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Proteins involved in mitochondrial metabolic functions and fertilization predominate in stallions with better motility

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ABSTRACT

Even in stallions with sperm quality within normal reference ranges at ejaculation, subtle differences in sperm quality exist that in many cases lead to reduced time frames for conservation of the ejaculate and/or reduced fertility. The spermatozoon is a cell highly suitable for proteomics studies, and the use of this technique is allowing rapid advances in the understanding of sperm biology. The aim of the present study was to investigate differences among stallions of variable sperm quality (based on motility and sperm velocities), although all horses had sperm characteristics within normal ranges. The proteome was studied using UHPLC/MS/MS and posterior bioinformatic and enrichment analysis; data are available via ProteomeXchange with identifier PXD025807. Sperm motility, linear motility and circular, straight line and average velocities (VCL, VSL, VAP) were measured using computer assisted sperm analysis (CASA). In stallions showing better percentages of motility, circular and average velocity predominated mitochondrial proteins with roles in the Citric acid cycle, pyruvate metabolism and oxidative phosphorylation. Interestingly, in stallions with better percentages of total motility, sperm proteins were also enriched in proteins within the gene ontology (G0) terms, single fertilization (G0: 0007338), fertilization (G0: 0009566), and zona pellucida receptor complex (GO:0002199). The enrichment of this proteins in samples with better percentages of total motility may offer a molecular explanation for the link between this parameter and fertility.

Significance: Proteomic analysis identified a high degree of specificity of stallion sperm proteins with discriminant power for motility, linear motility, and sperm velocities (VCL, VAP and VSL). These findings may represent an interesting outcome in relation to the molecular biology regulating the movement of the spermatozoa, and the biological meaning of the measurements that computer assisted sperm analysis (CASA) provide. Of a total of 903 proteins identified in stallion spermatozoa, 24 were related to the percentage of total motility in the sample; interestingly, gene ontology (G0) analysis revealed that these proteins were enriched in terms like single fertilization and fertilization, providing a molecular link between motility and fertility. Field studies indicate that the percentage of total motility is the CASA derived parameter with the best correlation with fertility in stallions.

1. Introduction

Horses have been selected based on performance in sports and/or for morphological traits, this kind of selection has had little impact on fertility in comparison with most domestic species in which indirect selection for fertility has been conducted. As a consequence, large differences among stallions in terms of sperm quality are common. Moreover, the tolerance of equine ejaculates to conservation over long periods also shows high variability [1]. Although progress has been made in the comprehension of the factors involved in sperm quality, there is little information regarding the molecular basis explaining this variability. Recent advances concerning the comprehension of the

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Table 1
Age, breed and fertility record of the stallions used in this study.

	Year of birth	Age when sample taken	Breed	Proven fertility	Number of offspring in stud books
ALT	2008	11	PRE (Purebred Spanish Horse)	Yes	100
AR	2008	11	Arab	Yes	12
AY	2000	19	CDE (Spanish Sport Horse)	Yes	At least 4
BAL	2011	8	PRE (Purebred Spanish Horse)	Yes	4
DC	2008	11	Lusitano	Yes	16
FP	2007	12	Anglo arab	Yes	68
ISL	2012	7	PRE (Purebred Spanish Horse)	Yes	Embryos
MCH	2013	6	Hispano-Arab	Yes	20
NB	2014	5	PRE (Purebred Spanish Horse)	Yes	4
ZED	2001	18	PRE (Purebred Spanish Horse)	Yes	7

All of them were of proven fertility with live foals registered. Note that differences in the number of foals registered depends of the popularity and demand of seminal doses from the stallion and not of its intrinsic fertility.

energetic regulation of the stallion spermatozoa have been particularly interesting, revealing the central role of mitochondria [2–9], and the understanding of redox regulation in these cells [10–13]. In addition, the introduction of proteomics is allowing rapid advances in the comprehension of the molecular biology of the stallion spermatozoa and revealing the molecular differences between stallions with different sperm quality [14], the differences between high and low motility fractions within the ejaculate [15], and differences among stallions with ejaculates which retain good quality after freezing and thawing [16,17]. Proteomic studies are also providing relevant information on other aspects of sperm functionality such as metabolism, capacitation, fertilization and the impact on early embryonic development in many different species, including humans [18–30]. However, to the best of our knowledge, subtle differences among stallions in terms of motility and velocity have not been investigated using proteomic approaches, thus the aim of this study was to identify specific proteins that may explain subtle differences among stallions with sperm characteristics within normal ranges. If specific proteins can be identified, useful markers of quality can be developed, and these proteins can help to understand molecular mechanisms regulating sperm motility and kinematics.

Table 2
Statistical model used in the Biomarker Workbench (https://qlucore.com) to identify variables (proteins) associated with CASA derived parameters of motility and velocity.

Explanatory variable Type	Explanatory variable Details	Eliminated factors	q < 0.05
Multi Group Comparison	BREED		19
Multi Group Comparison	stallion		43
Linear Regression	AGE[≠ 0]		0
Multi Group Comparison	motility		12
Multi Group Comparison	linear motility		4
Multi Group Comparison	VCL		11
Multi Group Comparison	VAP		7
Multi Group Comparison	VSL		4

The stallion, breed and age were also included without elimination of any factor in the model. The number of variables identified specifically for each trait with a q value <0.05 are given.

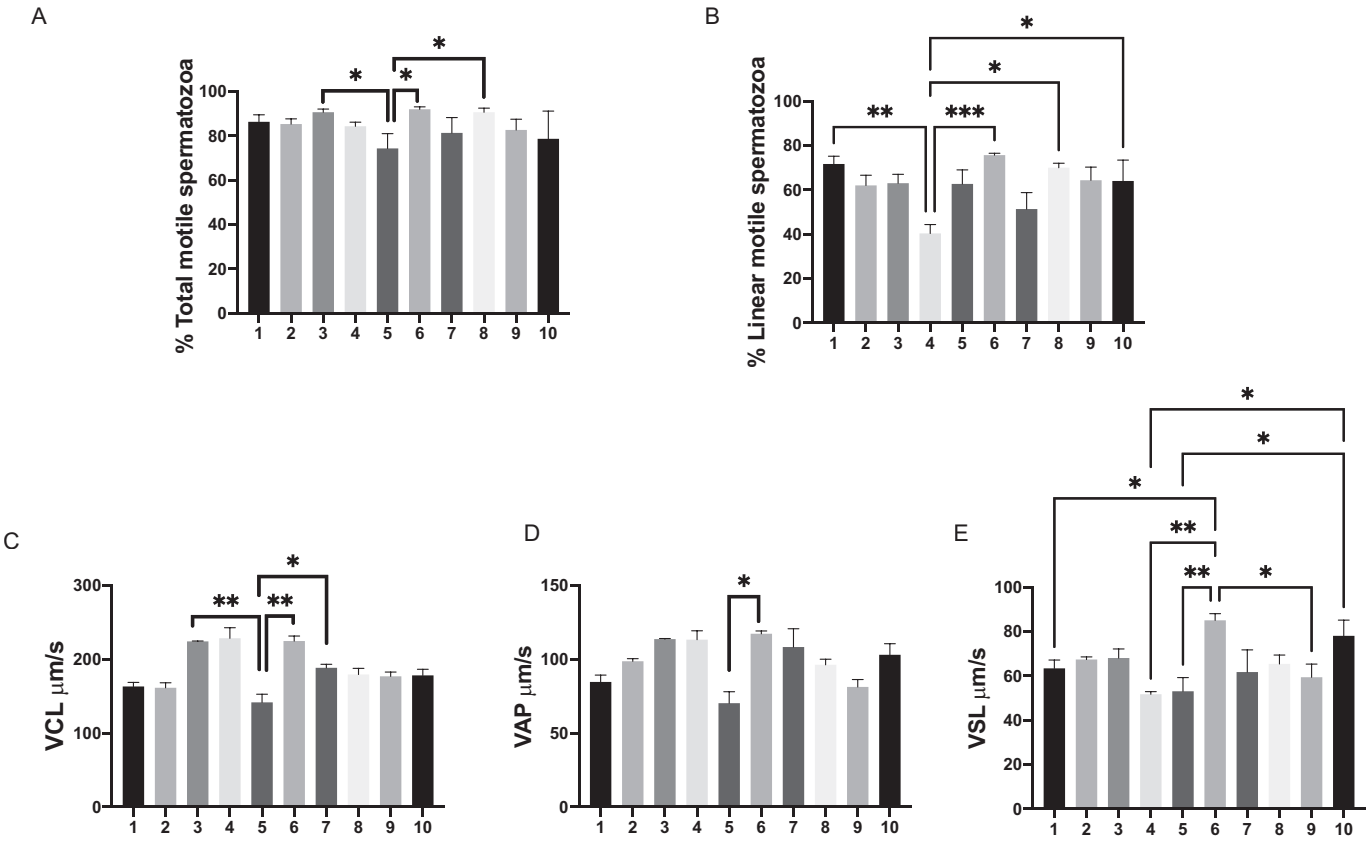


Fig. 1. Computer assisted sperm analysis (CASA), ejaculates were obtained and processed as described in material and methods and the percentages of total motile, linear motile, circular velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) in µm/s determined. A) percentages of total motile spermatozoa, B) percentage of linear motile spermatozoa, C) VCL, D) VAP, E) VSL * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ Data are expressed as means ± s.e.m. ($n = 30$ ejaculates).

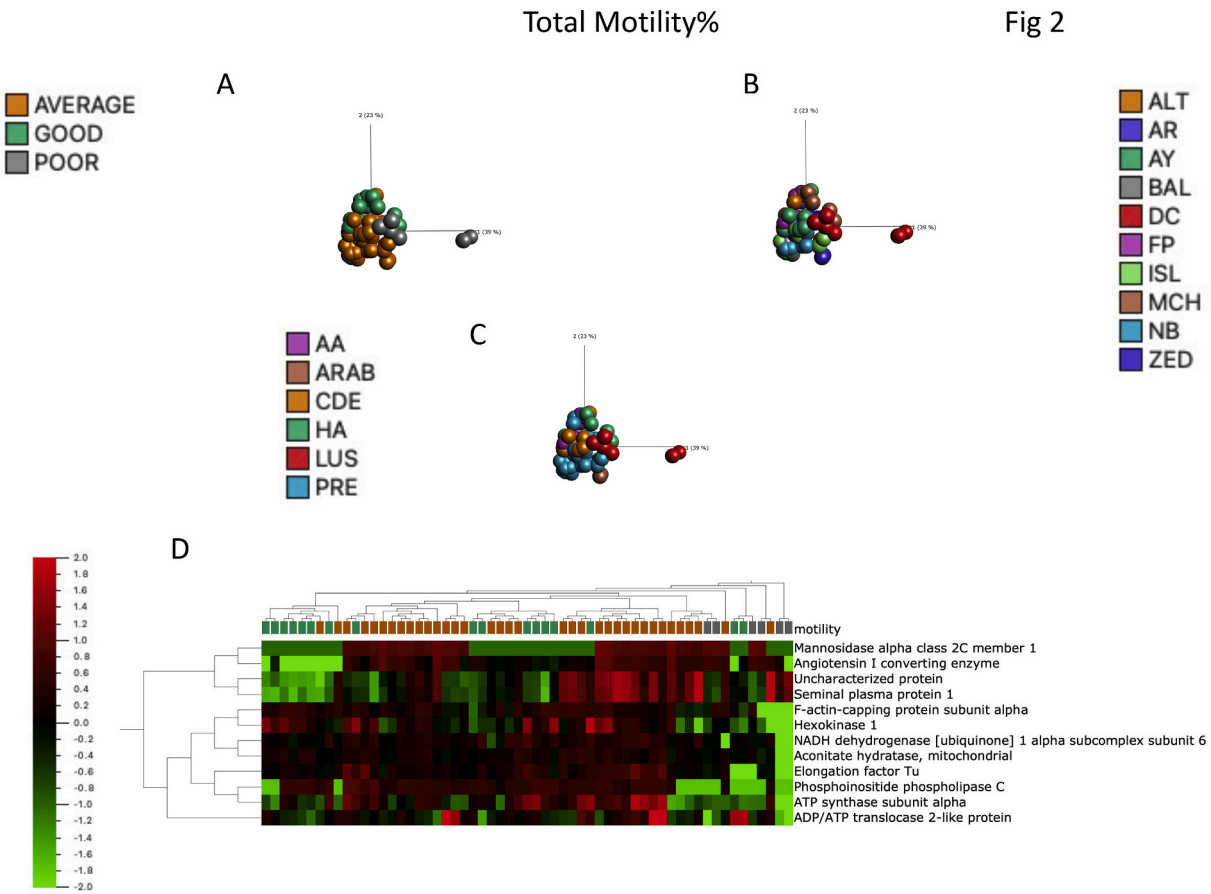


Fig. 2. Bioinformatic analysis of proteins differentially expressed in stallions with different percentages of total motility A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions, average, good, and poor B) 3D PCA overlayed with color codes for the different stallions, C) 3D PCA overlayed with color codes for the different breeds. D) Heat map showing different amounts of specific proteins in stallions with different percentages of total motility. Analysis was performed using Qlucore Omics Explorer (https://www.qlucore.com) (n = 30 ejaculates) (p = 6.30e-04, q = 0.045).

2. Material and methods

2.1. Reagents and media

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated.

2.2. Semen collection and processing

Semen was collected from 10 stallions of various breeds (5 Andalusians, 1 Spanish Sport Horse, 1 Lusitano, 1 Arab, 1 Anglo-Arabian and 1 Spanish-Arabian horse) maintained as indicated under 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 registered foals and embryos produced as indicated in Table 1 with a median age of 10.8 years old. 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 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 using an inline filter. Semen was immediately transported to the laboratory after collection for evaluation and processing. Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [35,36] to remove dead spermatozoa, seminal plasma and contaminating cells, and then was re-suspended in Tyrode's medium (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM $NaHCO_3$, 1 mM Na-Pyruvate, 21.6 mM Na-Lactate, 2 mM $CaCl_2$*2H2O, 3.1 mM KCl, 0.4 mM $MgSO_4$*7H2O, 0.3 mM NaH_2PO4*H2O, 0.3% BSA) 315 mOsm/kg and pH 7.4 [37]. An aliquot was immediately removed to measure motility and velocity assessment and the rest was processed for proteomic analysis.

2.3. Sperm preparation

Spermatozoa were washed three times in PBS (600 g × 10′) pelleted and kept frozen at −80 °C until analysis.

2.4. Protein solubilization

The pellet consisting of 200 × 10^6 spermatozoa was solubilized in lysis buffer formulated as follows (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7 M urea, 2 M thiourea and 40 mM Tris (pH 10.4). Per each 10 × 10^6 spermatozoa 20 μl of lysis buffer were added and the solution vortexed and incubated under constant rotation at 4 °C for 1 h.

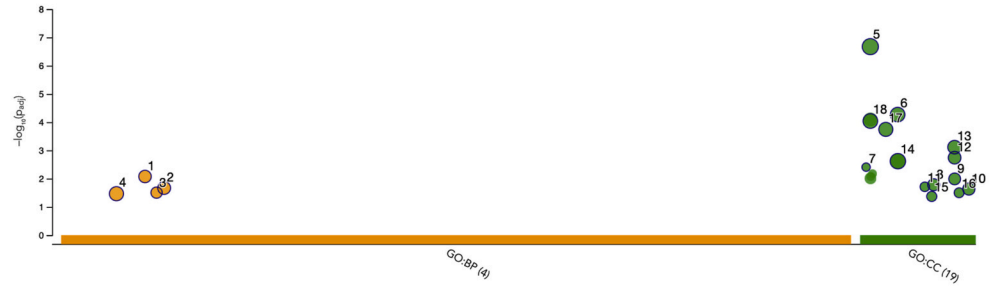
2.5. Protein quantification

The 2-D Quant Kit (GE Healthcare, Sevilla Spain) was used to quantify the amount of protein as described in [16] and all samples were normalized to obtain a final concentration of 100 μg of protein per sample.

2.6. In-solution Trypsin digestion

200 μL of solution obtained from the previous stage were mixed with

A

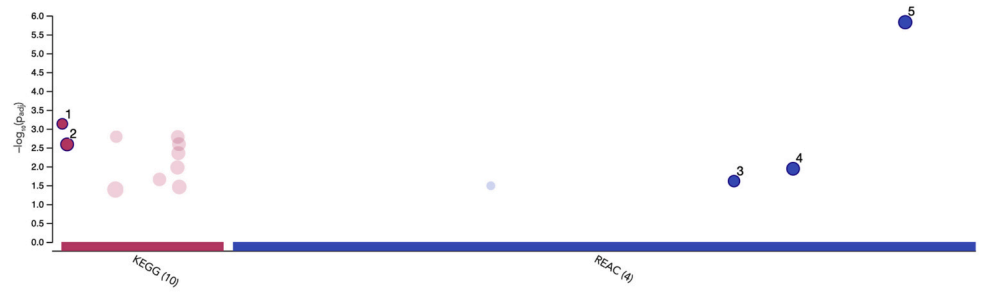


ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:BP	GO:0007338	single fertilization	8.373×10^{-3}
2	GO:BP	GO:0009566	fertilization	2.133×10^{-2}
3	GO:BP	GO:0009060	aerobic respiration	3.118×10^{-2}
4	GO:BP	GO:0006091	generation of precursor metabolites and energy	3.405×10^{-2}
5	GO:CC	GO:0005739	mitochondrion	2.118×10^{-7}
6	GO:CC	GO:0031966	mitochondrial membrane	5.392×10^{-5}
7	GO:CC	GO:0002199	zona pellucida receptor complex	3.904×10^{-3}
8	GO:CC	GO:0070469	respirasome	1.641×10^{-2}
9	GO:CC	GO:0098803	respiratory chain complex	1.017×10^{-2}
10	GO:CC	GO:1990204	oxidoreductase complex	2.319×10^{-2}
11	GO:CC	GO:0045277	respiratory chain complex IV	1.941×10^{-2}
12	GO:CC	GO:0098800	inner mitochondrial membrane protein complex	1.806×10^{-3}
13	GO:CC	GO:0098798	mitochondrial protein-containing complex	7.658×10^{-4}
14	GO:CC	GO:0031975	envelope	2.403×10^{-3}
15	GO:CC	GO:0070069	cytochrome complex	4.248×10^{-2}
16	GO:CC	GO:0101031	chaperone complex	3.151×10^{-2}
17	GO:CC	GO:0019866	organelle inner membrane	1.799×10^{-4}
18	GO:CC	GO:0005740	mitochondrial envelope	9.088×10^{-5}

version	e102_eg49_p15_7a9b4d6
date	16/3/2021 12:48:16
organism	ecaballus

g:Profiler

B



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	KEGG	KEGG:00020	Citrate cycle (TCA cycle)	7.384×10^{-4}
2	KEGG	KEGG:00190	Oxidative phosphorylation	2.590×10^{-3}
3	REAC	REAC:R-HSA-71406	Pyruvate metabolism and Citric Acid (TCA) cycle	2.437×10^{-2}
4	REAC	REAC:R-HSA-163200	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	1.145×10^{-2}
5	REAC	REAC:R-HSA-1428517	The citric acid (TCA) cycle and respiratory electron transport	1.491×10^{-6}

version	e102_eg49_p15_7a9b4d6
date	16/3/2021 13:16:17
organism	hsapiens

g:Profiler

Fig. 3. g:GOST multiquery Manhattan plot showing enrichment analysis of proteins present in different amounts in stallions with different percentages of total motility. The sperm proteome under each condition was queried against the equine proteome database. A) Gene Ontology (GO) terms for biological process (BP) are in orange, and those for cellular component (CC) in green. B) KEGG pathways are depicted in red, Reactome pathways are depicted in blue (using human orthologs). The *p* values are depicted on the y axis and in more detail in the results table below the image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

100 μL of 25 mM ammonium bicarbonate buffer with pH 8.5 (100 μg of protein in 300 μL 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 1 mM 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) into a 2 mL dark glass vial. Finally, samples were dried using a nitrogen current with the vial in a heating block at 35 °C. The dry samples were resuspended in 20 μL of buffer A, consisting of water/acetonitrile/formic acid (94.9:5:0.1).

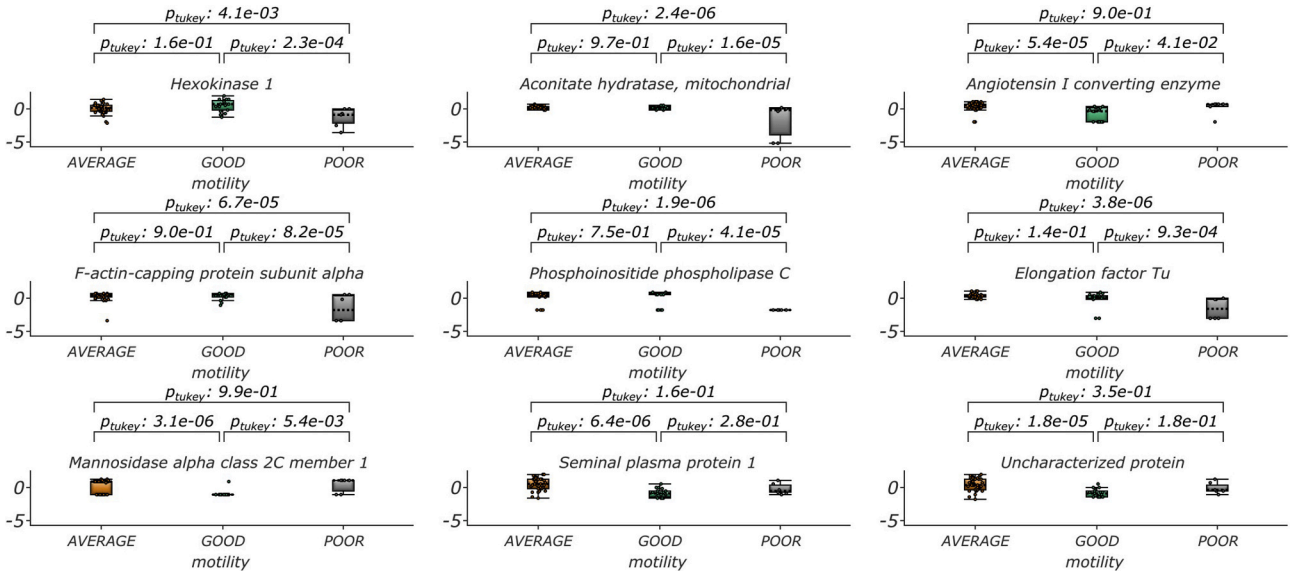


Fig. 4. Differences in the amount of specific representative proteins in stallions with different percentages of total motility. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category are represented by color codes. Proteins were normalized, Log_2 transformed and filtered by a fold change >4, ($p = 3.85e^{-4}$, q = 0.03) ($n = 30$ ejaculates).

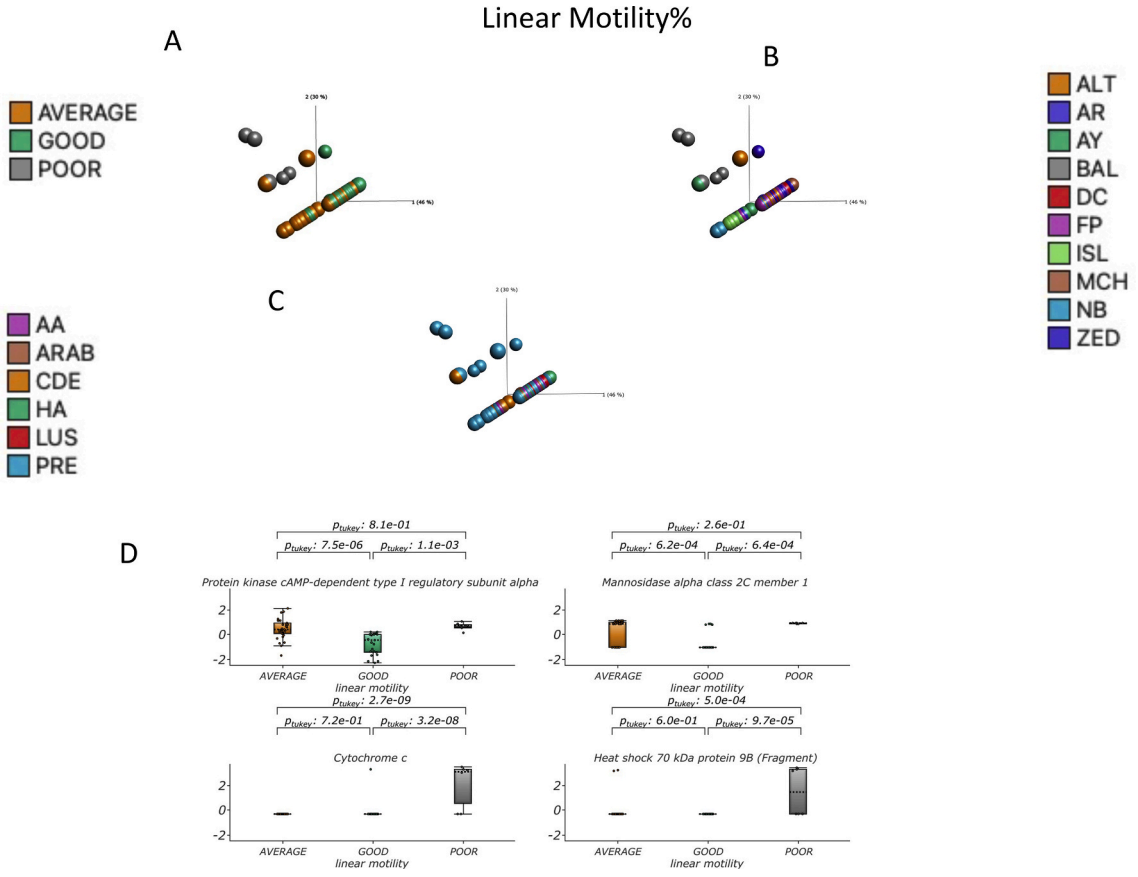


Fig. 5. Differences in the amount of specific representative proteins in stallions with different percentages of linear motility. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category are represented by color codes. Proteins were normalized Log_2 transformed and filtered by a fold change >2. A) 3-D principal component analysis (PCA) of the samples, showing different color codes for stallions showing average, good and poor percentages of linear motility. B) PCA overlayed with different color codes for the different stallions C) PCA overlayed with different color codes for different breeds D) Box plot showing different amounts of specific proteins in stallions with different percentages of linear motile spermatozoa, p = 3.8e-04, q = 0.033. (n = 30 ejaculates).

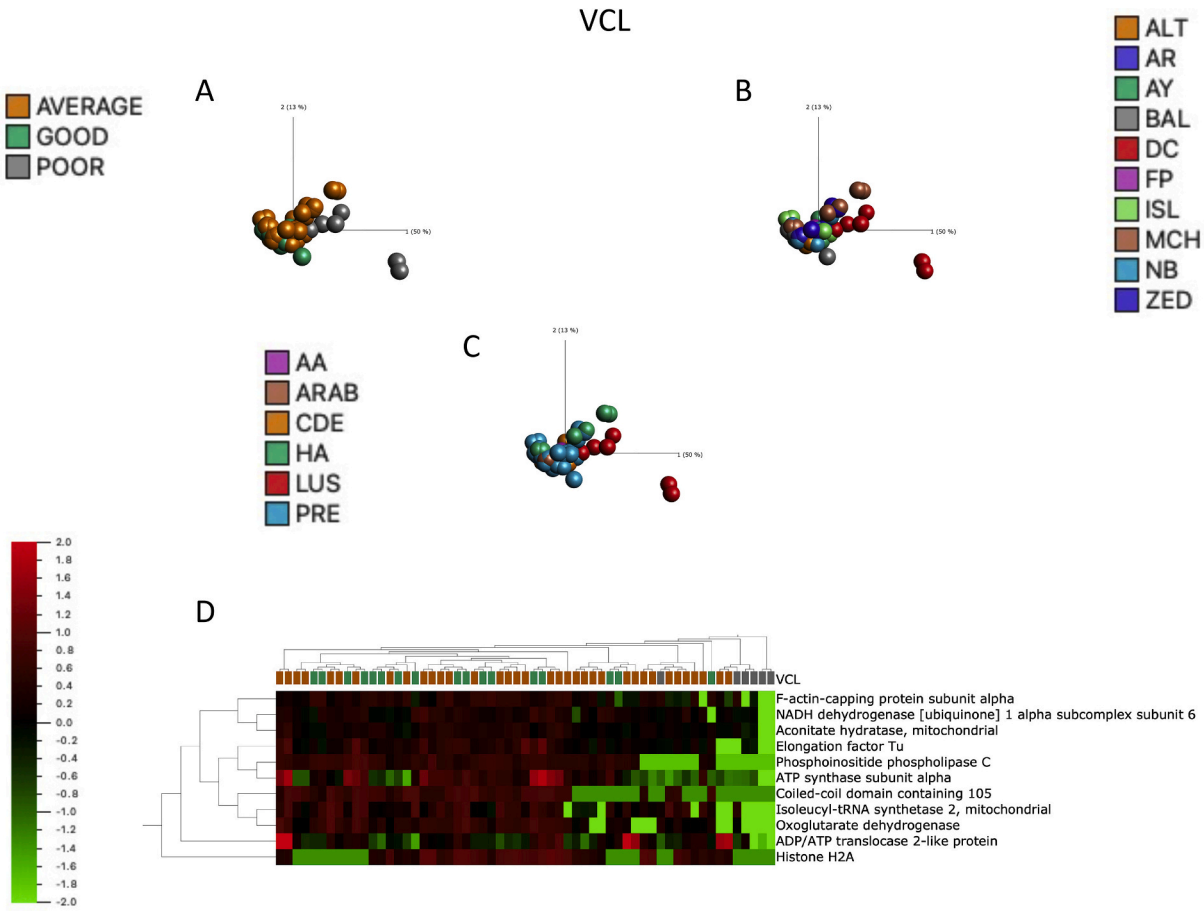


Fig. 6. Bioinformatic analysis of proteins differentially expressed proteins in stallions with different circular velocity (VCL) A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions B) 3D PCA for sperm proteins C) 3D PCA of the samples with different color codes for the breed. D) Heat Map showing different amounts of specific proteins in stallions with different VCL (p = 7.2e-04, q = 0.05). (n = 30 ejaculates) Analysis was performed using Qlucore Omics Explorer (https://www.qlucore.com). D).

2.7. UHPLC-MS/MS analysis

Separation and analysis of the samples was performed with 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 processed as previously described [38], in brief 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 then 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 300 °C. 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/s for MS and 3 spectra/s 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.

2.8. Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) as previously described [17]. Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH] + 50–10,000 m/z; maximum precursor charge +5; retention time and m/z tolerance ±60 s; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against Uniprot/Horse (https://www.uniprot.org/taxonomy/9796) was performed as follows: 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%). Protein polishing validation was then performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0%).

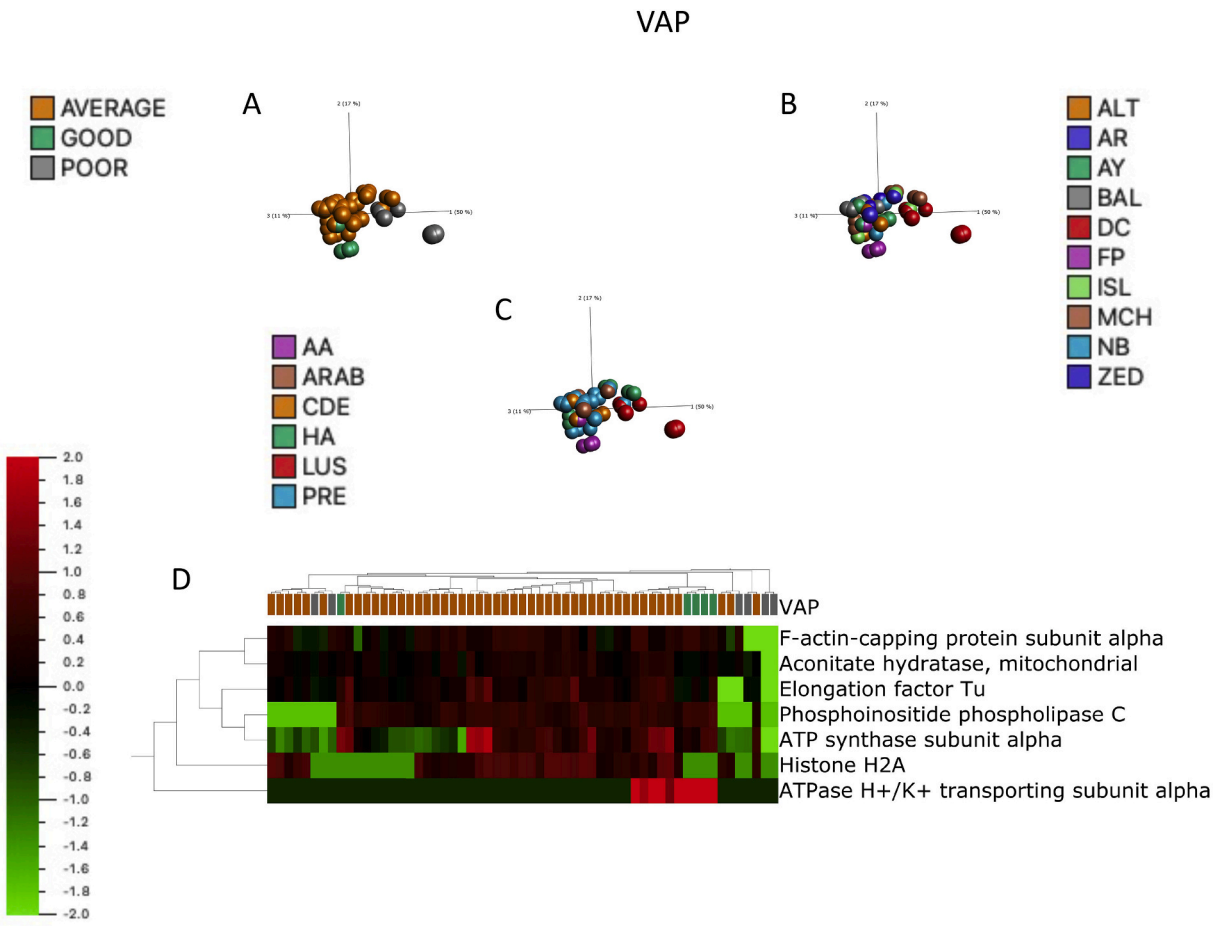


Fig. 7. Bioinformatic analysis of proteins differentially expressed in stallions with different average path velocity (VAP) A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions B) 3D PCA for sperm proteins C) Heat Map showing different amounts of specific proteins in stallions with different VAP (p = 1.84e-04, q = 0.024). Analysis was performed using Qlucore Omics Explorer (https://www.qlucore.com). D) (n = 30 ejaculates).

2.9. 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 [39]. 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 10 x 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.

2.10. Bioinformatic analysis

2.10.1. 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 [40] was used to determine the optimal filtering threshold.

2.10.2. Identifying discriminating variables

Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables with the highest significant difference in stallions showing different percentages for total and linear motility, and velocities (VCL, VAP and VSL). The identification of significantly different variables between the subgroups of stallions was performed by fitting a linear model for each variable with condition proteins of the spermatozoa from stallions showing different values of the above parameters as predictors, including the stallion, breed and age as nuisance covariates. *P*-values were adjusted for multiple testing using the Benjamini-Hochberg method [41,42] and variables with adjusted *p*-values (q values) below 0.1 were considered significant. Moreover, we also used the new function Biomarker Workbench in Qlucore version 3.7.21; this functionality allows the simultaneous analysis of multiple variables. We used a model including, age, breed, individual stallion, and the computer assisted sperm analysis (CASA) derived parameters: percentage of total motile spermatozoa, percentage of linear spermatozoa, circular velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL), in this analysis q values were adjusted to 0.05.

2.10.3. Enrichment analysis of pathways in the sperm proteome

PANTHER (http://www.pantherdb.org/pathway/pathwayList.jsp) and KEGG pathway (https://www.genome.jp/kegg/) [43–46] analysis was used to identify biological pathways likely to be active in the proteins enriched in each group. The significance of the presence of the protein list was queried against the equine proteome database using a FDR <0.05 and Fisher's exact test. g:Profiler was also used to perform an enrichment analysis [47]. Due to the increased depth of the human proteome in terms of annotation, the equine annotations were transformed to their human orthologs using g:Profiler (https://biit.cs.ut.ee/gprofiler/orth) and a pathway enrichment analysis was performed

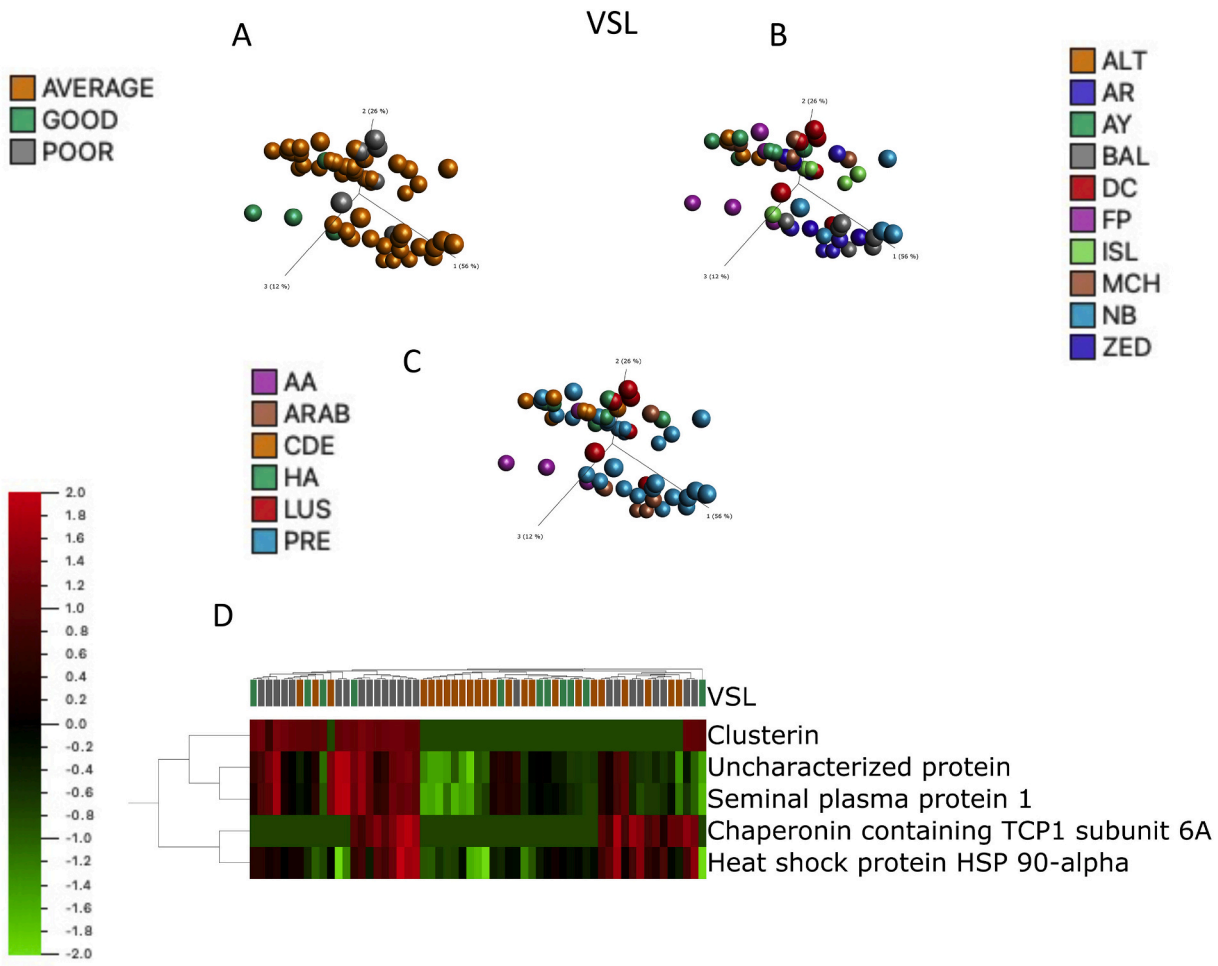


Fig. 8. Differences in the amount of specific representative proteins in stallions with different percentages of VSL. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category are represented by color codes. Proteins were normalized Log_2 transformed and filtered by a fold change >2 A) 3-D principal component analysis (PCA) of the samples, showing different color codes for stallions showing average, good and poor VSL. B) PCA overlayed with different color codes for the different stallions C) PCA overlayed with different color codes for different breeds D) Heat map different amounts of specific proteins in stallions with different VSL (n = 30 ejaculates).

again using g:Profiler and Reactome (https://reactome.org).

2.11. Western blotting

Proteins were extracted and denatured by boiling for 10 min at 70 °C in a loading buffer supplemented with 5% mercaptoethanol. Ten micrograms of sperm protein extract were loaded on a 10% polyacrylamide gel and resolved using SDS-PAGE. Immunoblotting was performed by incubating the membranes with blocking buffer containing primary antibodies (ATP5A1 from Biorbit, Cambridge UK, orb375551, HSP70, #4872, HSP90, #4874 and Cytochrome C, #4280, Cell Signaling, Danver Massachusetts, USA) following the instructions of the manufacturers overnight at 4 °C. Secondary antibodies were used at 0.27 μg/ml (anti-mouse) or 0.16 μg/ml (anti-rabbit) depending on the primary antibody used. Positive controls used were brain lysates for ATP5A1, and Hela cells for HSP70, HSP90 and cytochrome C. The detection of the specific signal was completed by using chemiluminescence. To generate this signal, the secondary antibodies were conjugated with horseradish peroxidase (HRP), an enzyme that emits light in the presence of the appropriate substrate (Clarity Western ECL Substrate, Bio-Rad). The intensity of the chemiluminescent signal and the relative molecular mass of the proteins were estimated by using the Bio-Rad chemidoc MP system. The signals were standardized by quantifying the protein concentration (Bradford assay) before loading an equal amount of proteins from the samples onto stain-free gels (Bio-Rad). After transfer, stain-free gels allowed an overall protein stain that were used for protein normalization.

2.12. Statistical analysis

The normality of motility and sperm velocities 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).

3. Results

3.1. Sperm motility and velocity

Sperm motilities and velocities differed between stallions; the percentage of total motile sperm was higher in stallion 3 (90.7 ± 1.4%), 6 (92.0 ± 1.15%) and 8 (90.7 ± 1.8%), that were classified as "good"; these values were significantly different from stallion 5 (74.3 ± 6.7; $P < 0.05$), that was classified as "poor". Stallions 1, 2, 4, 7, 9, and 10 presented motilities that were not statistically different from any other stallion and were classified as "average" (Fig. 1A). Differences among stallions were also evident in the percentages of linear motile

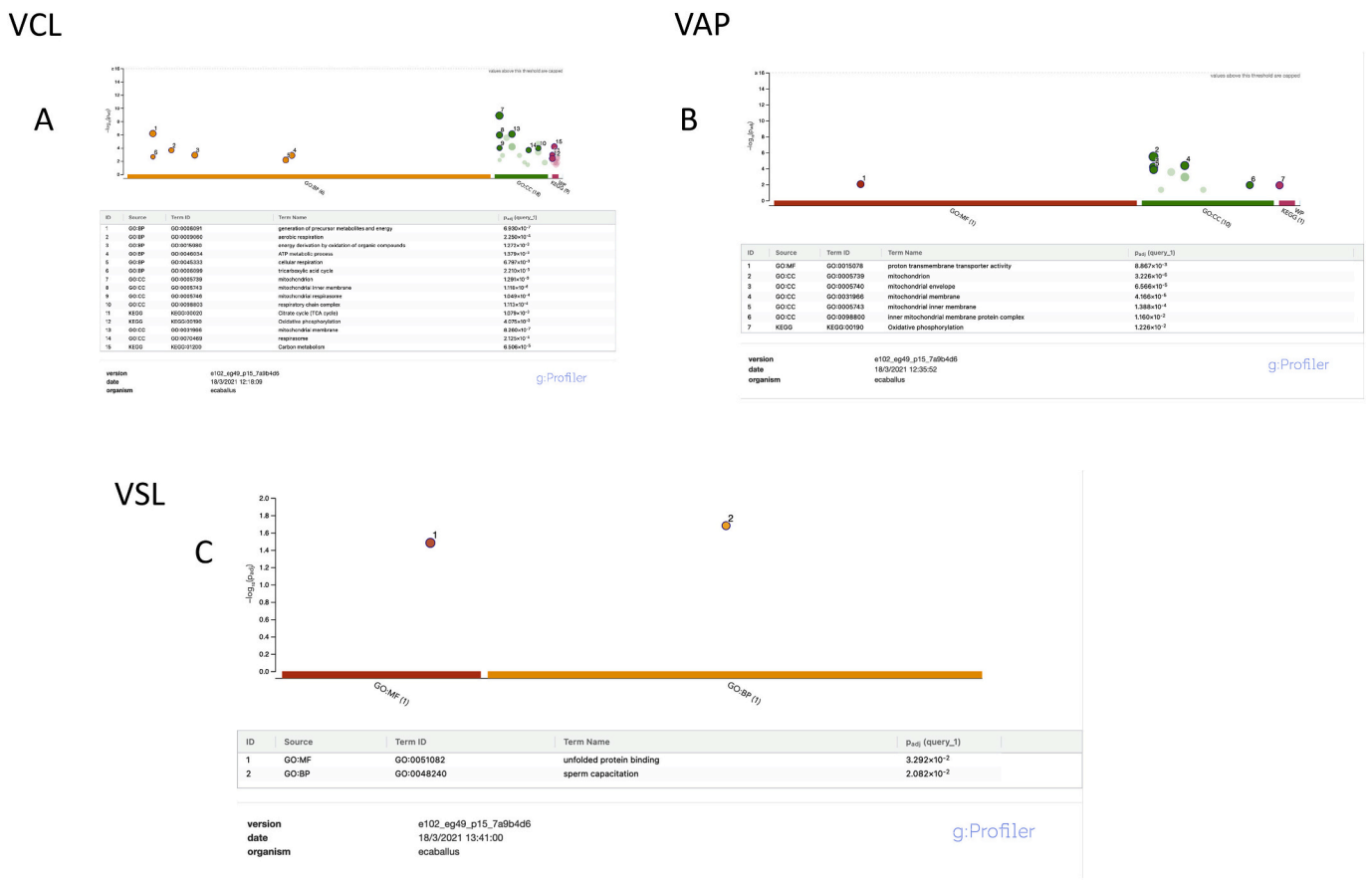


Fig. 9. g:GOST multiquery Manhattan plot showing enrichment analysis of proteins present in different amounts in stallions with different VCL, VAP and VSL. The sperm proteome was queried against the equine proteome database. A) Gene Ontology (GO) terms for biological process (BP) are in orange, and those for cellular component (CC) in green, KEGG pathways are depicted in red. The p values are depicted on the y axis and in more detail in the results table below the image (n = 30 ejaculates). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spermatozoa (Fig. 1B) with stallions 1 (71.7 ± 3.5%$P < 0.01$), 6 (75.7 ± 0.9; $P < 0.001$), 8 (70.0 ± 2.1%; $P < 0.05$) and 10 (64.0 ± 9.4%; $P < 0.05$) classified as "good" that were statistically different from stallion 4 (40.3 ± 4.0%), which was classified as "poor". Stallions 2,3,5, 7 and 9 were classified as "average", since the percentages of linear motilities were not significantly different from any other stallion. Sperm velocities also varied among stallions (Fig. 1A-D). Stallion 5 had the lowest circular velocity (141.1 ± 10.8 μm/s) and this difference was statistically significant from stallions 3 (224.0 ± 0.6 μm/s; $P < 0.01$), 6 (224.7 ± 6.7 μm/s; $P < 0.01$) and 7 (188.3 ± 4.9 μm/s; $P < 0.05$). Differences in average path velocity and straight line velocity were also observed among stallions (Fig. 1D-E).

3.2. Stallions with different sperm motility present different amounts of specific proteins

A total of 903 different sperm proteins were identified in stallion spermatozoa The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025807 [48] and in the accompanying data in brief article. The Biomarker workbench identified the individual stallion as the major source of variation, with 43 explanatory variables, breed retrieved 19 explanatory variables, motility 12 explanatory variables, VCL 11 explanatory variables, VAP 7 explanatory variables, VSL 5 explanatory variables and % linear motility 4 explanatory variables (Table 2). We constructed a Heat Map showing the differences in the amounts of the 12 proteins present in different amounts in stallions showing good, average and poor motility (p = 6.30e-04, q = 0.045; Fig. 2D). Principal component analysis (PCA) showed that most of the stallions presenting good, average and poor motility were grouped in well-defined clusters (Fig. 2A). Also, PCA analysis were used to see the impact of breed and stallion of these proteins, showing that only two replicates of one Lusitano stallion were located in a separate cluster in the PCA plot; in this case only one Lusitano stallion was included in the study (Fig. 2B and C). Enrichment analysis revealed that these proteins were related to important functions, mainly fertilization and generation of energy in the mitochondria through oxidative phosphorylation (Fig. 3A-B). In order to identify better discriminant proteins, a more restrictive statistical criteria (q = 0.03) was used (Fig. 4), *hexokinase 1, aconitate hydratase mitochondrial, phosphoinositide phospholipase C, elongation factor Tu and F actin capping protein subunit alpha* were more abundant in stallions showing good percentages of total motility. The search for discriminant variables for the percentages of linear motility, gave 4 proteins (3.80e-04; q = 0.033; Fig. 5D), the protein kinase cAMP dependent type I regulatory subunit alpha, mannosidase alpha class 2C member 1, cytochrome C and Heat Shock Protein 70 (Fig. 5D). PCAs were also constructed, and no effect of breed or individual stallion was evident in this case (Fig. 5A-C). Cytochrome C and heat shock protein 70 kDA protein 98 fragment, were present in higher amounts in stallions showing poor linear motility (p = 6.1e-4, q = 0.08; Fig. 5D).

3.3. Stallions with different sperm velocities differ in the amounts of specific proteins

Differences in protein composition were related to differences in

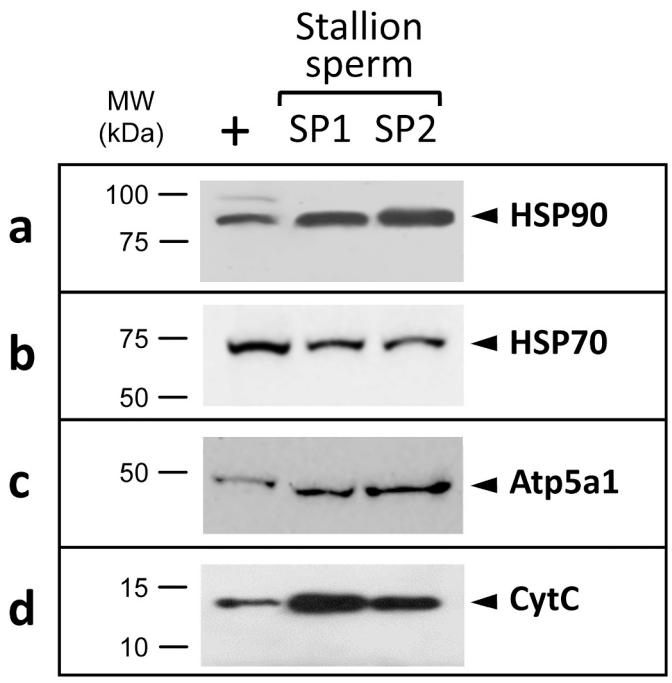


Fig. 10. Representative subset of proteins identified were validated through western blotting analysis.Sperm lysates from two different stallions were used. a) HSP90 b) HSP70 c) ATP5A1 and d) Cytochrome C. Positive controls used were brain lysates for ATP5A1, and Hela cells for HSP70, HSP90 and cytochrome C.

sperm velocities, especially in circular and average path velocities. Differences in circular velocity was related with different expression of 11 proteins (Fig. 7; $p = 7.2e\text{-}04$, q = 0.05), in average path velocity with different expression of 7 proteins (Fig. 8; $p = 1.84e\text{-}04$, q = 0.02) and straight line velocity with 5 different expressed proteins (Fig. 9; $p = 3.35e^{-}04$, q = 0.045). For each velocity, PCAs were constructed to visualize any possible effect of the stallion or breed; as in the case of total motility only two replicates of one stallion, the only Lusitano, were outside the main cluster in VCL (Fig. 6B-C) and VAP (Fig. 7B-C). Enrichment analysis revealed the predominance of mitochondrial proteins with roles in the electron transport chain and the Krebs cycle in relation to VCL and VAP (Fig. 9A andB). To the contrary, proteins in relation to straight line velocity were enriched in the gene ontology terms unfolded protein binding and sperm capacitation (Fig. 9C). Specific discriminant proteins present in higher amounts in stallions with higher circular velocity included the *ATP synthase subunit alpha, aconitase hydratase mitochondrial, phosphoinositide phospholipase c and the elongation factor tau* (Fig. 6D). Proteins acting as discriminant variables for stallions showing better average path velocity included *ATP synthase subunit alpha, aconitate hydratase mitochondrial, F-actin-capping protein subunit alpha, and the ATPase H^{+}/K^{+} transporting subunit* (Fig. 7D). Discriminant proteins for straight line velocity identified stallions with low VSL and were the *HSP-90 alpha, chaperonin containing TCP1 subunit 6A and seminal plasma protein 1* (Fig. 8D). A representative subset of proteins is presented in the WB in Fig. 10.

4. Discussion

In the present study we report compelling evidence that the proteome of the stallion spermatozoa clearly influences sperm motility. We were able to disclose proteins that were discriminant variables of good or poor motility and sperm velocity. Although the relation between the sperm proteome and motility have already been reported, most of these studies have been done comparing asthenospermic versus normospermic samples [27,49–53], or good quality versus low quality sperm after colloidal centrifugation to recover the high quality spermatozoa in the sample [14,15,53]. To the author's knowledge this is the first time that the proteome has been studied in stallions with different motilities, albeit, within normal ranges.

Proteomic analysis identified a high degree of specificity in relation to sperm proteins with discriminant power for motility, linear motility, as also occurred for the velocities herein studied, VCL, VAP and VSL. These findings may represent an interesting outcome in relation to the molecular biology regulating the movement of the spermatozoa, and the biological meaning of the measurements that computer assisted sperm analysis provide. Of a total of 903 proteins identified in stallion spermatozoa, 24 were related to the percentage of total motility in the sample; interestingly, gene ontology (G0) analysis revealed that these proteins were enriched in terms like single fertilization and fertilization, providing a molecular link between motility and fertility. Field studies indicate that the percentage of total motility is the CASA derived parameter with the best correlation with fertility in stallions [54,55].

Other gene ontology terms and reactome pathways revealed the predominance of mitochondrial proteins and metabolic pathways present in the mitochondria, the Krebs cycle and oxidative phosphorylation, with an important protagonist role in pyruvate metabolism in this highly specialized cell. These findings support the wide consensus in the scientific community in relation to the predominance of oxidative phosphorylation in the stallion spermatozoa in the production of ATP for motility [2,5,6,8,9]. Specific proteins were discriminant for motility; hexokinase-1 was present in higher amounts in stallions with significantly better motility. This protein has been previously identified as a marker of good motility in stallions [15]. This is one of the multiple isoforms of the protein phosphorylating glucose and other hexoses in the first step of glycolysis. It is located in the mitochondrial outer membrane and when there is increased glycolytic flux accumulation of glucose-6-phosphate may inhibit hexokinase and increase the production of mitochondrial reactive oxygen species (ROS) [56]. This mechanism may also exist in the spermatozoa, increased production of ROS has been related to increased glucose concentrations in extenders in our laboratory [57]. Apparently, this mitochondrial bound hexokinase-1 has a pivotal role in regulating mitochondrial redox homeostasis and its inhibition leads to increased production of ROS and activation of the mitochondrial apoptotic pathway [56].

Another protein found to be more abundant in stallions with better percentages of motility was the aconitase hydratase mitochondrial. This protein catalyzes the conversion of citrate to isocitrate via cis-aconitate. This finding underpins the importance of the mitochondrial production of ATP for sperm motility, supporting previous studies in the field [5,8,9,58]. Other proteins with significantly higher amounts in stallions showing better percentages of motility were the f actin-capping protein subunit alpha, phosphoinositide phospholipase C and the elongation factor tu. On the other hand, mannosidase alpha 2 class 2C member 1, and angiotensin I converting enzyme were discriminant proteins for stallions with poorer (although within normal ranges) percentages of total motile spermatozoa.

In relation to the percentages of linear motile spermatozoa, discriminant proteins for poor linear motility samples were identified. Interestingly cytochrome C and the heat shock 70 kd protein 9B (fragment) were much more abundant in samples with lower percentages of linear motility. The presence of higher amounts of cytochrome C underpins the role of apoptotic mechanisms in sperm damage; apoptotic phenomena [59–67] and more recently evidence of ferroptosis [13] are considered as major causes of sperm malfunction. The finding of higher amounts of cytochrome C confirms that the mitochondrial pathway of apoptosis is behind sperm malfunction [62,63]. Sperm protein composition also had a major influence on sperm velocities; with VCL and VAP showing a very similar profile, and VSL presenting a specific profile of differentially predominant proteins. Enrichment analysis in differentially expressed proteins in stallions showing differences in VCL and VAP showed enriched annotations of GO terms and Kyoto Encyclopedia of

Genes and Genomes (KEGG) pathways related to mitochondria and mitochondrial metabolism, oxidative phosphorylation and Citrate Cycle (TCA cycle). This is an interesting finding, since it indicates that the energy for sperm velocity is produced through mitochondrial metabolism. These findings argue against a key role of glycolysis in supporting sperm velocities; these concepts arose from the drop observed in sperm velocities when glycolysis was inhibited using the non-metabolizable analogue 2-deoxyglucose (2-DG). However the most likely explanation for this finding is ATP depletion due to futile phosphorylation of 2-DG by hexokinase, we have described that incubation of stallion of spermatozoa in a media devoid of glucose does not affect velocities provided that substrates for the TCA cycle are provided [9].

Proteins identified as discriminant variables for good VCL and VAP included the ATP synthase subunit alpha, the aconitase hydratase mitochondrial, and the phosphoinositide phospholipase C, other proteins specifically enriched in stallions with higher VCL and VAP included ATP synthase subunit alpha, and ADT/ATP translocase 2-like protein. In relation with straight line velocity, the proteins differentially present in samples with differing VSL were related to the GO terms unfolded protein binding and sperm capacitation. Proteins that were discriminant for poor VSL were identified and these were the heat shock protein HSP-90 alpha, and chaperonin containing TCP1 subunit 6A, and seminal plasma protein 1. As expected, and a according the type of genetic selection used in stallions [1], individual differences were much bigger that differences among breeds; the ones used in our study were very close genetically, Iberian horses (Andalusian, Lusitano, and Spanish Sport Horse), Arabian, and crosses between these groups, and reflect the more demanded breeds in the area of influence of our stallion station. We also validated specific proteins of interest using western blotting; we validated the ATP synthase, linked to better sperm quality, and three proteins linked to poor values HSP90, HSP70 and Cytochrome C. Although nowadays the approach of using WB to validate quantitative proteomics data is being questioned [68], and with more accurate MS technologies the focus is the control of the FDR [69], the proteins shown in the WB underpin the participation of mitochondria in sperm energetics [57], and the participation of apoptotic phenomena in sperm malfunction [66].

Overall, our findings provide further support of the theory of the high dependence of the stallion spermatozoa on mitochondrial metabolism (oxidative phosphorylation and TCA cycle) for motility and velocity, and provide new insights regarding the utility and the molecular mechanisms behind of the different CASA derived parameters. Results may suggest lower values of VSL are linked to capacitation like changes; a protein playing a major role in sperm capacitation like the HSP90 [70] is more abundant in stallions showing lower VSL. Furthermore, the GO term sperm capacitation was enriched in these stallions.

In conclusion, higher percentages of total motility as well as higher VCL and VAP were linked to mitochondrial proteins involved in the TCA cycle and oxidative phosphorylation, underpinning the importance of mitochondrial metabolism in the stallion spermatozoa. The opposite was found for percentages of linearly motile spermatozoa and VSL, discriminant variables identified stallions with poor values. Interestingly low percentages of linear motile spermatozoa were linked to higher levels of cytochrome C, supporting the link between the mitochondrial pathway for apoptosis and sperm malfunction. In addition, poor VSL is linked to increased levels of proteins participating in sperm capacitation, also suggesting a relation with lower straight-line velocity in capacitated spermatozoa.

Declaration of competing interest

The authors declare that there are no conflicts of interest that could be perceived to prejudice the reported research.

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