The Seminal plasma proteins *Peptidyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase and KIAA0825* are linked to better motility post thaw in stallions

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ABSTRACT

Seminal plasma plays an important role in sperm physiology. Seminal plasma proteins vehiculated in microvesicles, carry RNAs and proteins with a potential role in early embryo development. Additionally, proteins present in seminal plasma participate in redox regulation and energy metabolism. In view of these facts, we hypothesized that differences in protein composition of the seminal plasma among stallions may help to explain differences in freeze-ability seen among them. Three independent ejaculates from 10 different stallions of varying breeds were frozen using standard protocols in our laboratory. Aliquots of the ejaculate were separated and stored at -80º C until further proteomic analysis. Semen analysis was performed using computer assisted sperm analysis and flow cytometry. Significant differences in proteome composition of seminal plasma were observed in the group of stallions showing better motility post thaw. 3116 proteins were identified, and of these, 34 were differentially expressed in stallions with better motility post thaw, 4 of them were also differentially expressed in stallions with different percentages of linearly motile sperm post thaw and 1 protein, Midasin, was expressed in stallions showing high circular velocity post thaw.
Seminal plasma proteins may play a major role in sperm functionality; being vehiculated through extracellular vesicles and participating in sperm physiology. Bioinformatic analysis identifies discriminant proteins able to predict the outcome of cryopreservation, identifying potential new biomarkers to assess ejaculate quality.

Key words: stallion, spermatozoa, flow cytometry, CASA, proteomics, cryopreservation, seminal plasma

1.- Introduction

Seminal plasma, is composed of secretions from the accessory sex glands and has immunosuppressive/immunoregulatory functions[1, 2]. This fluid harbors numerous microvesicles, mainly prostasomes[3], with different functions nourishing spermatozoa and probably carrying RNAs and proteins with a role in early embryo development[4]. Despite its importance, equine seminal plasma has received little attention in comparison to the spermatozoa. However, the composition of equine seminal plasma has been the subject of several studies, some of them in relation to the freezeability of the ejaculate[5-8]. A detailed description of the proteome of equine seminal plasma has recently been published [9]. In this study, reactome and KEGG pathway analysis revealed an important

67 role of seminal plasma in metabolism and vesicle mediated transport. Moreover, a
68 specific protein in the seminal plasma, Annexin A2 served as a discriminant variable for
69 stallions that needed removal of seminal plasma to maintain the quality of their ejaculates
70 when conserved by refrigeration. However, little information is available regarding the
71 potential relationship between the proteins present in seminal plasma and the freeze-
72 ability of the ejaculates. While cryopreservation of stallion spermatozoa is a reproductive
73 technology of which use is increasing year after year, unresolved questions remain. One
74 of the major drawbacks is the high stallion-to-stallion variability, that precludes many
75 valuable stallions entering the international market for horse semen[10]. Undoubtedly,
76 increasing knowledge around the factors that are involved in this variability will open
77 new opportunities enabling its reduction. While sperm factors have received attention
78 [11-14], the role of seminal plasma has not been so extensively investigated in relation
79 with freeze-ability [6, 15, 16]. Previous reports indicate that equine seminal plasma plays
80 important roles in hexose metabolism and is rich in antioxidants [6, 9, 17].
81 Cryopreservation causes osmotic induced necrosis in a high proportion of spermatozoa,
82 while the surviving population experience a compromise in their energetic metabolism
83 and redox regulation [18, 19]. Since seminal plasma has roles in redox regulation and
84 energetic metabolism through prostasome and other micro-vesicles that vehiculate, we
85 hypothesized that differences in protein composition of the seminal plasma between
86 stallions may help to explain differences in freeze-ability seen among them. The objective
87 of this study was to identify proteins in seminal plasma that could be potential markers
88 of freeze-ability using potent bioinformatic tools.
89
90 **2.- Material and methods**
91
92 *2.1.- Reagents and media*
93
94 All chemicals were purchased from Sigma-Aldrich
95 (https://www.sigmaaldrich.com/spain), unless otherwise stated. JC-1,
96 monochlorobimane (MCB), Annexin V 647 conjugated, CellEvent® Caspase 3/7 Green
97 Detection Reagent, Hoechst 33342 and Ethidium homodimer (Eth-1) were purchased
98 from Thermofisher (https://www.thermofisher.com/es/es/home.html) DRAQ7 was
99 purchased from Beckman Coulter (https://www.beckmancoulter.com/es).
100

101 *2.2.- Experimental design*

102

103 Three independent ejaculates from 10 different stallions (n=30) were frozen using
104 standard protocols in our laboratory [13, 19, 20]. Aliquots of the same ejaculate used for
105 freezing semen were separated, and the seminal plasma removed by serial centrifugation
106 (2 x 1500g 10') and stored at -80º C until proteomic analysis. The absence of spermatozoa
107 or other contaminant cells in the samples was assessed under phase contrast microscopy.
108 Stallions were classified according to the outcome of cryopreservation relative to total
109 motility of their ejaculates post thaw (good > 35%). This threshold was based on current
110 recommendations for minimum quality for commercial doses of equine semen
111 *http://www.wbfsh.org/GB/Other%20activities/Semen%20standards.aspx)*, linear
112 motility post thaw (good > 30%) circular velocity post thaw (good > 115µm/s), viability
113 (good > 40% live spermatozoa at thawing) and mitochondrial membrane potential (> 40%
114 of spermatozoa showing high mitochondrial membrane potential at thawing). Ejaculates
115 were classified according of the number of good scores in the 5 different categories (0, 1,
116 2, 3, and 4), however there were no ejaculates which achieved the highest score in all five
117 categories.

118

119 ***2.3.**-Semen collection and processing*

120

121 Semen was collected from stallions of different breeds maintained as indicated by specific
122 institutional and European regulations for animal care (Law 6/2913 June 11th and
123 European Directive 2010/63/EU). The ethical committee of the University approved this
124 study. Ejaculates were collected using a warmed, lubricated Missouri model artificial
125 vagina and the gel fraction of the ejaculate was removed with an inline filter. Upon arrival
126 at the laboratory, the semen was processed through colloidal centrifugation [21, 22] and
127 seminal plasma removed. The ejaculate was extended in freezing media and frozen using
128 standard procedures that have been previously described by our laboratory [19]. In brief
129 semen was diluted in the Cáceres freezing medium (University of Extremadura, Cáceres,
130 Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 x10^6
131 spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle,
132 France), the straws were ultrasonically sealed with an UltraSeal 21® (Minitube of
133 America MOFA, Verona, Wisconsin, USA) machine and immediately placed in an
134 IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The

135 following freezing curve was used. Straws were kept at 20ºC for 15 min, and they were
136 then slowly cooled from 20ºC to 5ºC at a cooling rate of 0.1 ºC/min. Thereafter the
137 freezing rate was increased to -40ºC/min from 5ºC to -140ºC. The straws were then
138 plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a
139 water bath at 37°C for at least 30 sec.
140
141
142 ***2.4***.- *Protein solubilization*
143
144 Seminal plasma aliquots were solubilized in lysis buffer as previously described[9] and
145 20 microliters of lysis buffer was added, the solution was vortexed and incubated under
146 constant rotation at 4ºC for 1 hour.
147
148 ***2.5.-*** *Protein quantification*
149
150 Protein quantification was performed using the 2-D Quant Kit [9]. All samples were
151 normalized to obtain a final concentration of 100 μg of protein per sample.
152
153 ***2.6.-*** *In-solution trypsin digestion*
154
155 200 μL of the seminal plasma solution obtained from the previous stage were mixed with
156 100 μl of 25 mM ammonium bicarbonate buffer, pH 8.5 and the proteins were reduced
157 and alkylated. Digestion was performed by adding 1 μL of Trypsin Proteomics Grade as
158 previously described [9].
159
160 ***2.7.-*** *UHPLC-MS/MS analysis**.***
161
162 Separation and analysis of the samples was performed following the protocol described
163 in a previous study [9] with a UHPLC/MS system consisting of an Agilent 1290 Infinity
164 II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an
165 automated multisampler module and a High Speed Binary Pump, coupled to an Agilent
166 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an
167 Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the HPLC and

168 Q-TOF was via MassHunter Workstation Data Acquisition software (Agilent
169 Technologies, Rev. B.06.01).
170
171 ***2.8.-*** *Data processing*
172 Data processing and analysis was performed using Spectrum Mill MS Proteomics
173 Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) following
174 previously described protocols[9].
175
176 ***2.9.-*** *Computer-assisted sperm analysis (CASA)*
177
178 Sperm motility and velocity were assessed using a computer-assisted sperm analysis
179 (CASA) system (ISAS Proiser, Valencia, Spain) in fresh and frozen and thawed
180 spermatozoa according to standard protocols used at our center [23]. Semen samples were
181 loaded into a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands)
182 and placed on a stage warmed at 37°C. Analysis was based on an evaluation of 60
183 consecutive digitized images obtained using a 10x negative phase-contrast objective
184 (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields.
185 Spermatozoa with VAP > 35 µm/s were considered motile. Spermatozoa deviating < 45%
186 from a straight line were classified as linearly motile.
187
188 ***2.10.-*** *Flow cytometry*
189
190 Flow cytometry (FC) analyses were conducted using a Cytoflex®Sflow cytometer
191 (Beckman Coulter) equipped with violet, blue, yellow and red lasers. The instrument was
192 calibrated daily using specific calibration beads provided by the manufacturer. A
193 compensation overlap was performed before each experiment. Files were exported as
194 FCS files and analyzed using FlowjoV 10.7 Software (Ashland, OR, USA). Unstained,
195 single-stained, and Fluorescence Minus One (FMO) controls were used to determine
196 compensations and positive and negative events, as well as to set regions of interest as
197 described in previous publications by our laboratory [24, 25].
198
199 ***2.10.1.-*** *Measurement of GSH, viability and mitochondrial membrane potential in stallion*
200 *spermatozoa*
201

202 Intracellular GSH was measured adapting previously published protocols optimized for
203 GSH detection using flow cytometry[26] tailored to equine spermatozoa in our laboratory
204 [27]. Mitochondrial membrane potential and sperm viability were also simultaneously
205 assessed. In brief, sperm aliquots (1-5 x 10^6 sperm/mL) were stained with JC-1 1μM, (30
206 minutes in the dark at r.t.), DRAQ7 3μM and monochlorobimane (MCB) 10 μM (10
207 minutes in the dark at r.t). After assessing flow quality, doublets and debris were gated
208 out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP,
209 JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates)
210 and DRAQ7, at a peak excitation of 640, and emission of 690 nm.

211

212 ***2.10.2***.- *Assessment of caspase 3 activity and phosphatidylserine (PS) translocation*

213

214 Annexin V 647 conjugated and CellEvent® Caspase 3/7 Green Detection Reagent were
215 combined in a multiparametric test and evaluated by FC [20]. Samples were loaded with
216 Hoechst 33342 (0.3 μM) and CellEvent (2 μM) and incubated at room temperature for 15
217 minutes. Following this the samples were washed by a short centrifugation spin for 12"
218 and suspended in 200 μl of Annexin binding-buffer (solution in 10 mM HEPES, 140 mM
219 NaCl, 2.5 mM $CaCl_2$, pH 7.4). Five μL of Annexin V was added to 200 μL of sample.
220 After 15 minutes of incubation in the dark at room temperature, 400 μL of 1 × Annexin
221 binding-buffer was added before analysis using the flow cytometer (Cytoflex® S flow
222 cytometer, Beckman Coulter). To gate dead spermatozoa, samples were stained with 0.3
223 μM of Eth-1 and incubated for 5 minutes before they were immediately evaluated in a
224 flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). CellEvent staining was
225 validated as previously described [28].

226

227 2.11.- *Bioinformatic Analysis*

228

229 *2.11.1 .- Variance filtering and PCA*

230

231 Data were normalized and log_2 transformed using Qlucore Omics Explorer
232 (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the
233 data set in a three-dimensional space, after filtering out variables with low overall
234 variance to reduce the impact of noise and centering and scaling the remaining variables

235 to zero mean and unit variance. The projection score [29] was used to determine the
236 optimal filtering threshold.
237
238 *2.11.2.- Identifying discriminating variables*
239
240 Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating
241 variables that are most highly significantly different between good and poor freezers. The
242 identification was performed by fitting a linear model for each variable with condition
243 proteins in seminal plasma as a predictor of the outcome of cryopreservation and
244 including the stallion nuisance covariate. P-values were adjusted for multiple testing
245 using the Benjamini-Hochberg method [30, 31] and variables with adjusted *P*-values (q
246 value) below 0.1 were considered significant.
247
248 *2.12 .- Statistical analysis*
249 End points measured were, the percentage of total motile spermatozoa and circular
250 velocity after thawing, and the percentages of live spermatozoa, caspase 3 postitive
251 spermatozoa and the percentage of spermatozoa showing high mitochondrial membrane
252 potential.
253 The normality of the motility, sperm velocity and flow cytometry data were assessed
254 using the Kolmogorov-Smirnoff test. Paired t-tests and one-way ANOVA followed by
255 Dunnett's multiple comparisons test were performed using GraphPad Prism version 7.00
256 for Mac, La Jolla California USA, (www.graphpad.com).
257
258 **3.- Results**
259
260 *3.1.- Sperm quality post thaw differed between stallions*
261
262 *3.1.1.- Motility and velocities*
263
264 Significant differences were observed in total motility post thaw between the two groups
265 of stallions, good and poor freezers, with good freezers showing a mean percentage of
266 total motile spermatozoa of 37.7 ± 1.3 % while the group of poor stallions showed a
267 percentage of total motility post thaw of 21 ± 0.71% (P<0.001) (Figure 1A). A similar

268 trend was observed for the percentage of linear motile spermatozoa (Figure 1B). Sperm
269 velocity (circular velocity) was also significantly different between both groups, with
270 velocities of 125.6 ± 2.5 μm/s in good stallions, while VCL post thaw in poor freezers
271 was 107.2 ± 2.5 μm/s (*P*<0.0001) (Fig 1C).
272
273 *3.1.2.- Viability and mitochondrial membrane potential*
274
275 The percentage of live spermatozoa after thawing in the group of good stallions was 51.1
276 ± 1.6 % while in the group of poor freezers it was 34.2 ± 1.6% (*P*<0.0001) (Fig 2A). Poor
277 freezers showed higher percentages of caspase 3 positive spermatozoa (13.6 ± 0.6 vs 8.1±
278 04% in good freezers *P* <0.0001 Fig 2C). Good freezers showed a higher percentage of
279 spermatozoa with high mitochondrial membrane potential (49.1 ± 1.5 % vs 34.5 ± 1.1 in
280 poor freezers, P<0.0001, Fig 2B).
281
282 *3.2.- Protein composition of seminal plasma differs in stallions with better motility post*
283 *thaw*
284
285 To determine possible differences in the composition of seminal plasma in stallions
286 showing better values for motility, viability and mitochondrial activity post thaw,
287 independent volcano plots were constructed in a first step comparing the proteome of the
288 stallions showing significantly higher values in motility, viability and mitochondrial
289 membrane potential with the proteome of the rest of the stallions (Fig 3 A-C). Volcano
290 plots showed a different predominance of specific seminal plasma proteins among
291 stallions showing better results after cryopreservation in the three categories considered
292 (Fig 3 A-C). However, significant differences in the composition of the seminal plasma
293 proteome were only observed between the group of stallions showing better motility post
294 thaw. We identified 3116 proteins, and of these 34, were differentially expressed in
295 stallions with better motility post thaw (Fig 3D), 4 of them were also differentially
296 expressed in stallions with different percentages of linearly motile sperm post thaw and
297 1 protein, Midasin, was expressed in stallions showing high circular velocity post thaw
298 (Fig 3 D-E). Next, bioinformatic analysis was conducted to identify discriminant
299 variables; proteins in seminal plasma with potential to identify stallions that show good
300 motility post thaw. Qlucore Omics Explorer (https://qlucore.com) was used to identify

301 seminal plasma proteins that are most significantly different based on spectral counts in
302 stallions with better motility post thaw. Proteins of which amounts differed in seminal
303 plasma of stallions showing better motility after thawing were identified (Fig 4 C) with a
304 fold change > 2, P=0.009 and q=0.098. A further filter was applied to the analysis to
305 specifically identify proteins which were more abundant in stallions showing better
306 motility post thaw, and then a much stricter criteria was applied to select discriminant
307 proteins to obtain the most powerful discriminant variables. Proteins were filtered by a
308 fold change of at least 5 between both conditions, with a P=9.6e-04 and q=0.05. Six
309 proteins were identified as more abundant in the seminal plasma of stallions with better
310 motility post thaw (Fig 4D). These proteins were peptidyl arginine deiminase 2, rRNA
311 adenine N (6)-methyltransferase, KIAA0825, Rho guanine nucleotide exchange factor
312 28, endoplasmatic reticulum protein 44, and two uncharacterized proteins F6SCY and
313 A0A3Q2HPE3, one corresponding to the RAPGEF6 gene, with a guanyl nucleotide
314 exchange factor activity as molecular function, involved in small GTPase mediated signal
315 transduction. The second uncharacterized protein found corresponding to the
316 ARHGEF18 gene, has guanyl-nucleotide exchange factor activity and participates in
317 small GTPase mediated signal transduction processes.
318 In relation to the percentages of linear motile spermatozoa, significant differences were
319 observed in the proteome of stallions showing higher percentages of linear motile
320 spermatozoa post thaw (Fig 5). With a fold change >4.75, *P*= 8.7e-4 and q=0.071 four
321 proteins in the seminal plasma were identified as discriminant variables for stallions
322 showing different percentages of linearly motile spermatozoa post thaw (Fig 5 D). These
323 were *Peptitdyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase, KIAA0825*
324 *and an* Uncharacterized protein (A0A3Q2IAZ9), corresponding to the SCAF1 gene with
325 RNA polymerase II C-terminal domain binding molecular function.
326
327 *3.3.- Stallions showing an overall better outcome after cryopreservation show differences*
328 *in the composition of seminal plasma*
329
330 Bioinformatic analysis was performed to identify discriminant variables (proteins) in
331 seminal plasma potentially able to identify stallions with a better overall outcome after
332 cryopreservation and six proteins were identified as discriminant variables, being more
333 abundant in ejaculates scored in category 4 (p = 8.7e-4, q = 0.052 fold change > 4). These
334 proteins were the ATR serine/threonine kinase, peptidyldeiminase 2, rRNA adedine N(6)

335 methyltransferase, an uncharacterized protein (A0A3Q2IAZ9), KIAA0825 and the
336 Solute carrier family 25 member 37 (Fig 6).
337
338 **4.- Discussion**
339
340 In the present study the relationship between the proteome of seminal plasma in stallions
341 and sperm quality post thaw were investigated. Post thaw sperm quality was defined in
342 terms of the percentages of total and linear motility, circular velocity (VCL) in µm/s and
343 percentages of viable sperm and spermatozoa showing high mitochondrial membrane
344 potential. Three proteins were significantly enriched in the seminal plasma of stallions
345 showing better motility (total and linear) post thaw, *peptidyl arginine deiminase 2, rRNA*
346 *adenine N (6)-methyltransferase and KIAA0825.* Other proteins were observed in
347 stallions with better motility post thaw including th*e Rho guanine nucleotide exchange*
348 *factor 28, endoplasmatic reticulum protein 44,* and two uncharacterized proteins *F6SCY*
349 *and A0A3Q2HPE3, one corresponding to the RAPGEF6 gene,* with a guanyl nucleotide
350 exchange factor activity as molecular function, involved in small GTPase mediated signal
351 transduction. The second uncharacterized protein found corresponded to the *ARHGEF18*
352 gene, which has guanyl-nucleotide exchange factor activity and participates in small
353 GTPase mediated signal transduction processes. *Peptidyl arginine deiminase 2* catalyzes
354 the deamination of arginine residues of proteins leading to citrulline. Citrullination is a
355 poorly understood post translational modification that has been related to modulation of
356 epigenetic events, immunity, and transcriptional regulation[32-34]. This protein has
357 potential roles in fertility, since it has been reported that the female human knockout
358 phenotype for the gene coding for a similar protein (PADI6) is sterile due to a cleavage
359 failure of their fertilized eggs [35, 36]. The possibility that the spermatozoa vehiculates
360 this protein to the oocyte must be considered and warrants further investigation, taking
361 into account that a set of embryo proteins are exclusively of paternal origin [4]. The *Rho*
362 *guanine nucleotide exchange factor 28* belong to the family of guanine nucleotide
363 exchange factors, this family of proteins have key regulatory roles in embryo
364 development [37], through regulation of differentiation, proliferation and morphogenesis.
365 *KIAA0825* (A0A3Q2HFS8) was also more abundant in the seminal plasma of stallions
366 showing better total and linear motility post thaw. This is a protein of unknown function,
367 however the murine ortholog is known to be expressed during limb development. It has
368 also been reported that variants of this gene are linked to post axial polydactyly in

369 humans[38]. Other proteins enriched in the seminal plasma of stallions showing better
370 motility post thaw were *rRNA adenine N(6)-methyltransferase*, this protein is involved in
371 rRNA methylation as codified by the transcription factor B1 mitochondrial (TFB1M)
372 gene. The knockouts for this gene show altered mitochondrial function, reduced ATP
373 production and increased levels of reactive oxygen species (ROS) in response to cellular
374 stress [39], providing a potential link between the presence of higher levels of this protein
375 and resistance to cryopreservation. The protein A0A3Q2IAZ9 was also more abundant
376 in the seminal plasma of stallions showing higher percentages of linearly motile sperm
377 post thaw, this protein corresponds to the SCAF1 gene. This gene codifies for a protein
378 with RNA polymerase II C-terminal domain binding. Potential functions in early embryo
379 development could be attributed to this protein, considering recent developments
380 indicating the importance of paternal proteins vehiculated in semen and seminal plasma
381 for early embryo development [4]. We also searched for variables able to discriminate
382 between stallions showing better overall performance post thaw. Four proteins were also
383 discriminant for good motility post thaw, peptidyl arginine deiminase 2, rRNA, adenine
384 N(6) methyltransferase, KIAAA0825 and an uncharacterized protein corresponding to
385 the SCAR-1 gene, as well as two specific proteins from this group consisting of an ATR
386 serine/threonine kinase and the solute carrier family 25 member 37 (SLC25A37). The
387 ATR serine/threonine kinase is activated in the presence of single stranded DNA[40],
388 participating in DNA repair and playing an important role in meiosis in the male germinal
389 epithelium [41], probably linking the higher presence of this protein with better sperm
390 quality, and thus more resistance to the stresses of cryopreservation. The mitochondrial
391 metal transporter mitoferrin1 (SLC25A37), plays a major role in mitochondrial iron
392 homeostasis, as well as in the functionality of oxidative phosphorylation proteins[42],
393 this protein is probably related with improved mitochondrial functionality and thus cryo-
394 resistance.

395 In conclusion, seminal plasma proteins may play a major role in sperm functionality, in
396 spite of limited time of contact between seminal plasma proteins and spermatozoa during
397 semen processing, these proteins may be vehiculated through extracellular vesicles that
398 rapidly attach to sperm membranes [43, 44], participating in sperm physiology.
399 Bioinformatic analysis identifies discriminant proteins with potential to predict the
400 outcome of cryopreservation, pointing out the potential for the development of new
401 biomarkers reflecting the quality of the ejaculates after further validation.
402

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411

412 **CONFLICT OF INTEREST**

413 The authors have no conflicts of interest to disclose

414

415 **AUTHOR`S CONTRIBUTIONS**

416 GG-P performed experiments and writing (native English speaker), FEM-C, JMO-R, AS-
417 R, ES-R performed experiments, CG and CO-F supervision and data analysis, FJP
418 conceived the study, data analysis and interpretation, writing and funding acquisition.

419

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421

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Figure legends

Figure 1.- Average values of motility (total and linear) and velocity (circular velocity) after freezing and thawing in ejaculates from 10 different stallions (3 replicates per

574 stallion n=30), classified as good (>35% total motility post thaw) or poor (<35% total
575 motility post thaw). Semen was collected and processed as indicated in material and
576 methods and the percentage of total motile spermatozoa (A), the percentage of linear
577 motile spermatozoa (B) and the circular velocity (VCL) µm/s (C) were measured using
578 computer assisted sperm analysis (CASA). Data are presented as means ± s.e.m. and
579 derived from 3 identical replicates from each of the stallions (n=30 ejaculates) **
580 $P<0.01$, ****$P<0.00001$.
581
582 Figure 2.- Viability and mitochondrial membrane potential after freezing and thawing in
583 ejaculates from 10 different stallions (3 replicates per stallion n=30), classified as good
584 (>40 viability) or poor (<40% viability post thaw). Semen was collected and processed
585 as indicated in material and methods and the percentage of live spermatozoa (A),
586 percentage of spermatozoa showing high mitochondrial membrane potential (B) and
587 the percentage of caspase 3 positive spermatozoa (C) were measured using flow
588 cytometry. D-F are representative cytograms of the assays. Data are presented as means
589 ± s.e.m. and derived from 3 identical replicates from each of the stallions (n=30
590 ejaculates) ***$P<0.001$, ****$P<0.00001$.
591
592 Figure 3.- Volcano plots showing seminal plasma proteins differentially expressed in
593 ejaculates with better quality post thaw in terms of motility (A), viability (B) and
594 mitochondrial membrane potential (C). Proteins which were more abundant are
595 presented on the right-hand side of the volcano plot, proteins less abundant are
596 presented on the left-hand side of the volcano plot. The difference in protein content
597 (Log_2 fold change) is plotted against the significance of the difference $-Log_{10}$ (P)
598 between the two conditions. D and E: Venn diagram showing different amounts of
599 proteins in ejaculates showing better motility, linear motility and velocity post thaw (3
600 independent ejaculates from 10 different stallions in addition to two technical replicates
601 (n = 60 samples were used to derive results from proteomic analysis).
602
603 .
604 Figure 4.- Bioinformatic analysis of the proteins in stallion seminal plasma (3
605 independent ejaculates from 10 different stallions in addition to two technical replicates
606 (n = 60 samples were used to derive results from proteomic analysis), showing different
607 amounts present in stallions with good and poor motility post thaw. A) Principal

608 component analysis of the samples (ejaculates) B) Principal component analysis
609 (variables) C) Heat map showing the different amounts of proteins in seminal plasma of
610 stallions with good and poor motility post thaw D) Seminal plasma proteins identified
611 as potential discriminant variables for stallions showing good (>35%) and poor motility
612 (<35%) post thaw. Proteins were filtered by a fold change of at least 5 between both
613 conditions, with a P=9.6e-04 and q=0.05.
614
615
616 Figure 5.- Bioinformatic analysis of the proteins in stallion seminal plasma (3
617 independent ejaculates from 10 different stallions in addition to two technical replicates
618 (n = 60 samples were used to derive results from proteomic analysis), showing different
619 amounts in stallions with good and poor linear motility post thaw. A) Principal
620 component analysis of the samples (ejaculates) B) Principal component analysis
621 (variables) C) Heat map showing the different amounts of proteins in seminal plasma of
622 stallions with good and poor linear motility post thaw D) Seminal plasma Proteins
623 identified as discriminant variables for stallions showing good and poor linear motility
624 post thaw. With a fold change >4.75, *P*= 8.7e-4 and q=0.071 four proteins in the
625 seminal plasma were identified as discriminant variables.
626
627 Figure 6.- Bioinformatic analysis of the proteins in stallion seminal plasma showing
628 different amounts in stallions classified in 5 categories according to post thaw sperm
629 quality, with 0 being the worst and 4 the best. Seminal plasma proteins identified as
630 discriminant variables for stallions classified in category number 4. Six proteins were
631 identified as discriminant variables, being more abundant in ejaculates scored in
632 category 4 (p = 8.7e-4, q = 0.052, fold change > 4).
633
634