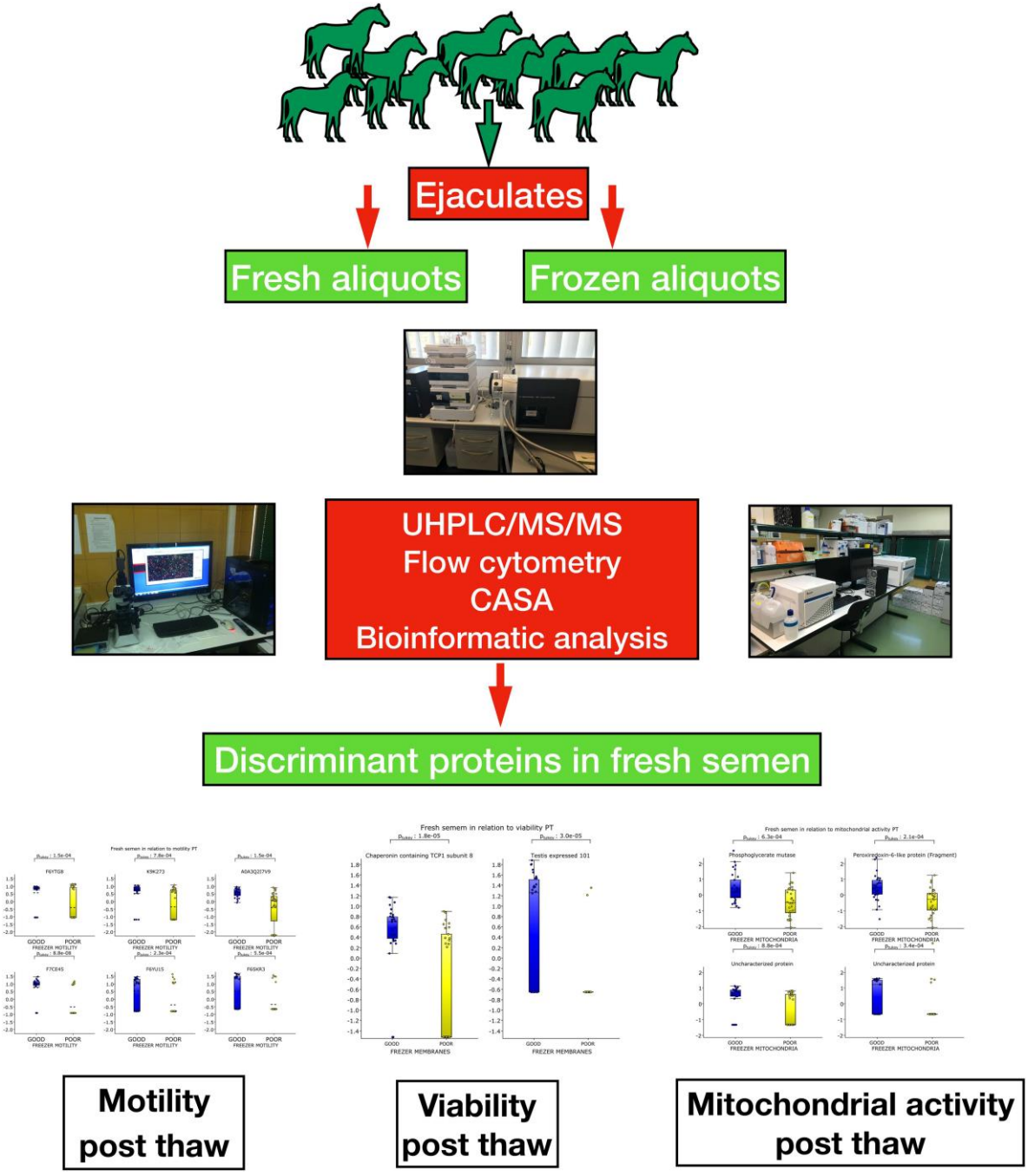
Acknowledgements

The authors received financial support for this study from the Ministry of Science-FEDER, Madrid, Spain, grants, AGL2017-83149-R and PID2019-107797RA-I00 AEI/10.13039/501100011033, Junta de Extremadura-FEDER (GR 18008 and IB16030). JMOR holds a PhD grant from the Junta de Extremadura- FEDER (PD 18005), Mérida, Spain. GG-P holds a PhD grant from the Ministry of Science, Madrid, Spain (PRE2018-083354).

Abstract

The identification of stallions and or ejaculates that will provide commercially acceptable quality post-thaw before cryopreservation is of great interest, avoiding wasting time and resources freezing ejaculates that will not achieve sufficient quality to be marketed. Our hypothesis was that after bioinformatic analysis, the study of the stallion sperm proteome can provide discriminant variables able to predict the post-thaw quality of the ejaculate. At least three ejaculates from 10 different stallions were frozen following a split sample design. Half of the ejaculate was analyzed as a fresh aliquot and the other half was frozen and then analyzed as a frozen-thawed aliquot. Computer-assisted sperm analysis and flow cytometry were used to analyze sperm quality. Detailed proteomic analysis

was performed on fresh and frozen and thawed aliquots, and bioinformatic analysis was used to identify discriminant variables in fresh samples able to predict the outcome of cryopreservation. Those with a fold change > 3, a P = 8.2e-04, and a q = 0.074 (equivalent to False discovery rate (FDR)) were selected, and the following proteins were identified in fresh samples as discriminant variables of good motility post-thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15, and F6SKR3. Other discriminant variables were also identified as predictors of good mitochondrial membrane potential and viability post-thaw. We concluded that proteomic approaches are a powerful tool to improve current sperm biotechnologies.



Key words: spermatozoa, UHPLC/MS/MS, proteins, artificial insemination, horse, flow cytometry, cryopreservation, CASA.

Introduction

Cryopreservation is still the most widely used technology for the long-term preservation of male gametes. This technique is applied both in human medicine and animal breeding. In particular, in the equine breeding industry it supports an important international trade of the genetics of the highest value stallions, either due to their morphological traits and/or performance in sports[1]. In spite of the fact that the technique has been in use since the second half of the past century [2], there are a number of unresolved problems, including, among others, the important breed and stallion to stallion variability regarding the capacity of ejaculates to maintain acceptable sperm quality after thawing [3]. During the cryopreservation process, spermatozoa are exposed to the toxicity of the cryoprotectants [4], to hyperosmotic shock during the process of freezing and to hypoosmotic shock during thawing [5, 6]. As a consequence, many spermatozoa succumb to osmotic induced necrosis during the procedure. A high percentage of the surviving population experience damage in their flagellar machinery, plasma membranes, and mitochondria leading to reduced functionality [1]. The molecular mechanisms behind cryodamage are osmotic induced necrosis and alteration of the redox regulation and metabolism in the surviving population of spermatozoa that leads to accelerated sperm senescence and eventually death. Osmotic stress disturbs mitochondrial membranes, leading to increased superoxide production. Once the redox equilibrium is lost, the excess of reactive oxygen species (ROS) attacks the lipids found in the membranes increasing the production of lipoperoxides that lead to oxidative damage in DNA and proteins causing sperm malfunction and/or demise. Moreover, non-lethal damage to spermatozoa may impact offspring [7]. The last decade has been witness to the incorporation of new techniques into the study of the sperm biology, particularly the application of proteomics is allowing a rapid advance in the knowledge of the molecular biology of these cells [8-11]. Proteomic analysis of sperm proteins has allowed numerous aspects of sperm biology to be unveiled, widening our understanding of these particular cells. These studies have revealed numerous changes in the sperm proteome related to different variables, including fertile vs infertile patients [12], identification of new roles of sperm proteins controlling early embryo development [13], new endogenous metabolic pathways [14] and have also identified differences in the proteome of fractions of the ejaculate with high and low motility [15-17]. Changes in the proteome in relation to cryopreservation have been described in different species [11, 18-20]. However, there is no data currently available on how the proteome of the ejaculated spermatozoa is related to the ability of the ejaculates to withstand cryopreservation. Identification of ejaculates that will present commercially acceptable quality after freezing and thawing will be of great interest to the equine

industry. Freezing ejaculates that will have to be discarded post thaw due to unacceptable quality post thaw causes a significant waste of time, money and resources that could be avoided if adequate markers of post thaw quality can be developed. Since proteomics is a powerful tool, it was hypothesized that cryopreservation may impact the proteome of good and poor freezers differently, and that the study of the proteome can detect specific proteins that can be used as discriminant variables in fresh samples to identify ejaculates with superior capacity to survive the cryopreservation process.

MATERIAL AND METHODS

Reagents and media

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated. Hoechst 33342, Ethidium homodimer, JC-1 and CellEvent™ reagents for flow cytometry were purchased from Thermofisher (Carlsbad, Ca USA). DRAQ7 was purchased from Beckman Coulter (Brea, Ca USA).

Semen collection and processing

Semen was collected from 10 stallions of various breeds maintained as indicated in specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). All stallions were of proven fertility, with a median age of 10.8 years old (range 5 to 19 years old), including 5 Andalusians, 1 Spanish Sport Horse, 1 Lusitano, 1 Arab, 1 Anglo-Arabian and 1 Spanish-Arabian horse. Semen was collected from all stallions on a regular basis (2-3 times per week), and ejaculates used in this study were collected after depletion of the extragonadal sperm reserves. The ethical committee of the University approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina. The gel was removed with an inline filter. Semen was immediately transported to the laboratory after collection for evaluation and processing. The experimental design employed a split sample approach, with single ejaculates divided in two subsamples (fresh and frozen thawed experimental groups). Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [21, 22] to remove dead spermatozoa, seminal plasma and contaminating cells, and then one aliquot was re-suspended in Tyrodes medium (20mM HEPES, 5mM Glucose, 96mM NaCl, 15mM $NaHCO_3$, 1mM Na-Pyruvate, 21.6 mM Na-Lactate, 2mM $CaCl_2$*2H2O, 3.1mM KCl, 0.4mM $MgSO_4$*7H2O, 0.3mM NaH_2PO4*H2O, 0.3% BSA) 315

mOsm/kg and pH 7.4 [23] (fresh extended semen), and the other aliquot was re-suspended in freezing media and frozen using standard procedures that have been previously described by our laboratory (frozen thawed semen) [24]. In brief the aliquot was diluted in the freezing medium, Cáceres (University of Extremadura, Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 $x10^6$ spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were sealed ultrasonically with UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) and immediately placed in an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The following freezing curve was used. Straws were kept for 15 min at 20ºC, and they were then slowly cooled from 20ºC to 5ºC at a cooling rate of 0.1 ºC/min. Thereafter the freezing rate was increased to -40ºC/min from 5ºC to -140ºC. The straws were then plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a water bath at 37°C for at least 30 s.

Experimental design

Three independent ejaculates from 10 different stallions were collected and processed as follows. Half of the ejaculate was frozen using standard protocols in our laboratory (frozen thawed (FT)), the other half was processed as fresh spermatozoa (Fresh).

Sperm preparation

The spermatozoa (fresh and FT samples) were washed three times in PBS (600g x 10') and fresh and FT samples pelleted and kept frozen at -80ºC until analysis.

Protein solubilization

Isolated spermatozoa (200 $x10^6$ spermatozoa) were solubilized in lysis buffer (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7M urea, 2M thiourea and 40 mM Tris (pH 10.4). 20 microliters of lysis buffer was added per each 10 x 10^6 spermatozoa vortexed and incubated under constant rotation at -4ºC for 1 h.

Protein Quantification

Protein quantification was performed using the 2-D Quant Kit (GE Healthcare, Sevilla Spain) following the manufacturer's instructions: https://www.gelifesciences.co.jp/tech_support/manual/pdf/80648622.pdf. All samples were normalized to obtain a final concentration of 100 µg of protein per sample.

In-solution Trypsin digestion.

200 µL of solution obtained from the previous stage were mixed with 100 µL of 25 mM ammonium bicarbonate buffer pH 8.5 (100µg of protein in 300µL of solution). In this solution, the proteins were reduced by adding 30 µL of 10 mM DTT and incubated at 56 °C for 20 min. The proteins were then alkylated by adding 30 µL of 20 mM IAA and incubated for 30 min at room temperature in the dark. Finally, digestion was performed by adding 1 µL of Trypsin Proteomics Grade (Sigma) (Trypsin solution: 1 µg/µL in 1mM HCl) for at least 3 h to overnight at 37 °C. The reaction was stopped with 10 µL of 0.1% formic acid and filtered through 0.2 µm (hydrophilic PTFE) to 2 mL dark glass vial. Finally, samples were dried using a nitrogen current with the vial in a heating block at 35ºC. The dry samples were resuspended in 20 µL of buffer A, consisting of water/acetonitrile/formic acid (94.9:5:0.1)

UHPLC-MS/MS analysis.

The separation and analysis of the samples were performed with a UHPLC/MS system consisting of an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high speed binary pump, and coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the UHPLC and Q-TOF was using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). The sample was injected onto an Agilent AdvanceBio Peptide Mapping UHPLC column (2.7 µm, 150 × 2.1 mm, Agilent technologies), thermostatted at 55 °C, at a flow rate of 0.4 ml/min. The gradient program started with 2% of B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) that remained in isocratic mode for 5 min and then increased linearly up to 45% B over 40 min, increasing up to 95% B over 15 min and remaining constant for 5 min. After this 65 min of run, 5 min of post-time followed using the initial condition for conditioning of the column for the next run. The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 l/min at a temperature of 250°C, and the sheath gas flow was set to

12 l/min at a temperature of 300 ºC. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500 V, 340 V and 750 V respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range were 50-1700 m/z and scan rates were 8 spectra/sec for MS and 3 spectra/sec for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of -4.8. The same ion was rejected after two consecutive scans.

Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH]+ 50–10000 m/z; maximum precursor charge +5; retention time and m/z tolerance ± 60 seconds; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against the appropriate and updated protein database (in this case: Uniprot/Horse) was performed using the following criteria: non fixed modifications were selected and as a variable modification: carbamidomethylated cysteines and tryptic digestion with 5 maximum missed cleavages were selected. ESI-Q-TOF instrument with minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation of reversed database scores. The autovalidation strategy used was auto-threshold, in which the peptide score is automatically optimized for a target % FDR (1.2%). Then the protein polishing validation was performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0 %).

Computer-Assisted Sperm Analysis (CASA)

Sperm motility and velocity were assessed in fresh and frozen thawed samples using a Computer-Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to standard protocols used at our center [25]. Semen samples were loaded into a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37°C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random

fields. Spermatozoa with a VAP > 35 μm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

Flow cytometry

Flow cytometry analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter, Brea Ca USA) equipped with violet, blue, yellow and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowjoV 10.6.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory [26, 27]

Measurement viability and mitochondrial membrane potential in stallion spermatozoa

Mitochondrial membrane potential and sperm viability were assessed simultaneously. Sperm aliquots (1-5 x 10^6 sperm/mL) were stained with JC-1 1μM, (30 minutes in the dark at r.t.) and DRAQ7 3μM (10 minutes in the dark at r.t). Briefly, after assessment of the flow quality, doublets and debris were gated out. JC-1 was detected at a peak excitation of 488 nm and emission 525 nm (monomers) and excitation of 511 nm and emission of 596 nm (aggregates) and DRAQ7, at a peak excitation of 640 nm, and emission of 690 nm.

Assessment of viability and caspase 3 activity

Samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent™ (2 μM) and incubated at room temperature for 25 minutes. To gate dead spermatozoa samples were stained with 0.3 μM of Eth-1 and incubated for a further 5 minutes before they were immediately evaluated in a flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). CellEvent™ staining was validated as previously described [28].

Bioinformatic Analysis

Variance filtering and PCA. Data were normalized and $\log_2$ transformed using Qlucore Omics Explorer (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the data

set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [29] was used to determine the optimal filtering threshold.

***Identifying discriminating variables*.** Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables with the highest significant difference in fresh and frozen thawed spermatozoa in stallions showing better parameters for motility, viability and mitochondrial activity post thaw. The identification of significantly different variables between the subgroups of stallions showing good and poor sperm functionality post thaw from each individual ejaculate was performed by fitting a linear model for each variable with condition proteins in fresh samples from stallions showing good motility, viability and mitochondrial activity post thaw as predictors, including the stallion, breed and age as nuisance covariates. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [30, 31] and variables with adjusted p-values below 0.1 were considered significant.

Statistical analysis

The normality of the motility, sperm velocity and flow cytometry data were assessed using the Kolmogorov-Smirnoff test. Paired t-tests and One-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 8.00 for Mac, La Jolla California USA, (www.graphpad.com).

Results

Sperm characteristics in good and poor freezers

Stallions were classified according to total motility (CASA) post thaw, with those showing values for total motility > 35% considered as good and those showing values < 35% considered as poor (Fig 1). This threshold was based on current recommendations for minimum quality for commercial doses of equine semen *(http://www.wbfsh.org/GB/Other%20activities/Semen%20standards.aspx).*
Highly significant differences were observed between stallions showing good and poor motility post thaw in all motility and velocity parameters (Fig 1). Four stallions were classified as good and 6 as poor in terms of motility post thaw. Additionally, two further categories were established [32-34]. The first depended on the viability at thawing, good showing > 40% intact membranes post thaw and

poor < 40 % intact membranes, with five stallions in each category. The third category considered mitochondrial activity at thawing, with good freezers showing > 40% of spermatozoa displaying high mitochondrial membrane potential at thawing, and poor freezers displaying < 40% of spermatozoa with high mitochondrial membrane potential at thawing. In this category, 5 stallions were classified as good and 5 as poor. Only one stallion was classified as good in all three categories. Average values for groups of good and poor freezers in these categories are given in fig 2.

Motility in fresh samples does not predict the outcome after cryopreservation.

Motility in fresh semen was not different in stallions showing good and poor outcomes after cryopreservation (Fig 3). Total motility in the group of stallions classified as good was 84.3 ± 1.8% and 84.8 ± 2.6% in the poor group (Fig 3 B). The percentage of linear motile spermatozoa was higher in the group or poor stallions 66.6 ± 2.2% than in the group of good stallions 56.3 ± 4.1% ($P<0.05$) (Fig 3 C). There was no difference in sperm velocities between both groups of stallions (Fig 3 D-F). However, differences in the proteome of fresh spermatozoa in stallions showing ejaculates with good and poor outcomes after cryopreservation in motility, viability and mitochondrial membrane potential post thaw, were evident in Volcano plots (Fig 3 A-G-H). Moreover, a Venn diagram was constructed showing changes between fresh and frozen thawed sperm, and those present in stallions showing good outcomes after cryopreservation in each of the three categories defined (Fig 4).

The amounts of specific proteins differ in spermatozoa from stallions showing good and poor outcomes after cryopreservation.

Motility post thaw. Initially the way in which cryopreservation modifies the proteome in different ways in good and poor freezers was identified. The complete list of proteins identified is provided as a supplementary file. Qlucore Omics explorer was used to identify the discriminant variables with the highest significant difference between the subgroups of fresh and frozen thawed samples in stallions showing good and poor motility post thaw. With a q-value cut-off of 0.1 and a fold change >2, significant differences in the response to cryopreservation between the groups were observed (Fig 5 A-B). Using Venn diagrams, 73 proteins present in both groups (good and poor freezers) were identified, 24 proteins were present only in stallions showing good motility post thaw, and 22 proteins were present only in stallions showing poor motility post thaw (Fig 5C). Then, in order to reduce the number of proteins and obtain fewer proteins with the highest discriminant power, the most significant variables from fresh samples were selected in order to identify predictive discriminant

variables able to distinguish stallions showing good motility post thaw. Those variables with a fold change >3, a P= 8.2e-04 and a q=0.074 (equivalent to FDR) were selected, and the following proteins were identified as discriminant variables able to identify stallions showing good motility post thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15 and F6SKR3 (Fig. 6), corresponding to *Mannosidase alpha class 2C member 1, Mitochondrial NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit 9-like protein, Isoleucyl-tRNA synthetase 2, mitochondrial, Acetyl-CoA acetyltransferase 1, Latherin, and Ubiquitin specific peptidase 43*.

Mitochondrial activity post thaw. The response to cryopreservation was studied in terms of mitochondrial potential post thaw. Changes between fresh and frozen samples in stallions showing good and poor mitochondrial activity post thaw, with P=0.01, q= 0.1 and a fold change > 2 were identified. Both stallions showing good mitochondrial activity (Fig 7A), and poor mitochondrial activity post thaw (Fig 7B) showed increased amounts of some proteins and reduced amounts of others. The Venn diagram showed that 94 proteins were present in both groups, but 34 proteins were only present in stallions showing good mitochondrial activity post thaw and 35 proteins were only present in those which did not present good mitochondrial activity after thawing (Fig 7 C). Following this, a search was performed for discriminant variables in fresh semen able to forecast good mitochondrial membrane potential post thaw. Variables with a fold change > 3, P=4.38e-04 and q=0.099 were identified in fresh samples showing better percentages of active mitochondria post thaw. The discriminant proteins were, F7A616, K9KDP8, A0A3Q2HAZ2, A0A3Q2L2U8, corresponding to *Phosphoglycerate mutase, Peroxiredoxin 6 like protein,* an uncharacterized protein similar to *actin-1* and a second uncharacterized protein similar to the transmembrane protein named *GRAM domain containing 1A*. (Fig 8)

Viability post thaw. Samples showing percentages over or below 40% intact membranes post thaw showed different changes in the sperm proteome as a consequence of cryopreservation. Differences in fresh and frozen thawed samples in both groups were observed with a fold change > 2, p=0.01 and q=0.1 (Fig 9 A-B). Venn diagrams revealed 92 proteins present in stallions showing good and poor viability post-thaw, with 19 proteins only present in poor freezers and 64 which were present only in good freezers (Fig 9 C). Following this, a search was performed for discriminant variables in fresh semen able to identify stallions likely to show good viability post-thaw (> 40% intact membranes at thawing). Variables with a fold change >3, p= 0.001 and q= 0.01 were selected, and two proteins were retrieved, serving as discriminant variables, *Chaperonin containing TCP1 subunit 8 and testis expressed 101* (Fig 10).

Age does not cause a major impact on the outcome of cryopreservation

The mean age of the stallions used in this study was 10.8 years old, but ages ranged from 5 to 19 years old. In order to determine the possible influence of age on the outcome of cryopreservation, ages in good and poor stallions in the three categories considered were compared and no significant differences were detected between good and poor freezers in any of them (Fig 11 -1). Moreover, a PCA analysis was conducted including age and the outcome of cryopreservation, showing that age is not a factor causing major effects on the outcome of cryopreservation (Fig 11-2). Only three samples belonging to the youngest stallions were identified outside the main population.

Discussion

The present study investigated changes in the proteome in ejaculates showing both good and poor outcomes after cryopreservation, and how the proteome can be used to discriminate between them. These outcomes were classified into three categories, motility, viability and mitochondrial membrane potential post thaw. We studied whether the impact of cryopreservation on the proteome differed between groups and whether specific proteins in fresh spermatozoa can be used as discriminant variables to differentiate between stallions showing good and poor outcomes after cryopreservation of their ejaculates. Notably, sperm motility and velocities in fresh samples were not good predictors of motility post thaw, and unexpectedly, the percentage of linear motility in fresh samples was even higher in stallions with poor outcomes (Fig 3), neither did age have a major impact on the outcome of cryopreservation. However, differences in the amounts of proteins in fresh samples were evident between poor and good freezers. In addition, cryopreservation had a different impact on the proteome of the stallion spermatozoa in both groups.

Although cryopreservation had a similar impact in both groups, causing reduction of the levels of some proteins and increases in others, there were specific proteins associated with stallions producing ejaculates with better motility post thaw. Six proteins were identified in fresh samples, capable of identifying the group of stallions showing better motility post thaw. Three of them were mitochondrial proteins (K9K273, A0A3Q2I7V9 and F7CE45), stressing the importance of these organelles for sperm function [35, 36], and in particular producing ATP for sperm motility through oxidative phosphorylation. The F6YTG8 (*alpha mannosidase*) is a protein with a role in the catabolism of oligosaccharides [37]. Alpha mannosidase activity prevents accumulation of

oligosaccharides. More recently a role in preventing mitochondrial dependent apoptosis has been proposed [38]. Since an important proportion of the damage occurring during cryopreservation involves a mitochondrial apoptotic pathway [39, 40], the aforementioned function of this protein provides an explanation for our findings. Moreover, a study in ovine semen found a positive correlation between alpha mannosidase and a positive outcome after cryopreservation [41]. F7CE45, *Acetyl- CoA acetyltransferase 1*, catalyzes the last step in the mitochondrial beta oxidation pathway [42], and also plays a major role in ketone body metabolism [43]. Spermatozoa are able to obtain energy for motility using the beta oxidation pathway [10, 14] providing an explanation for the link between a major presence of this protein in fresh samples and better motility post thaw. Finally, the presence of *Latherin* (F6YU15) was described for the first time in the spermatozoa. This is present in the saliva and sweat of horses and has strong surfactant properties [44, 45]. Its activity is responsible for the foam formed in the skin of horses during vigorous exercise. It is not clear what the possible function of this protein is in the spermatozoa, although antibacterial properties inhibiting the growth of biofilms [46] have been attributed to latherin. A potential contribution of sperm latherin to endometrial health is a tempting possibility that warrants further research.

Cryopreservation also caused a different impact in ejaculates showing good and poor mitochondrial activity post thaw. The Venn diagram revealed 94 common proteins, 34 proteins specific to the group showing high mitochondrial membrane potential post thaw, and 35 to the group showing poor mitochondrial membrane post thaw. Next a search was performed for discriminant variables in fresh semen able to predict mitochondrial activity post thaw, and four proteins were found to be potent discriminant variables for the prediction of good mitochondrial membrane potential post thaw. The *peroxiredoxin like 6 protein* was more abundant in the ejaculates showing better mitochondrial activity post thaw. Peroxiredoxin 6 is considered as one of the major antioxidant defenses of the spermatozoa [47, 48], and taking into account that a high percentage of cryodamage come from oxidative stress [49, 50], is not surprising that samples richer in this antioxidant protein are able to better withstand the cryopreservation process. A glycolytic enzyme, *Phosphoglycerate mutase (PGAM)*, was also more abundant in good freezers. This enzyme is upregulated in many cancer cells [51] and catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) during glycolysis. In cancer cells that overexpress this protein, there is an increase in 2-PG and a decrease in 3-PG. Also, these cells express higher levels of lactate and increased flux through the pentose phosphate pathway [51], thus producing more reducing power in the form of NADPH. This mechanism may also explain the enhanced cryo-survival of ejaculates with higher levels of PGAM in stallions and warrants further research on the interaction between redox metabolism and redox

regulation in the spermatozoa. The *GRAM domain containing 1A* was also more abundant in the spermatozoa of stallions showing good mitochondrial membrane potential post thaw; this is a cholesterol transfer protein, with a role in the early stages of autophagosome formation [52]. These functions may explain the major presence of this protein in good freezers since mitophagy has recently been related to sperm quality [53]. Finally, an uncharacterized protein similar to *actin-1* was also more abundant in good freezers; a tempting possibility is that this protein is also related to mitophagy. Actin structures cage damaged mitochondria during mitophagy [54], however further research is warranted to characterize this protein and identify its role in the spermatozoa.

Cryopreservation also had a different impact in the groups showing good and poor membrane integrity post thaw. The Venn diagram revealed 92 common proteins in both groups, 64 specific to the group showing high viability post thaw and 19 specific to the group showing poor motility post thaw. Bioinformatic analysis to reveal discriminant variables in fresh semen able to predict viability after thawing was then performed.

The proteins *Chaperonin containing TCP1 subunit 8 and testis expressed 101* were more abundant in samples showing better membrane integrity post thaw. The chaperonin containing TCP1 complex plays a role in mediating sperm-oocyte interaction [55-57], thus playing a major part in the early stages of fertilization. The testis expressed 101 also plays a role in fertilization, mediating binding of sperm to the zona pellucida, as well as in the migration of spermatozoa within the oviduct [58, 59]. The presence of these proteins with direct and major roles in fertilization in the ejaculates of stallions showing better viability post thaw, underpins the need for proper assessment of sperm membranes in the andrological evaluation of stallions.

In conclusion, cryopreservation impacts different sperm functions and structures in good and poor freezers in a number of different manners. Changes in specific proteins occur between these groups. On the other hand, specific proteins in fresh samples can be used as discriminant variables to potentially predict the response of specific ejaculates to cryopreservation. It is noteworthy that many of these proteins were mitochondrial, stressing the importance of these organelles for spermatozoa functionality. Interestingly, proteins related to redox regulation and energetic metabolism were also more abundant in good freezers. The data reported here provide a strong basis for further research into the molecular damage occurring during cryopreservation and paves the way for the development of simple assays which can be used prior to cryopreservation to assess whether an ejaculate will be of sufficient quality post thaw to be marketed.

References

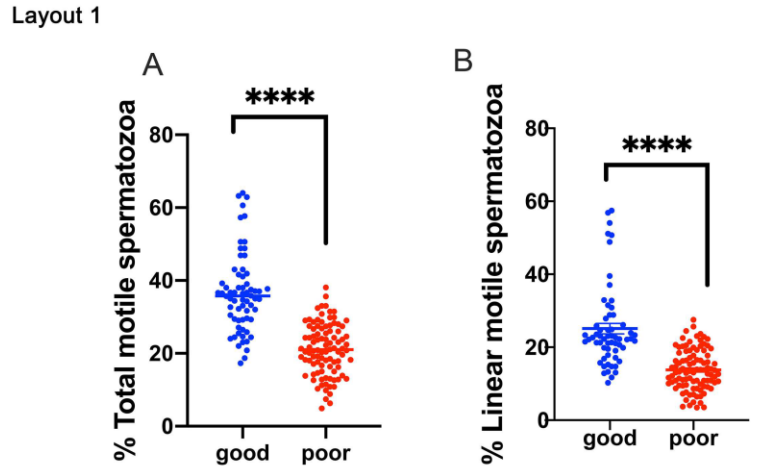
1. Pena FJ, Garcia BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology 2011; 76:1177-1186.
2. Brinsko SP, Varner DD. Artificial insemination and preservation of semen. Vet Clin North Am Equine Pract 1992; 8:205-218.
3. Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008; 105:119-128.
4. Macias Garcia B, Ortega Ferrusola C, Aparicio IM, Miro-Moran A, Morillo Rodriguez A, Gallardo Bolanos JM, Gonzalez Fernandez L, Balao da Silva CM, Rodriguez Martinez H, Tapia JA, Pena FJ. Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology 2012; 77:1280-1289.
5. Garcia BM, Moran AM, Fernandez LG, Ferrusola CO, Rodriguez AM, Bolanos JM, da Silva CM, Martinez HR, Tapia JA, Pena FJ. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. J Androl 2012; 33:105-113.
6. Cole JA, Meyers SA. Osmotic stress stimulates phosphorylation and cellular expression of heat shock proteins in rhesus macaque sperm. J Androl 2011; 32:402-410.
7. Ortiz-Rodriguez JM, Ortega-Ferrusola C, Gil MC, Martin-Cano FE, Gaitskell-Phillips G, Rodriguez-Martinez H, Hinrichs K, Alvarez-Barrientos A, Roman A, Pena FJ. Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. PLoS One 2019; 14:e0213420.
8. Bogle OA, Kumar K, Attardo-Parrinello C, Lewis SE, Estanyol JM, Ballesca JL, Oliva R. Identification of protein changes in human spermatozoa throughout the cryopreservation process. Andrology 2017; 5:10-22.
9. Griffin RA, Baker M, Aitken RJ, Swegen A, Gibb Z. What makes a fertile sperm? Unique molecular attributes of stallion fertility. Reproduction 2019; 158:R125-R137.
10. Swegen A, Curry BJ, Gibb Z, Lambourne SR, Smith ND, Aitken RJ. Investigation of the stallion sperm proteome by mass spectrometry. Reproduction 2015; 149:235-244.
11. Martin-Cano FE, Gaitskell-Phillips G, Ortiz-Rodriguez JM, Silva-Rodriguez A, Roman A, Rojo-Dominguez P, Alonso-Rodriguez E, Tapia JA, Gil MC, Ortega-Ferrusola C, Pena FJ. Proteomic profiling of stallion spermatozoa suggests changes in sperm metabolism and compromised redox regulation after cryopreservation. J Proteomics 2020; 221:103765.
12. Jodar M, Soler-Ventura A, Oliva R, Molecular Biology of R, Development Research G. Semen proteomics and male infertility. J Proteomics 2017; 162:125-134.
13. Jodar M, Attardo-Parrinello C, Soler-Ventura A, Barrachina F, Delgado-Duenas D, Civico S, Calafell JM, Ballesca JL, Oliva R. Sperm proteomic changes associated with early embryo quality after ICSI. Reprod Biomed Online 2020; 40:700-710.
14. Amaral A, Castillo J, Estanyol JM, Ballesca JL, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. Mol Cell Proteomics 2013; 12:330-342.
15. D'Amours O, Frenette G, Bourassa S, Calvo E, Blondin P, Sullivan R. Proteomic Markers of Functional Sperm Population in Bovines: Comparison of Low- and High-Density Spermatozoa Following Cryopreservation. J Proteome Res 2018; 17:177-188.

16. Amaral A, Paiva C, Attardo Parrinello C, Estanyol JM, Ballesca JL, Ramalho-Santos J, Oliva R. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. J Proteome Res 2014; 13:5670-5684.
17. Griffin RA, Swegen A, Baker M, Aitken RJ, Eskerret-Birne D, Silva Rodriguez A, Martín-Cano FE, Nixon B, Peña FJ, Delehedde M, Sergeant N, Gibb Z. Mass spectrometry reveals distinct proteomic profiles in high- and low-quality stallion spermatozoa. Reproduction 2020; 160:695-707.
18. Parrilla I, Perez-Patino C, Li J, Barranco I, Padilla L, Rodriguez-Martinez H, Martinez EA, Roca J. Boar semen proteomics and sperm preservation. Theriogenology 2019; 137:23-29.
19. Perez-Patino C, Barranco I, Li J, Padilla L, Martinez EA, Rodriguez-Martinez H, Roca J, Parrilla I. Cryopreservation Differentially Alters the Proteome of Epididymal and Ejaculated Pig Spermatozoa. Int J Mol Sci 2019; 20.
20. Pini T, Rickard JP, Leahy T, Crossett B, Druart X, de Graaf SP. Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. J Proteomics 2018; 181:73-82.
21. Morrell JM, Garcia BM, Pena FJ, Johannisson A. Processing stored stallion semen doses by Single Layer Centrifugation. Theriogenology 2011; 76:1424-1432.
22. Ortega-Ferrusola C, Garcia BM, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Rodriguez-Martinez H, Tapia JA, Pena FJ. Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim Reprod Sci 2009; 114:393-403.
23. Hall SE, Aitken RJ, Nixon B, Smith ND, Gibb Z. Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa. Biol Reprod 2017; 96:107-121.
24. Ortega Ferrusola C, Anel-Lopez L, Ortiz-Rodriguez JM, Martin Munoz P, Alvarez M, de Paz P, Masot J, Redondo E, Balao da Silva C, Morrell JM, Rodriguez Martinez H, Tapia JA, et al. Stallion spermatozoa surviving freezing and thawing experience membrane depolarization and increased intracellular Na(). Andrology 2017; 5:1174-1182.
25. Ortega-Ferrusola C, Macias Garcia B, Suarez Rama V, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Tapia JA, Rodriguez-Martinez H, Pena FJ. Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. Reprod Domest Anim 2009; 44:419-423.
26. Gallardo Bolanos JM, Balao da Silva CM, Martin Munoz P, Morillo Rodriguez A, Plaza Davila M, Rodriguez-Martinez H, Aparicio IM, Tapia JA, Ortega Ferrusola C, Pena FJ. Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction 2014; 148:221-235.
27. Martin Munoz P, Ortega Ferrusola C, Vizuete G, Plaza Davila M, Rodriguez Martinez H, Pena FJ. Depletion of Intracellular Thiols and Increased Production of 4-Hydroxynonenal that Occur During Cryopreservation of Stallion Spermatozoa Lead to Caspase Activation, Loss of Motility, and Cell Death. Biol Reprod 2015; 93:143.
28. Ortiz-Rodriguez JM, Balao da Silva C, Masot J, Redondo E, Gazquez A, Tapia JA, Gil C, Ortega-Ferrusola C, Pena FJ. Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase 3. PLoS One 2019; 14:e0211994.
29. Fontes M, Soneson C. The projection score--an evaluation criterion for variable subset selection in PCA visualization. BMC Bioinformatics 2011; 12:307.
30. Tamhane AC, Hochberg Y, Dunnett CW. Multiple test procedures for dose finding. Biometrics 1996; 52:21-37.
31. Viskoper RJ, Laszt A, Oren S, Hochberg Y, Villa Y, Drexler I, Bregman L, Mishal J. The antihypertensive effect of atenolol and bopindolol in the elderly. Neth J Med 1989; 35:185-191.

32. Vidament M. French field results (1985-2005) on factors affecting fertility of frozen stallion semen. Anim Reprod Sci 2005; 89:115-136.
33. Battut IB, Kempfer A, Lemasson N, Chevrier L, Camugli S. Prediction of the fertility of stallion frozen-thawed semen using a combination of computer-assisted motility analysis, microscopical observation and flow cytometry. Theriogenology 2017; 97:186-200.
34. Munoz PM, Ferrusola CO, Lopez LA, Del Petre C, Garcia MA, de Paz Cabello P, Anel L, Pena FJ. Caspase 3 Activity and Lipoperoxidative Status in Raw Semen Predict the Outcome of Cryopreservation of Stallion Spermatozoa. Biol Reprod 2016; 95:53.
35. Aitken RJ, Gregoriatis D, Kutzera L, Towney E, Lin M, Wilkins A, Gibb Z. Patterns of MTT reduction in mammalian spermatozoa. Reproduction 2020.
36. Davila MP, Munoz PM, Bolanos JM, Stout TA, Gadella BM, Tapia JA, da Silva CB, Ferrusola CO, Pena FJ. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. Reproduction 2016; 152:683-694.
37. Paciotti S, Persichetti E, Klein K, Tasegian A, Duvet S, Hartmann D, Gieselmann V, Beccari T. Accumulation of free oligosaccharides and tissue damage in cytosolic alpha-mannosidase (Man2c1)-deficient mice. J Biol Chem 2014; 289:9611-9622.
38. Wang L, Suzuki T. Dual functions for cytosolic alpha-mannosidase (Man2C1): its down-regulation causes mitochondria-dependent apoptosis independently of its alpha-mannosidase activity. J Biol Chem 2013; 288:11887-11896.
39. Ortega-Ferrusola C, Sotillo-Galan Y, Varela-Fernandez E, Gallardo-Bolanos JM, Muriel A, Gonzalez-Fernandez L, Tapia JA, Pena FJ. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl 2008; 29:213-221.
40. Ortega-Ferrusola C, Anel-Lopez L, Martin-Munoz P, Ortiz-Rodriguez JM, Gil MC, Alvarez M, de Paz P, Ezquerra LJ, Masot AJ, Redondo E, Anel L, Pena FJ. Computational flow cytometry reveals that cryopreservation induces spermptosis but subpopulations of spermatozoa may experience capacitation-like changes. Reproduction 2017; 153:293-304.
41. Rickard JP, Leahy T, Soleilhavoup C, Tsikis G, Labas V, Harichaux G, Lynch GW, Druart X, de Graaf SP. The identification of proteomic markers of sperm freezing resilience in ram seminal plasma. J Proteomics 2015; 126:303-311.
42. Fukao T, Yamaguchi S, Tomatsu S, Orii T, Frauendienst-Egger G, Schrod L, Osumi T, Hashimoto T. Evidence for a structural mutation (347Ala to Thr) in a German family with 3-ketothiolase deficiency. Biochem Biophys Res Commun 1991; 179:124-129.
43. Wakazono A, Fukao T, Yamaguchi S, Hori T, Orii T, Lambert M, Mitchell GA, Lee GW, Hashimoto T. Molecular, biochemical, and clinical characterization of mitochondrial acetoacetyl-coenzyme A thiolase deficiency in two further patients. Hum Mutat 1995; 5:34-42.
44. McDonald RE, Fleming RI, Beeley JG, Bovell DL, Lu JR, Zhao X, Cooper A, Kennedy MW. Latherin: a surfactant protein of horse sweat and saliva. PLoS One 2009; 4:e5726.
45. Beeley JG, Eason R, Snow DH. Isolation and characterization of latherin, a surface-active protein from horse sweat. Biochem J 1986; 235:645-650.
46. Gakhar L, Bartlett JA, Penterman J, Mizrachi D, Singh PK, Mallampalli RK, Ramaswamy S, McCray PB, Jr. PLUNC is a novel airway surfactant protein with anti-biofilm activity. PLoS One 2010; 5:e9098.
47. Fernandez MC, O'Flaherty C. Peroxiredoxin 6 is the primary antioxidant enzyme for the maintenance of viability and DNA integrity in human spermatozoa. Hum Reprod 2018; 33:1394-1407.

48. Ozkosem B, Feinstein SI, Fisher AB, O'Flaherty C. Absence of Peroxiredoxin 6 Amplifies the Effect of Oxidant Stress on Mobility and SCSA/CMA3 Defined Chromatin Quality and Impairs Fertilizing Ability of Mouse Spermatozoa. Biol Reprod 2016; 94:68.
49. Gibb Z, Griffin RA, Aitken RJ, De Iuliis GN. Functions and effects of reactive oxygen species in male fertility. Anim Reprod Sci 2020:106456.
50. Ertmer F, Oldenhof H, Schutze S, Rohn K, Wolkers WF, Sieme H. Induced sub-lethal oxidative damage affects osmotic tolerance and cryosurvival of spermatozoa. Reprod Fertil Dev 2017; 29:1739-1750.
51. Hitosugi T, Zhou L, Elf S, Fan J, Kang HB, Seo JH, Shan C, Dai Q, Zhang L, Xie J, Gu TL, Jin P, et al. Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. Cancer Cell 2012; 22:585-600.
52. Laraia L, Friese A, Corkery DP, Konstantinidis G, Erwin N, Hofer W, Karatas H, Klewer L, Brockmeyer A, Metz M, Scholermann B, Dwivedi M, et al. The cholesterol transfer protein GRAMD1A regulates autophagosome biogenesis. Nat Chem Biol 2019; 15:710-720.
53. Aparicio IM, Espino J, Bejarano I, Gallardo-Soler A, Campo ML, Salido GM, Pariente JA, Pena FJ, Tapia JA. Autophagy-related proteins are functionally active in human spermatozoa and may be involved in the regulation of cell survival and motility. Sci Rep 2016; 6:33647.
54. Kruppa AJ, Buss F. Actin cages isolate damaged mitochondria during mitophagy. Autophagy 2018; 14:1644-1645.
55. Dun MD, Smith ND, Baker MA, Lin M, Aitken RJ, Nixon B. The chaperonin containing TCP1 complex (CCT/TRiC) is involved in mediating sperm-oocyte interaction. J Biol Chem 2011; 286:36875-36887.
56. Redgrove KA, Anderson AL, Dun MD, McLaughlin EA, O'Bryan MK, Aitken RJ, Nixon B. Involvement of multimeric protein complexes in mediating the capacitation-dependent binding of human spermatozoa to homologous zonae pellucidae. Dev Biol 2011; 356:460-474.
57. Byrne K, Leahy T, McCulloch R, Colgrave ML, Holland MK. Comprehensive mapping of the bull sperm surface proteome. Proteomics 2012; 12:3559-3579.
58. Fujihara Y, Tokuhiro K, Muro Y, Kondoh G, Araki Y, Ikawa M, Okabe M. Expression of TEX101, regulated by ACE, is essential for the production of fertile mouse spermatozoa. Proc Natl Acad Sci U S A 2013; 110:8111-8116.
59. Li W, Guo XJ, Teng F, Hou XJ, Lv Z, Zhou SY, Bi Y, Wan HF, Feng CJ, Yuan Y, Zhao XY, Wang L, et al. Tex101 is essential for male fertility by affecting sperm migration into the oviduct in mice. J Mol Cell Biol 2013; 5:345-347.

Figures



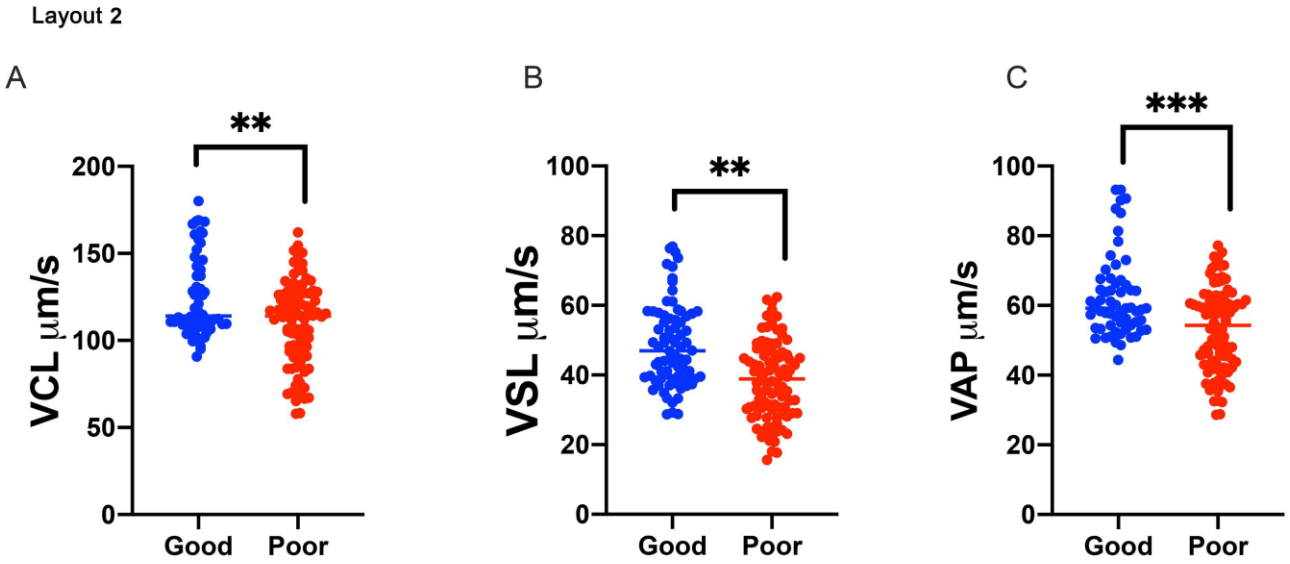


Fig 1.- (Layout 1) Thirty stallion ejaculates were frozen and thawed as described in material and methods. Based in their post thaw motility they were classified as good (> 35% total motility) or poor freezers (<35 % total motility post thaw). Computer Assisted Sperm Analysis (CASA) was used to analyze sperm quality after thawing. a) percentage of total motile spermatozoa in good and poor freezers b) percentage of linear motile spermatozoa in good and poor freezers. Layout 2. A) Circular velocity (VCL) B) Straight line velocity (VSL) and C) Average path velocity (VAP) in good and poor freezers. Data are means ± s.e.m. **** $P<0.001$, ** $P<0.01$.

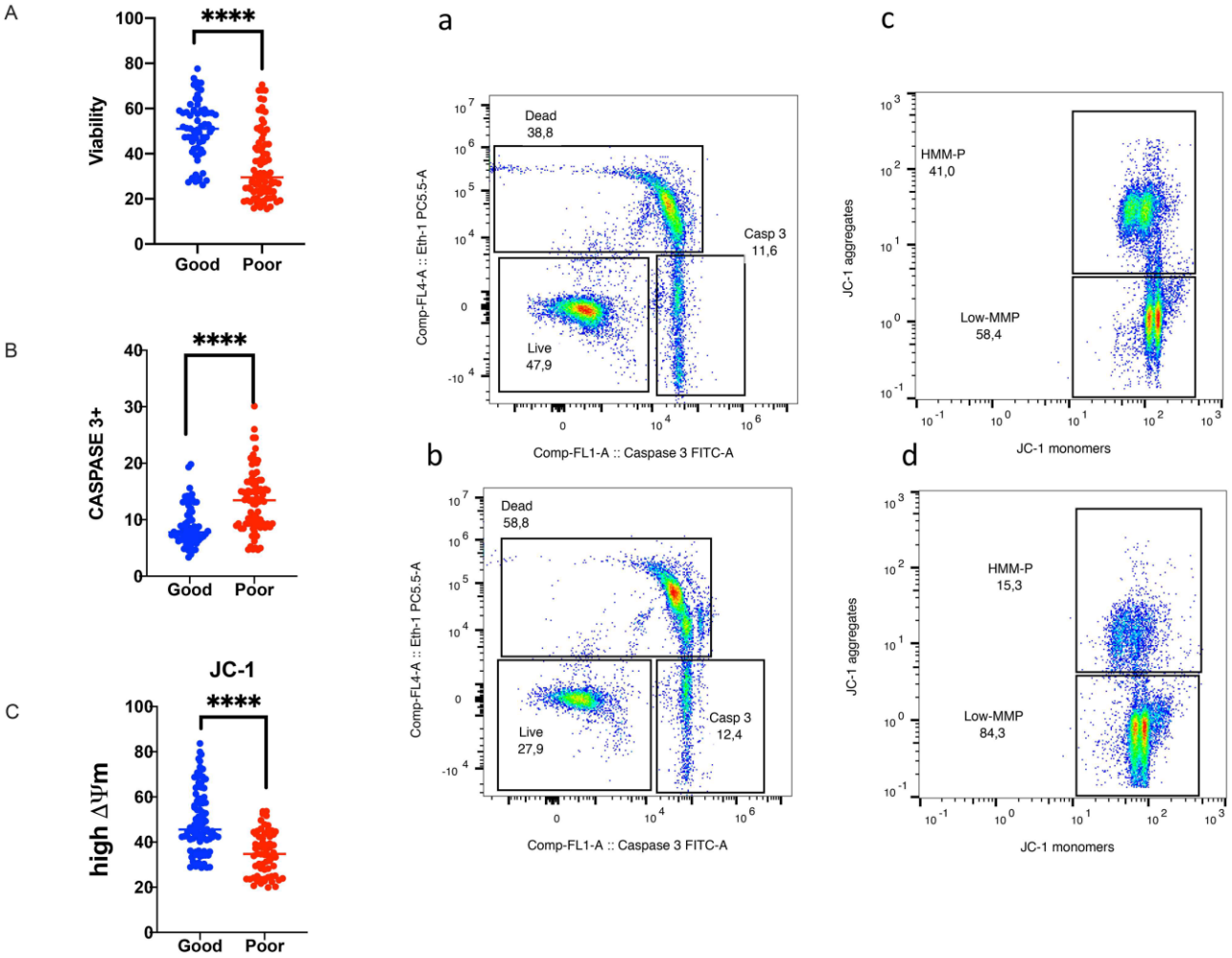


Fig 2.- Viability and mitochondrial membrane potential in stallions showing good and poor viability and mitochondrial activity post thaw. 30 stallion ejaculates were frozen and thawed as described in material and methods. Samples were processed for flow cytometry analysis as described in material and methods. A) Viability (intact membranes and negative for caspase 3 spermatozoa) B) Caspase 3 positive spermatozoa C) Spermatozoa depicting high mitochondrial membrane potential in good and poor freezers. a-b) representative cytograms of viability analysis, c-d) representative cytograms of mitochondrial membrane potential analysis. Data are means ± s.e.m. **** $P< 0.001$.

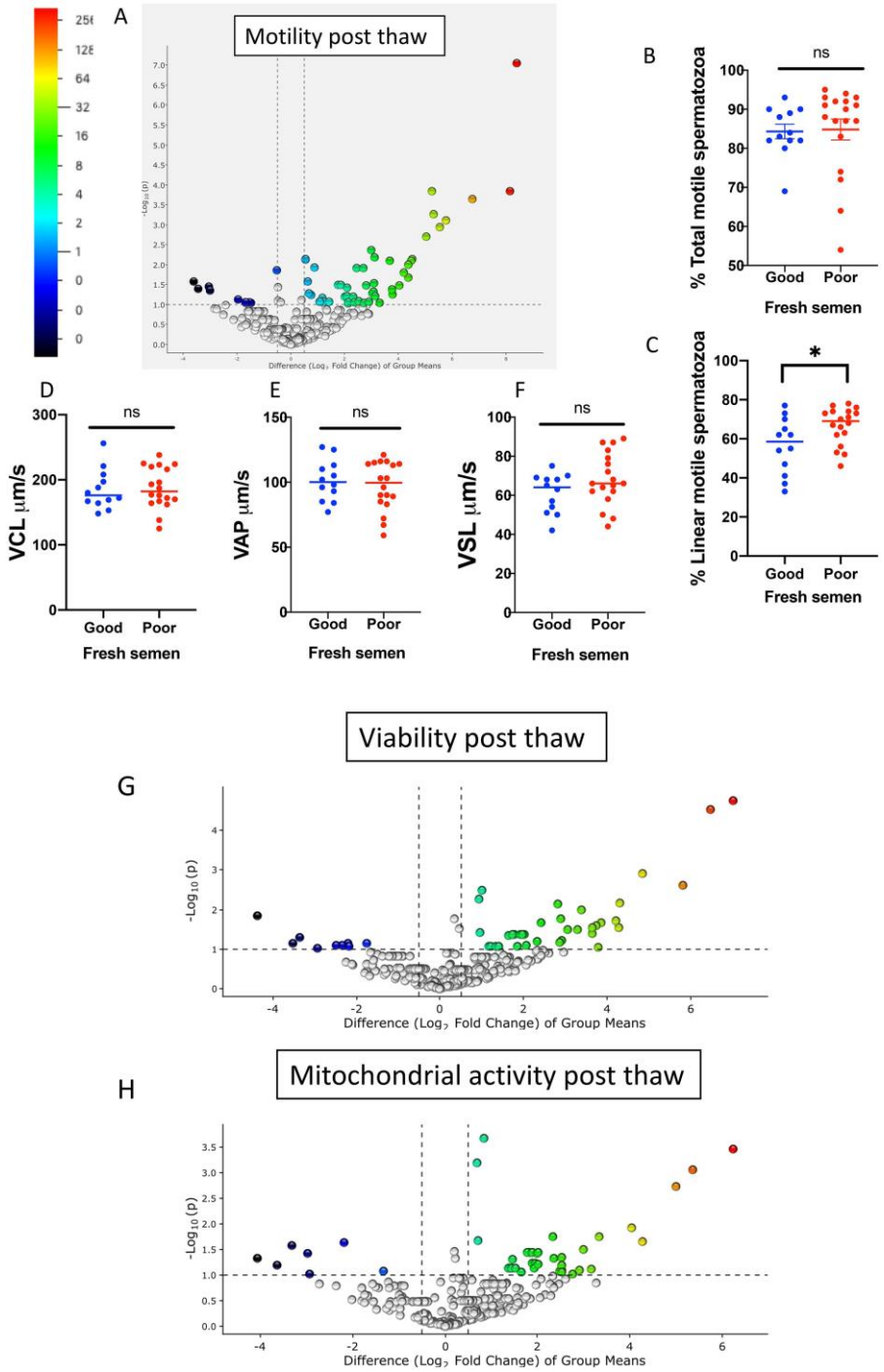


Fig 3.- Computer-assisted Sperm Analysis and shot gun proteomic analysis in pre-freezing samples. A) Volcano plot showing differences in protein expression in fresh samples in stallions showing good and poor motility post thaw B) Percentages of total motile spermatozoa in fresh samples of good and poor freezers C) Percentages of linear motile spermatozoa in fresh samples of good and poor freezers D) Circular velocity E) Average path velocity E) Straight line velocity. Data are means ± s.e.m. * $P<0.05$ 2.- Volcano plots showing the proteome in fresh spermatozoa in stallions showing good and poor viability (G) and mitochondrial activity post thaw (H).; x-axis (log_2 fold change) y axis ($-log_{10}$ *P* value).

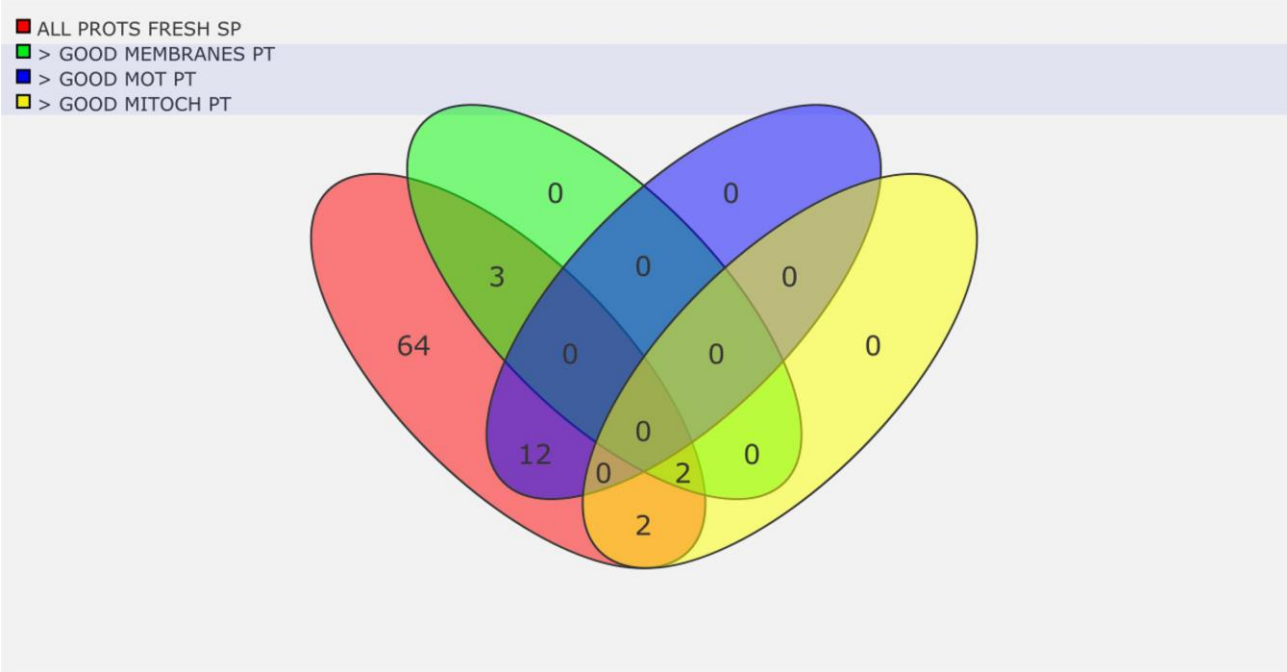


Fig 4.- Venn diagram showing proteins significantly enriched (discriminant variables) in fresh spermatozoa and those in each category for stallions showing good motility, good mitochondrial membrane potential and good viability post thaw.

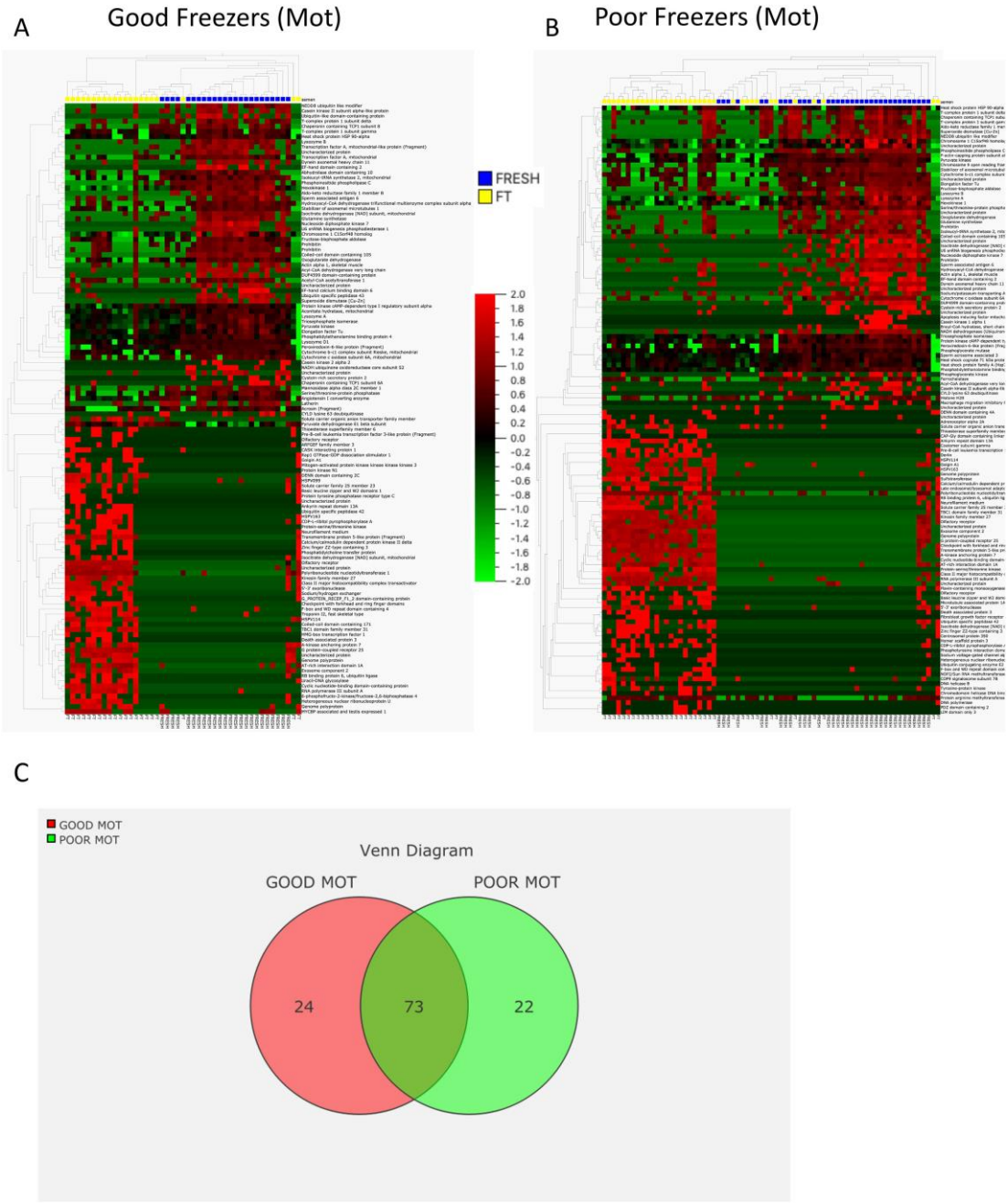


Fig 5. Layout 1- Heat map showing the impact of cryopreservation on the proteome of stallions showing good or poor motility post thaw. Proteins are classified following a hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing larger amounts of protein and green areas smaller amounts of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good freezers B) poor freezers Layout 2- Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in amounts of proteins in stallions showing good and poor motility post thaw. 73 proteins were present in both groups, 24 proteins were only present in the group of good freezers and 22 proteins were present only in the group of poor freezers.

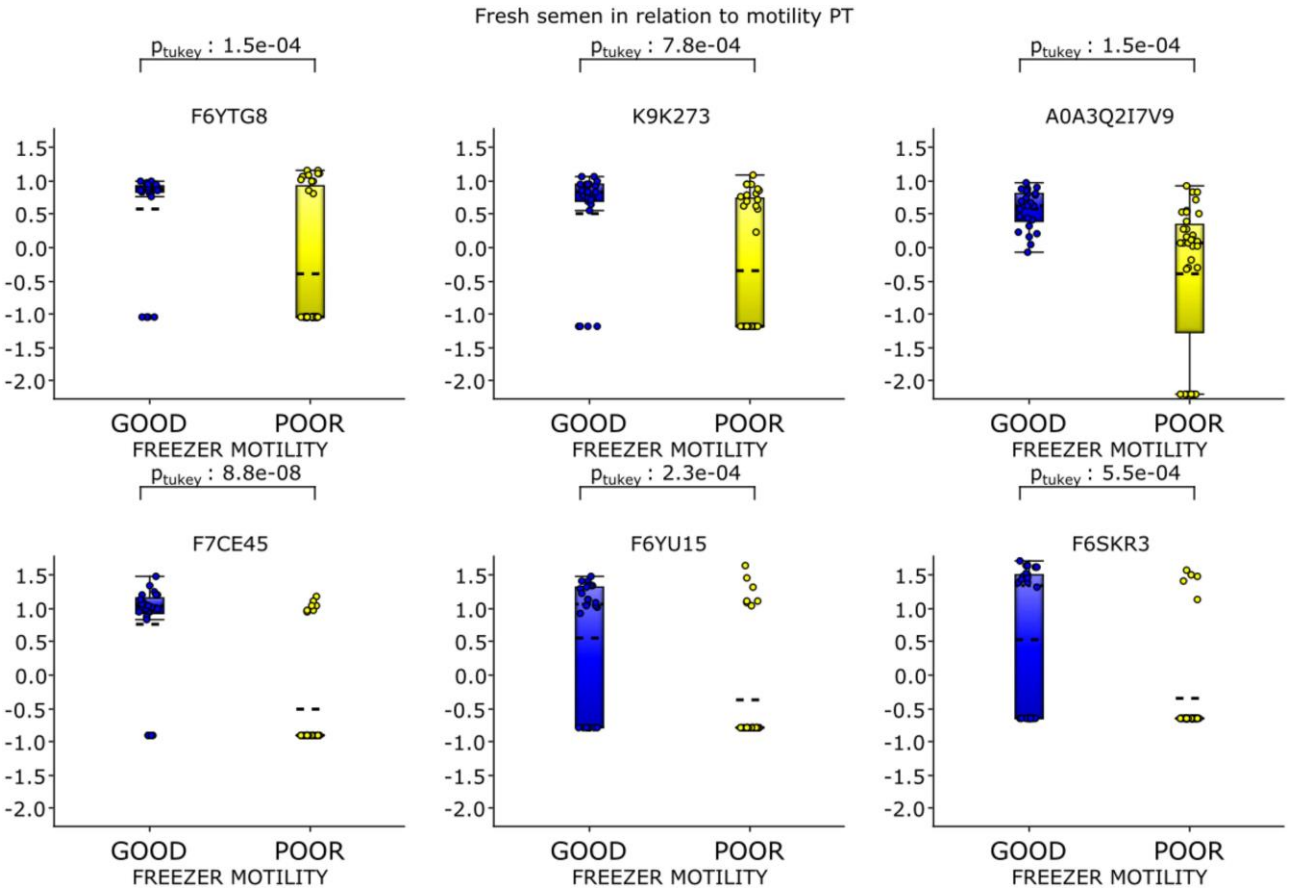


Fig 6.- Discriminant variables between good and poor stallions in terms of motility post thaw F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15 and F6SKR3 corresponding to *Mannosidase alpha class 2C member 1, Mitochondrial NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex subunit 9-like protein, Isoleucyl-tRNA synthetase 2, mitochondrial, Acetyl-CoA acetyltransferase 1, Latherin and Ubiquitin specific peptidase 43*. Proteins showing a fold change >3, a P= 8.2e-04 and a q=0.074 (equivalent to FDR)

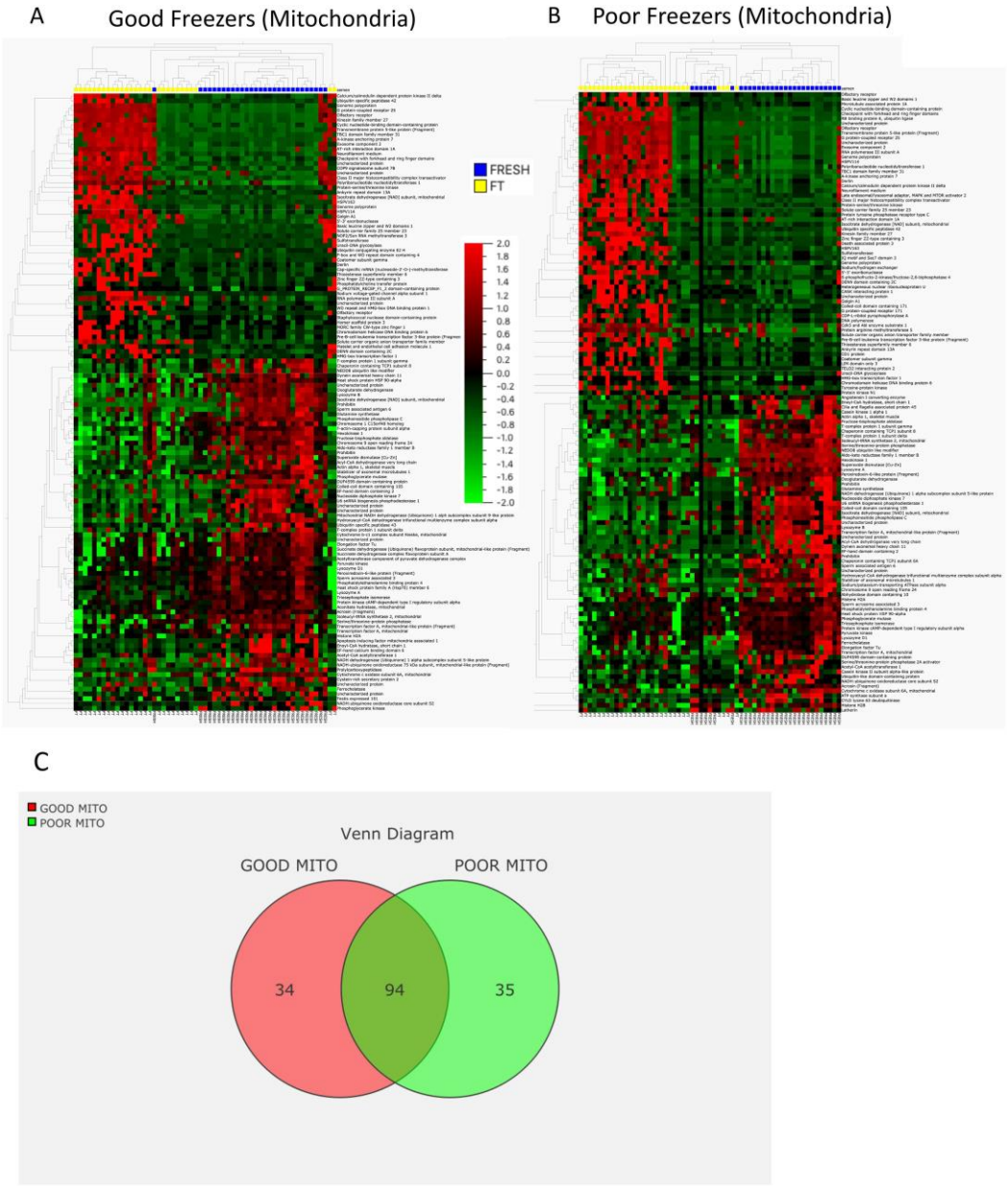


Fig 7 Layout 1.- Heat map showing the impact of cryopreservation in the proteome of stallions showing good or poor mitochondrial membrane potential post thaw. Proteins are classified following hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing larger amounts of protein and green areas represent smaller amounts of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good mitochondrial membrane post thaw B) poor mitochondrial membrane post thaw. Layout 2 Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in amounts of proteins in stallions showing good and poor mitochondrial membrane potential post thaw. 94 proteins were present in both groups, 34 proteins were only present the group presenting good mitochondrial membrane potential post thaw and 35 proteins were present only in the group presenting poor mitochondrial membrane potential post thaw.

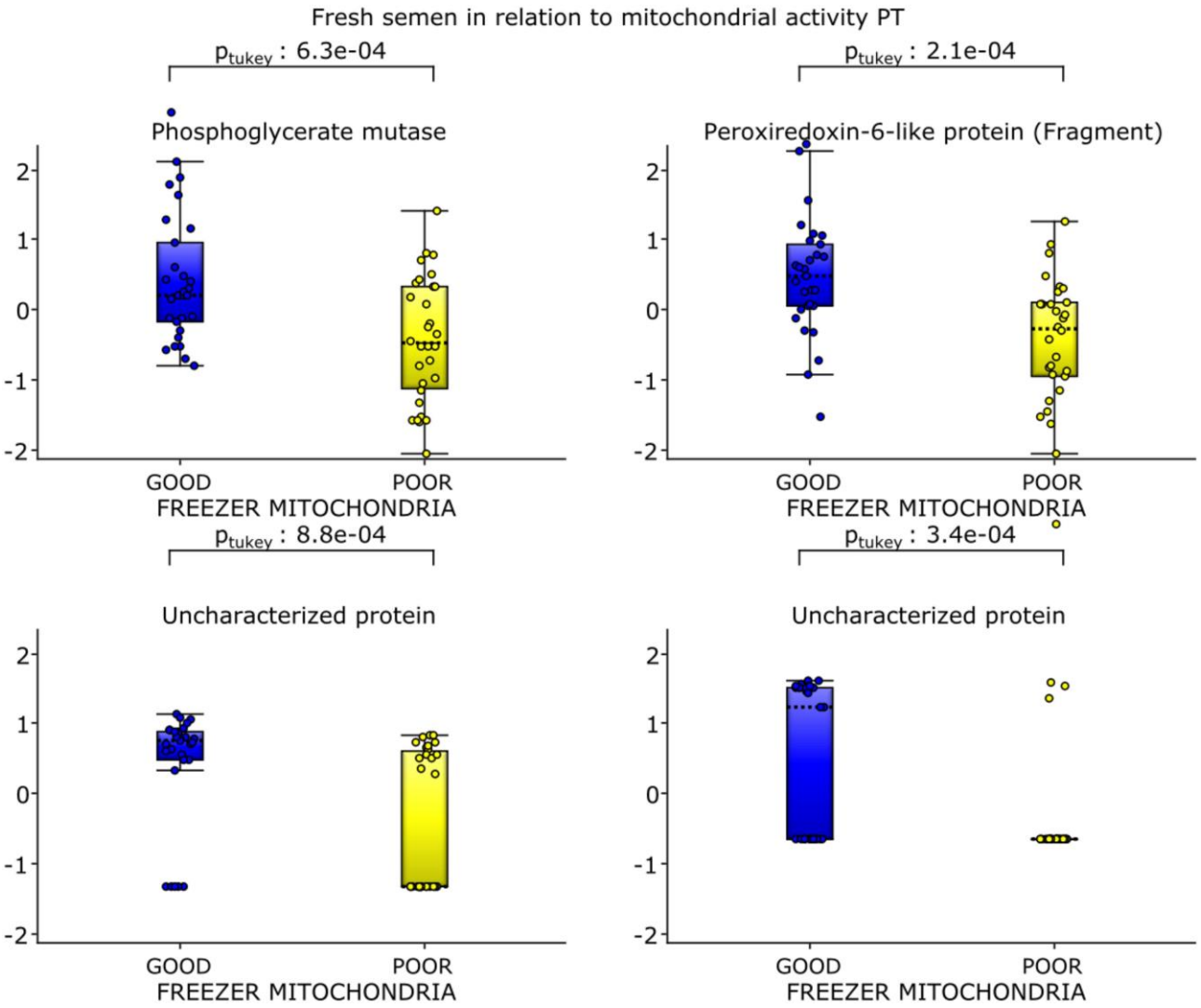


Fig 8.- Discriminant variables between good and poor freezers in terms of mitochondrial membrane potential post thaw. The discriminant proteins were, F7A616, K9KDP8, A0A3Q2HAZ2, A0A3Q2L2U8, corresponding to *Phosphoglycerate mutase, Peroxiredoxin 6 like protein,* an uncharacterized protein similar to actin-1 and an uncharacterized protein similar to the transmembrane protein named *GRAM domain containing 1A.* Variables showing a fold change >3, p=4.38e-04 and q=0.099.

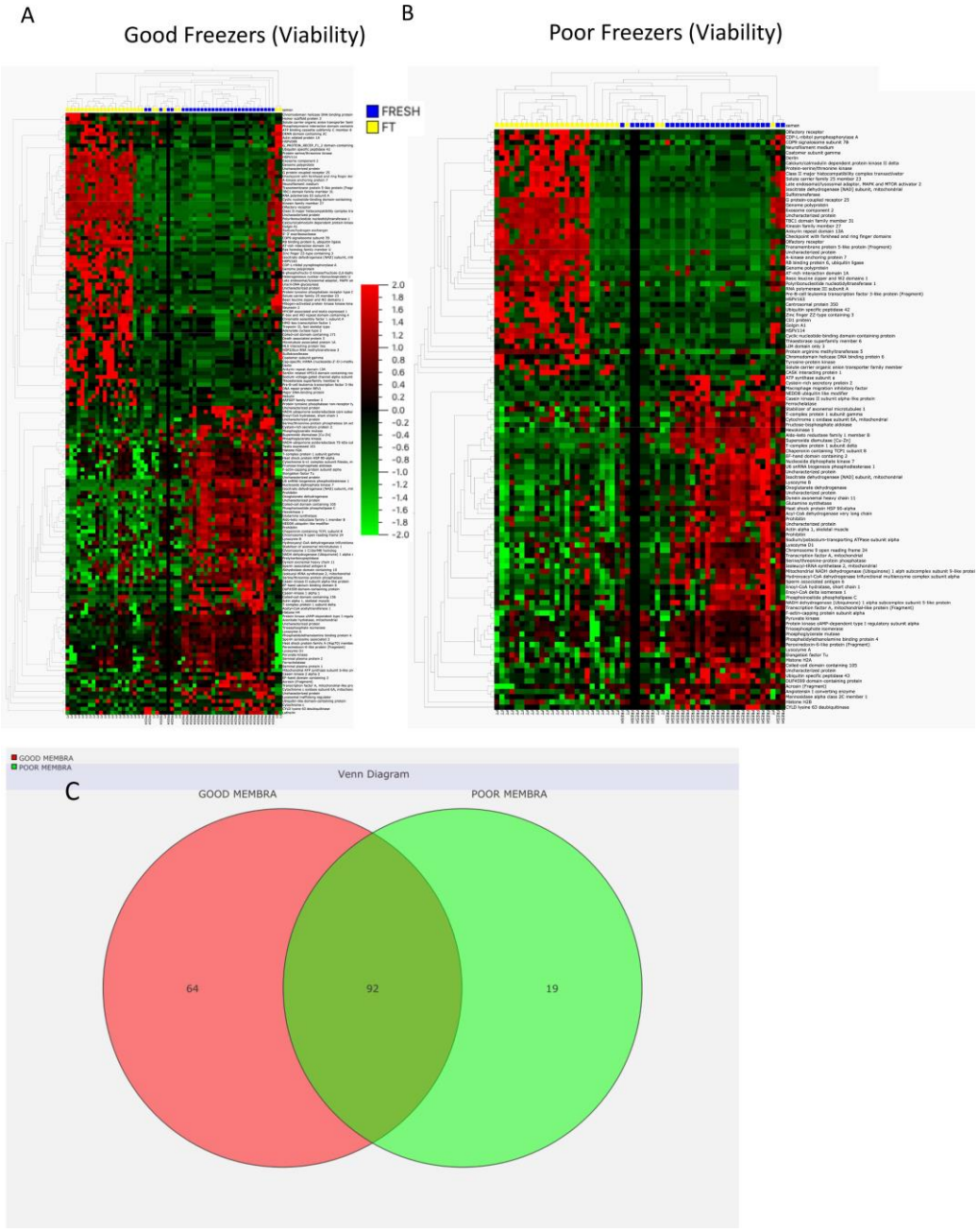


Fig 9.- Heat map showing the impact of cryopreservation on the proteome of stallions showing good and poor membrane integrity post thaw. Proteins are classified following hierarchical clustering. Fresh samples correspond to the blue marks, frozen thawed samples correspond to the yellow marks. The heat map code is present with red areas representing larger amounts of protein and green areas represent smaller amounts of protein. Proteins were normalized, filtered by a fold change >2 with p=0.01 and q=0.1 A) good viability post thaw B) poor viability post thaw. Layout 2.- Venn Diagram showing significant changes caused by cryopreservation (q-value cut-off of 0.1 and a fold change >2) in the amounts of proteins in stallions showing good and poor membrane integrity post thaw. 92 proteins were present in both groups, 64 proteins were only present in the group presenting good membrane viability post thaw and 19 proteins were only present in the group presenting poor membrane viability post thaw.

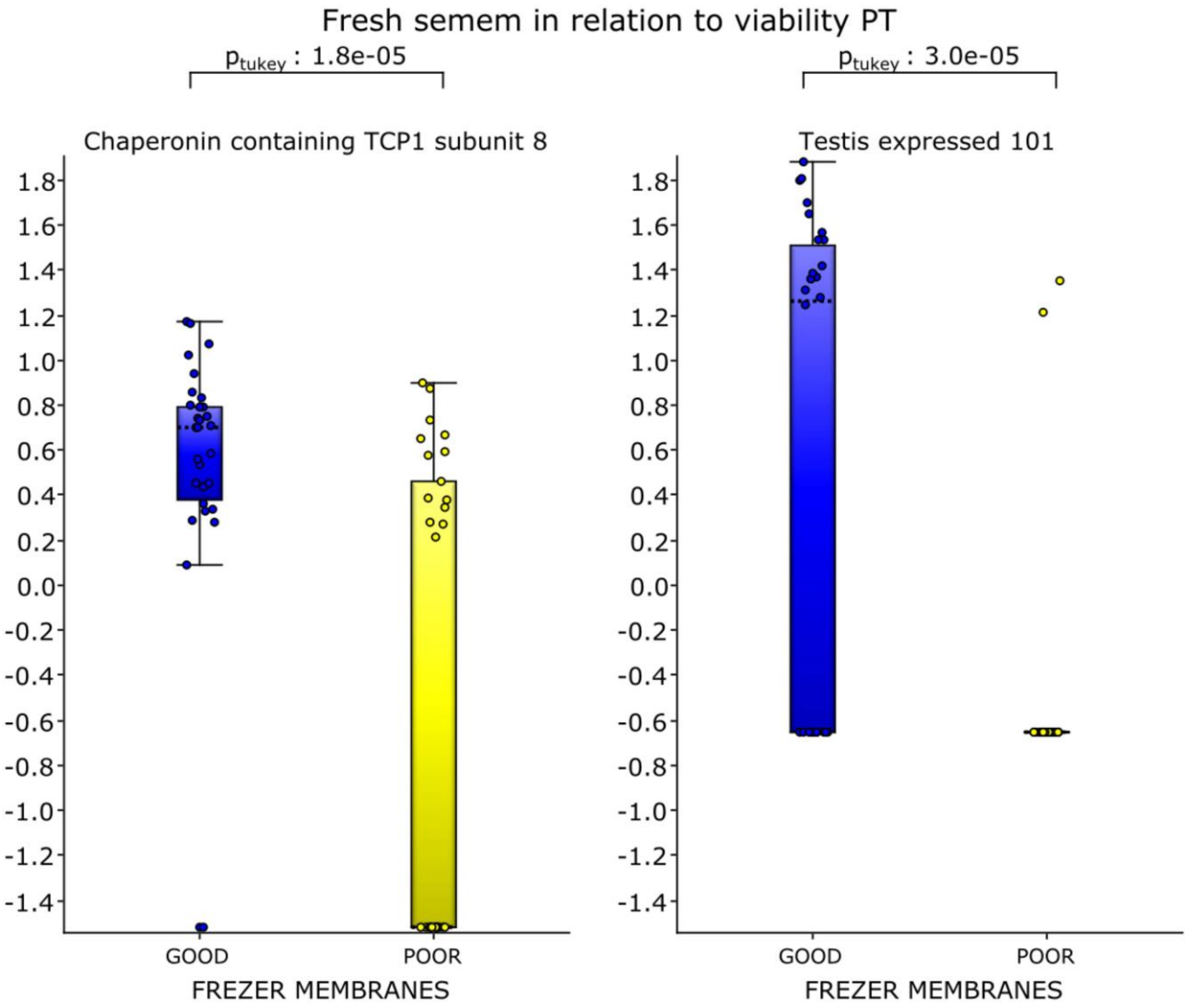


Fig 10.- Discriminant variables between good and poor freezers in terms of viability post thaw, *Chaperonin containing TCP1 subunit 8 and testis expressed 101.* Fold change >3, p= 0.001 and q= 0.01

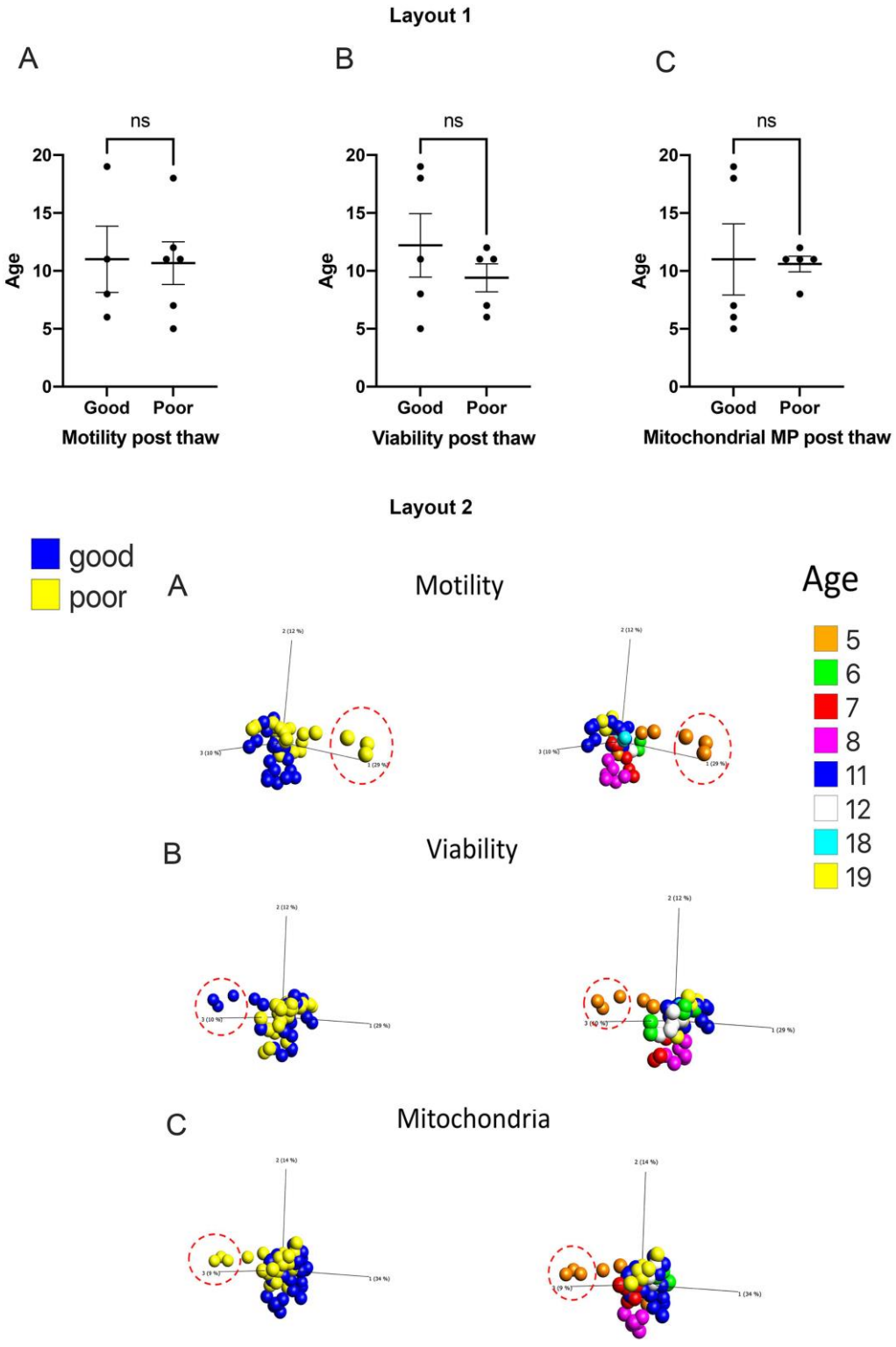


Fig 11 (Layout 1) Effect of age on the outcome of cryopreservation; stallion ejaculates were collected and processed as described in material and methods. The effect of age on motility, viability and mitochondrial membrane potential post thaw was analyzed. No significant differences were observed (two sided t test). Layout 2.- 3D principal component analysis of the stallions' outcome after cryopreservation (left) in the three categories considered, and of the stallion's age (right). Color codes identify both the outcome of cryopreservation and the age of the stallions.