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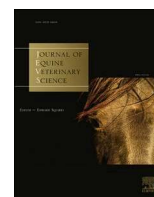


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Original Research



ProAKAP4 as a predictor of semen freezability in Asturcon ponies

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ABSTRACT

Background: Cryopreservation is a valuable tool in equine reproduction, although its success remains highly variable among stallions. The proAKAP4 protein has been proposed as a molecular marker of sperm quality and cryotolerance.

Aims/objectives: This study aimed to evaluate the relationship between proAKAP4 concentration and sperm cryotolerance in ejaculates from Asturcon ponies, and to assess whether proAKAP4 levels in fresh semen can predict post-thaw sperm quality parameters before and after cryopreservation.

Methods: Twenty-four ejaculates from eight different stallions were used (three ejaculates per animal). Fresh and thawed ejaculates, as well as those frozen with BotuCRIO and FAO extender, were classified into two groups based on proAKAP4 concentration (low or high) using complete linkage hierarchical cluster analysis. Sperm quality was assessed using computer-assisted sperm analysis and flow cytometry, including evaluations of motility, viability, morphology, acrosome integrity, and plasma membrane lipid disorder. ProAKAP4 concentration was measured using a commercial ELISA kit.

Results: Fresh and frozen ejaculates with high proAKAP4 concentrations exhibited higher total motility, progressive motility, and sperm membrane integrity ($P < 0.05$) than ejaculates with lower proAKAP4 concentrations. Ejaculates frozen with BotuCRIO and FAO extender and classified as having high proAKAP4 concentrations also displayed improved ($P < 0.05$) total and progressive motility. BotuCRIO samples with high proAKAP4 levels showed higher ($P < 0.05$) plasma and acrosomal membrane integrity, and lower ($P < 0.05$) membrane lipid disorder compared to those with lower proAKAP4 levels. A pre-freeze ProAKAP4 concentration threshold of 70.33 ng/10⁶ spermatozoa was identified as predictive of good freezability, and a post-thaw threshold of 40 ng/10⁶ spermatozoa was associated with acceptable quality.

Conclusion: These findings confirm that proAKAP4 is a promising biomarker for semen cryotolerance and support its integration into routine semen evaluation protocols for stallion selection.

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Table 1
Means ± SEM of total and progressive motility and proAKAP4 concentration in fresh and thawed ejaculates using two extenders (BotuCRIO and FAO).

	ProAKAP4 (ng/10M spz)	Total Motility (%)	Progressive Motility (%)
Fresh ejaculate	83.3 ± 11.8 ^b	39.7 ± 6.4 ^b	24.9 ± 4.7 ^b
BotuCRIO	22.9 ± 5.0 ^a	17.4 ± 3.8 ^a	5.9 ± 2.1 ^a
FAO	21.6 ± 4.6 ^a	14.7 ± 3.7 ^a	3.5 ± 1.8 ^a

Different letters (a, b) indicate significant differences ($P \leq 0.05$) between groups within each semen type. $n = 24$ ejaculates.

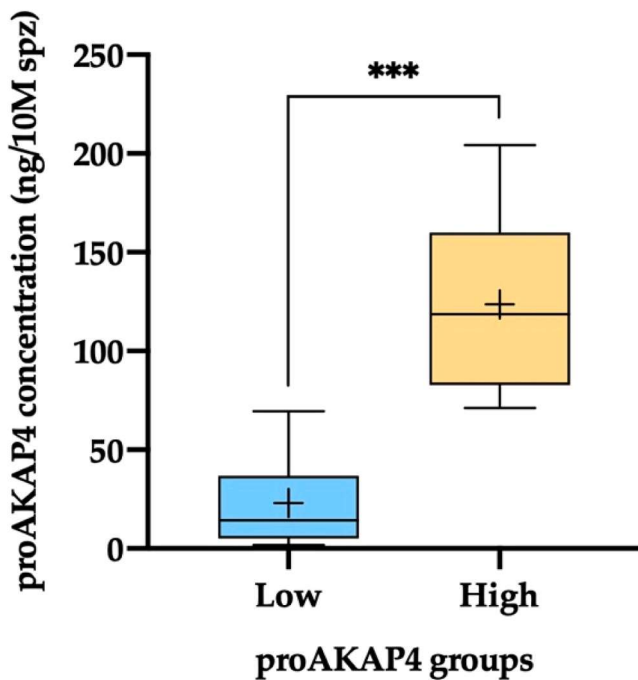


Fig. 1. Box-whisker plot showing the proAKAP4 concentration (ng/10M spz) of fresh and thawed Asturcon ponies' ejaculates as having low ($n = 58$; sky blue) or high ($n = 14$; orange) proAKAP4 concentrations. The cross indicates the mean, the line indicates the median, the boxes enclose the 25th and 75th percentiles, and the whiskers extend to the 5% and 95% percentiles (***) $P \leq 0.001$.

1. Introduction

The Asturcon, or Asturian pony, is a small, rustic horse breed from Asturias and Leon in northern Spain. Traditional breeds support

extensive farming due to their adaptability and robustness, thriving in low-input agricultural settings [1–3]. Losing such breeds threatens animal husbandry, agrobiodiversity, and overall biodiversity [4]. The genetic diversity of endangered breeds could provide solutions to climate challenges like extreme weather conditions, escalating water scarcity, and changes in feed availability [5]. In this context, germplasm and frozen semen banks are essential for preserving the genetic material of breeds and species at risk of extinction [6].

Over the years, a consistent challenge in stallion semen cryopreservation has been that some stallions are classified as poor freezers. Their ejaculates are considered unsuitable for insemination if progressive motility is below 35% [7–9]. The literature reports that about 20-40% of stallions fall into this "poor freezers" group [10]. There are currently no reliable tools to predict whether an ejaculate will freeze adequately. Sperm quality and fertility can be assessed using several parameters, including motility, membrane integrity, acrosome integrity, chromatin integrity, and mitochondrial function. These parameters are widely recognized as predictors of fertilization potential [11]. Advanced approaches, such as omics technologies and molecular assays, require specialised equipment and technical expertise. However, in the field, stallion semen cryopreservation is often performed without comprehensive sperm quality assessments [12].

There is growing interest in finding biomarkers that predict sperm quality, freezability, and/or fertility. These could help selecting ejaculates suitable for cryopreservation [8]. Fertility is positively linked to metabolism-related proteins and cysteine-rich secretory protein 3 (CRISP3), whereas negatively linked to kallikrein-1E2 (KLK2), clusterin, and seminal plasma proteins 1 (SP1) and 2 (SP2) [13]. Some proteins, including secreted phosphoprotein 1, fructose-bisphosphate aldolase, and malate dehydrogenase 1, have been linked to reduced sperm motility and velocity [14]. By contrast, proteins like glutathione peroxidase and triosephosphate isomerase were enriched in samples with higher straight-line velocity [8]. Annexin A2 is linked to reduced semen tolerance to cooling. Transcobalamin-2 and ST3 beta-galactoside alpha-2,3-sialyltransferase 1 were more abundant in semen resistant to cooling. Still, their roles as cryosurvival biomarkers remains unclear [15]. Aquaporins have emerged as promising biomarkers because they transport water and cryoprotectants across cell membranes and may help protect sperm before freezing [16–18].

Another key semen quality biomarker is the anchoring protein AKAP4 [19]. As the main structural protein of the spermatozoa flagellum, it regulates sperm motility and the acrosome reaction. Its precursor form, proAKAP4, is essential for the signalling pathway that maintains motility and fertility [20]. Blommaert et al. [21] reported that in stallions, proAKAP4 concentration reflects post-thaw motility, whereas Carracedo et al. [20] suggested that it may serve as an indicator of potential motility reserves. This finding was later confirmed in stallions [22]. Several studies have shown that proAKAP4 concentration

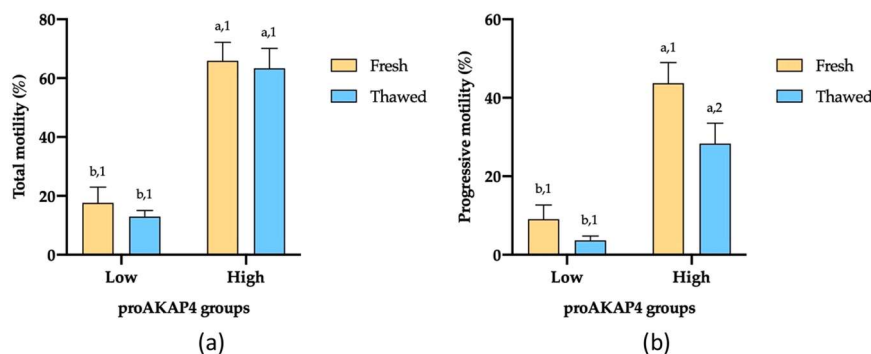


Fig. 2. Mean ± SEM of the percentages of total motile (a) And progressive (b) Sperm with low and high proAKAP4 concentration of fresh and thawed Asturcon ponies' ejaculates. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each semen type. (1,2) Different numbers indicate significant differences ($P \leq 0.05$) between semen types within each group.

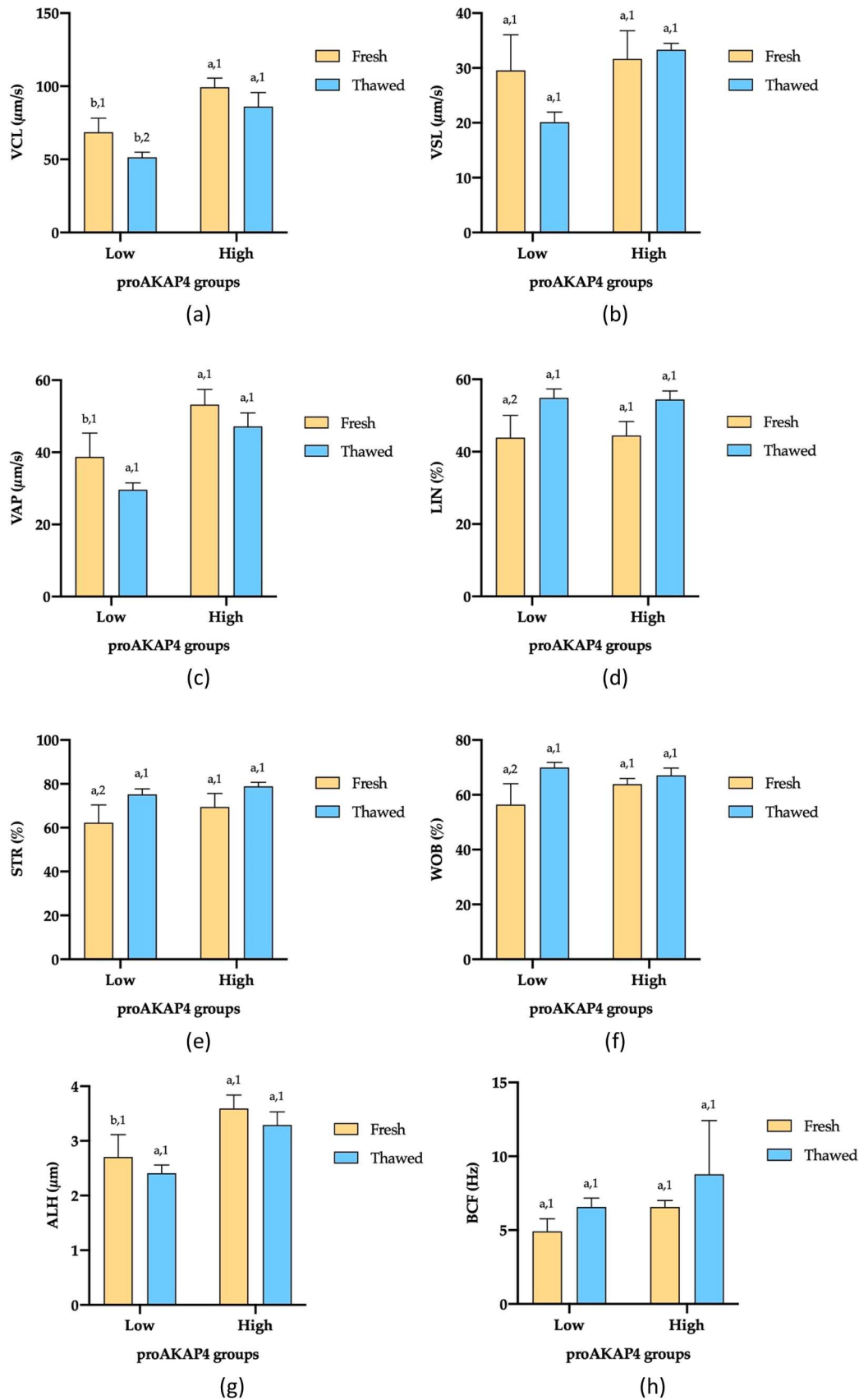


Fig. 3. Mean \pm SEM of the kinematic parameters (curvilinear velocity (VCL, a), straight line velocity (VSL, b), average path velocity (VAP, c), linearity coefficient (LIN, d), straightness coefficient (STR, e), wobble coefficient (WOB, f), amplitude of lateral head displacement (ALH, g), and beat-cross frequency (BCF, h)) with low and high proAKAP4 concentration of fresh and thawed Asturcon ponies ejaculates. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each semen type. (1,2) Different numbers indicate significant differences ($P \leq 0.05$) between semen types within each group.

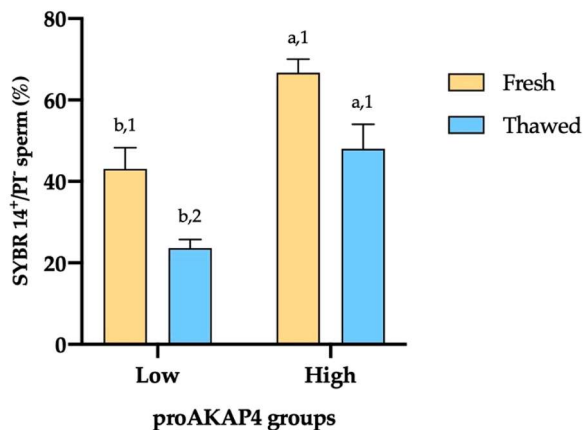


Fig. 4. Mean \pm SEM of the percentage of sperm with intact plasma membrane (SYBR14⁺/PI⁻) and with low and high proAKAP4 concentration of fresh and thawed Asturcon ponies' ejaculates. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each semen type. (1,2) Different numbers indicate significant differences ($P \leq 0.05$) between semen types within each group.

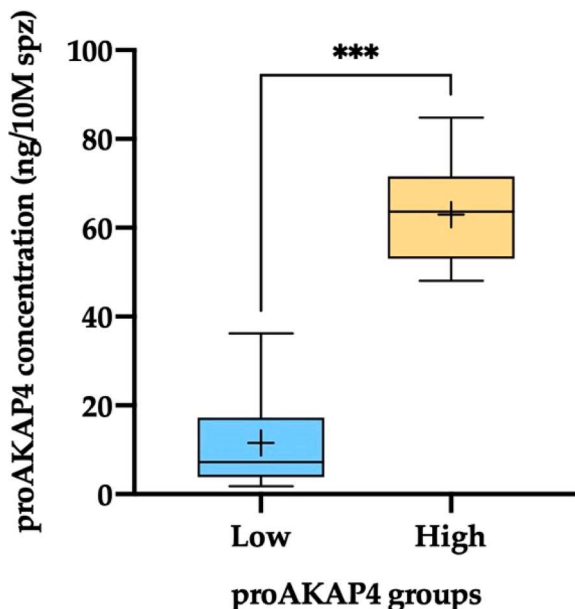


Fig. 5. Box-whisker plot showing the proAKAP4 concentration (ng/10M spz) of Asturcon ponies' ejaculates frozen with BotuCRIO and FAO extender as having low ($n = 38$; sky blue) or high ($n = 10$; orange) proAKAP4 concentrations. The cross indicates the mean, the line indicates the median, the boxes enclose the 25th and 75th percentiles, and the whiskers extend to the 5% and 95% percentiles (***) $P \leq 0.001$.

correlate with total and progressive post-thaw motility in stallion semen [21–24], including associations with sperm velocity parameters [22,24]. Moreover, the slowest spermatozoa subpopulation in stallions has been negatively correlated with proAKAP4 concentration. Ejaculates with a higher percentage of slow spermatozoa exhibited a lower proAKAP4 concentration [24]. Notably, proAKAP4 concentration varies depending on the extender used to freeze equine semen [23] but is unaffected by cryoprotectant during the first 3 hours after thawing [22,25].

The aim of this study was to evaluate the relationship between proAKAP4 concentration and equine sperm cryotolerance, and to determine whether proAKAP4 levels in fresh ejaculates can serve as a predictive biomarker of post-thaw sperm quality, including motility and membrane functionality parameters assessed by flow cytometry.

2. Materials and methods

2.1. Experimental group

Eight Asturcon ponies, owned by ACPRA (*Asociación de Criadores de Ponis de Raza Asturcón*) and with recognized offspring, were kept at Serida (www.serida.org) in Villaviciosa (Asturias), northern Spain. They were housed in individual boxes and fed with cereals and hay. During the semen collection, their weight ranged from 218 and 240 kg, and their age ranged from 8 to 24 years.

Each pony underwent three semen collections, following the same experimental protocol. All protocols complied with the ARRIVE guidelines and regulations (Spanish Royal Decree 53/2013, Directives 86/609/EEC, 91/628/EEC, 92/65/EEC, and 2010/63/EU) on the use of animals in research.

2.2. Semen collection, evaluation, and cryopreservation

All collections were performed using a dummy and a Hannover artificial vagina. Each ejaculate was filtered after collection. Once the semen was filtered, the concentration was determined using a spectrophotometer (Accucell®, IMV Technologies, Aigle, France). Afterward, motility descriptors were determined using the CASA system (section 2.3), and viability was evaluated by eosin-nigrosin staining [26]. Before starting the freezing protocol, 2 ml of raw semen were placed inside an Eppendorf® tube and directly plunged into the liquid nitrogen for further proAKAP4 analysis.

Cryoconservation of each ejaculate began with the dilution of the whole ejaculate at 1:2 with INRA96® prewarmed at 37°C and placed in 50ml tubes. Two tubes of each ejaculate were centrifuged for 10 minutes at 1700 rpm at 20°C. Once the supernatant was discarded, the pellet was resuspended in freezing media at a concentration of 100×10^6 spermatozoa/mL; one tube with BotuCRIO and the other with FAO. Every batch was packed with 0.5 ml straws previously marked and placed inside the freezer, DigitCool IMV (IMV technologies, l'Aigle, France). Semen was cryopreserved according to the manufacturer's instructions for equine semen freezing.

2.3. Semen motility analysis

Sperm motility in fresh ejaculate and in post-thawed conditions was evaluated using a computer-assisted sperm-analysis (CASA) system (ISAS; Proiser; Barcelona; Spain). After collection, 2 μ L of fresh semen were placed directly into a Makler chamber prewarmed at 37°C to perform the analysis. Frozen samples were thawed at 37°C in a water bath for 1 min; 20 μ L of sperm sample were placed into a Makler® chamber prewarmed at 37°C. For each sample, different fields were analysed, taking at least 1000 spermatozoa/sample. The following motility descriptors were evaluated: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μ m/s), straight-line velocity (VSL, μ m/s), average path velocity (VAP, μ m/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μ m), and frequency of head displacement (BCF, Hz).

CASA settings were: magnitude of x200 with 10 randomly fields; 60 images captured per second; 30 sequences taken in each analyse; particle area 3 pixels; connectivity: 6; minimum number of images to calculate the ALH: 10. Cut-off value for motile spermatozoa was VAP ≥ 20 μ m/s, and for progressive motile spermatozoa was STR $\geq 80\%$ and VAP ≥ 30 μ m/s.

2.4. Flow cytometry analysis in post-thawed conditions

The CytoFLEX® flow cytometer from Beckman Coulter was used for this analysis. The side- and forward-scatter channels were calibrated with 10- μ m diameter flow-control fluorospheres (Beckman Coulter). Parameters studied included sperm membrane integrity (SYBR-14/

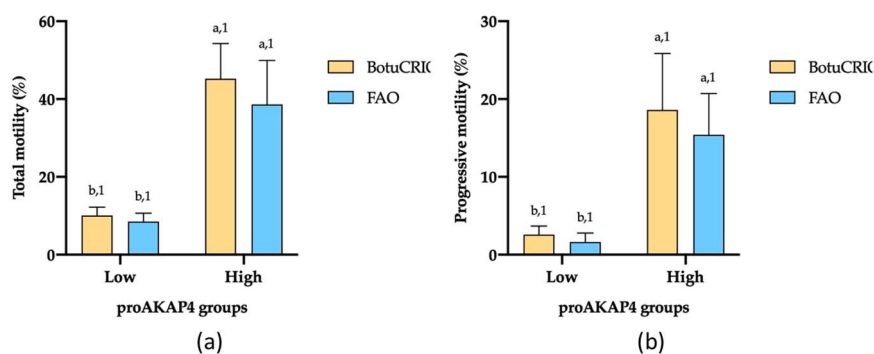


Fig. 6. Mean \pm SEM of the percentages of total motile (a) And progressive (b) Sperm with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCRIO and FAO extenders. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each extender type. No significant differences ($P > 0.05$) between extender types within each group.

propidium iodide [PI]), acrosome integrity (PNA-FITC)/PI, plasma membrane lipid disorder (M540)/YO-PRO-1, mitochondrial membrane potential (JC-1/ [LIVE/DEAD far red]), intracellular levels of total ROS (dichlorodihydrofluorescein diacetate [H_2DCFDA]/PI), and superoxides (dihydroethidium [HE]/Yo-Pro-1). Information on flow cytometry analyses is provided in accordance with the MIFlowCyt guidelines [27].

Sperm were specifically selected from other particles/cells by using the Forward Scatter Detector (FSD), which analyses the size of particles; and the Side Scatter Detector (SSD), which detects their roughness. The combination of both detectors enabled discrimination between sperm from other particles, such as debris and cell aggregates. All samples were excited with the blue laser (488 nm), except the ones labelled with the LIVE/DEAD fixable far red dead cell stain, which were excited with the red laser (638 nm). The fluorescence emitted by SYBR-14, Yo-Pro-1, PNA-FITC, DCF, and JC-1 monomers was collected through the FITC channel (bandpass 524/40 nm); the fluorescence emitted by JC-1 aggregates was detected via PE channel (bandpass 585/42 nm); the fluorescence emitted by the LIVE/DEAD fixable far red fluorochrome was detected through the APC channel (bandpass 660/20 nm); and the fluorescence of PI and E was measured with the PC5.5 channel (bandpass 690/50 nm). For each sample, two technical replicates of at least 5000 sperm per replicate were evaluated.

2.4.1. Assessment of plasma membrane integrity

Sperm plasma membrane integrity (viability) was determined using the LIVE/DEAD viability kit (Molecular Probes; Eugene, OR, United States), according to the protocol of Garner and Johnson [28], with adaptations for horse sperm. In summary, semen samples were incubated with SYBR14 (final concentration: 31.5 nM) and PI (final concentration: 7.6 μ M) at 38°C in the dark for 10 min. Staining with both fluorochromes resulted in four different populations: (i) viable sperm (SYBR-14⁺/PI⁻); (ii) moribund sperm (SYBR-14⁺/PI⁺); (iii) non-viable sperm (SYBR-14⁻/PI⁺); and (iv) debris particles (SYBR-14⁻/PI⁻). The percentage of debris particles (SYBR14⁻/PI⁻) was used to correct the viable sperm percentage.

2.4.2. Assessment of acrosome integrity

The integrity of sperm acrosomes was evaluated using a combination of PNA-FITC and PI, following the procedure outlined by Rathi et al. [29] with minor modifications. Semen samples were incubated in the dark at 38°C for 10 min, with PNA conjugated to FITC (final concentration: 1.17 μ g/mL) and PI (final concentration: 5.6 μ M). Four different sperm populations were characterized: (1) viable membrane-intact sperm (PNA-FITC⁻/PI⁻), (2) viable sperm with a damaged plasma membrane (PNA-FITC⁺/PI⁻), (3) non-viable sperm with a damaged plasma membrane and fully lost outer acrosome membrane (PNA-FITC⁻/PI⁺), and (4) non-viable sperm with a damaged plasma membrane that presented an outer acrosome membrane that could not

be fully intact (PNA-FITC⁺/PI⁺). Viable sperm with an intact acrosomal membrane (PNA-FITC⁻/PI⁻) were used to evaluate acrosome integrity.

2.4.3. Assessment of plasma membrane lipid disorder

The analysis of plasma membrane lipid disorder in sperm was conducted using the combination of the fluorochromes M540 and YO-PRO-1, following the protocol outlined by Rathi et al. [29], with minor modifications. In brief, semen samples were subjected to incubation in the absence of light at 38°C for 10 min with M540 (final concentration: 2.5 μ M) and YO-PRO-1 (final concentration: 25 nM). Four different sperm populations were characterized: (1) non-viable sperm with high plasma membrane lipid disorder (M540⁺/YO-PRO-1⁺), (2) viable sperm with high plasma membrane lipid disorder (M540⁺/YO-PRO-1⁻), (3) non-viable sperm with low plasma membrane lipid disorder (M540⁻/YO-PRO-1⁺), and (4) viable sperm with low plasma membrane lipid disorder (M540⁻/YO-PRO-1⁻).

2.4.4. Assessment of intracellular total ROS levels

To assess intracellular total ROS levels, a combination of the fluorochromes H_2DCFDA and PI was used [30]. The samples were incubated for 20 minutes at 38°C with H_2DCFDA (final concentration: 0.35 μ M). The oxidation of H_2DCFDA (which is not fluorescent) to DCF (a highly fluorescent molecule) that emits green fluorescence indicates the presence of highly oxidative molecules. After the incubation period, the fluorochrome PI (final concentration: 6 μ M) was added to differentiate sperm viability, and the samples were incubated for an additional 5 minutes. For each sample, total ROS levels were expressed as the intensity of DCF in live sperm (PI⁻).

2.4.5. Assessment of intracellular superoxide levels

For the analysis of intracellular superoxide (O_2^-) levels, the samples were exposed to the fluorochromes HE (final concentration: 5 μ M) and YO-PRO-1 (final concentration: 31.25 nM) for 30 minutes at 38°C in the absence of light as described by Guthrie and Welch [30]. The oxidation of HE to ethidium (E), which emits red fluorescence, indicates the presence of O_2^- . Four different sperm populations were distinguished: (1) non-viable sperm with low O_2^- levels (E⁻/Yo-Pro-1⁺), (2) viable sperm with low O_2^- levels (E⁻/Yo-Pro-1⁻), (3) non-viable sperm with high O_2^- levels (E⁺/Yo-Pro-1⁺), and (4) viable sperm with high O_2^- levels (E⁺/Yo-Pro-1⁻).

2.4.6. Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated after staining with JC-1 (final concentration: 750 nM) and the LIVE/DEAD fixable far red dead cell fluorochrome (Molecular Probes; Eugene, OR, United States), diluted at 1:8000 (v:v) in PBS, following the protocol of Llawanera et al. [31] with minor modifications. Samples were stained at 38°C in the dark for 30 min. When sperm have a high mitochondrial membrane potential,

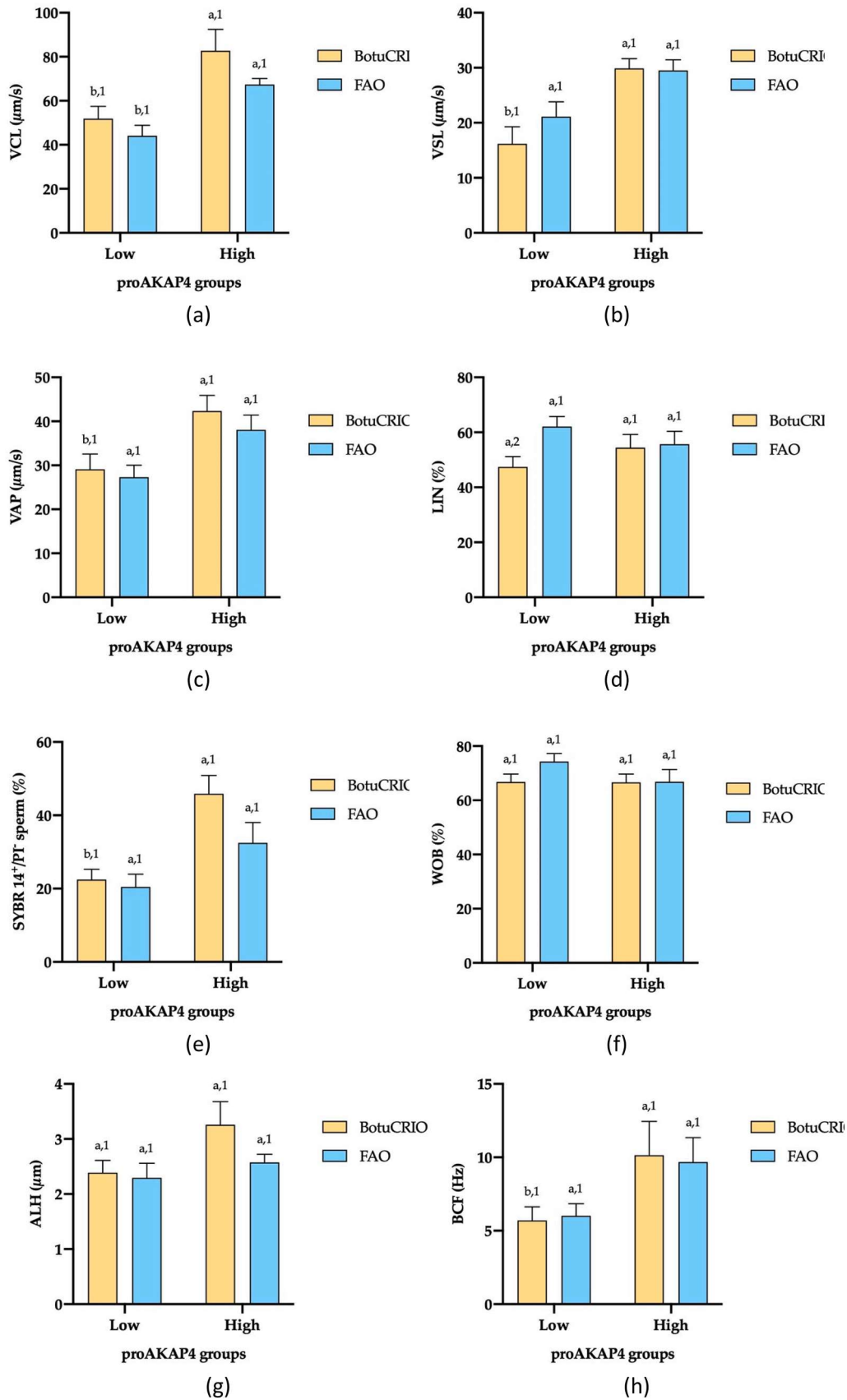


Fig. 7. Mean ± SEM of the kinematic parameters (curvilinear velocity (VCL, a), straight line velocity (VSL, b), average path velocity (VAP, c), linearity coefficient (LIN, d), straightness coefficient (STR, e), wobble coefficient (WOB, f), amplitude of lateral head displacement (ALH, g), and beat-cross frequency (BCF, h)) with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCRIO and FAO extenders. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each extender type. No significant differences ($P > 0.05$) between extender types within each group.

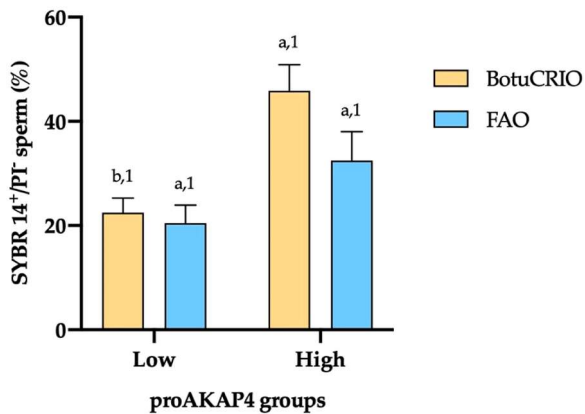


Fig. 8. Mean ± SEM of the percentage of sperm with intact plasma membrane (SYBR14⁺/PI⁻) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each extender type. No significant differences ($P > 0.05$) between extender types within each group.

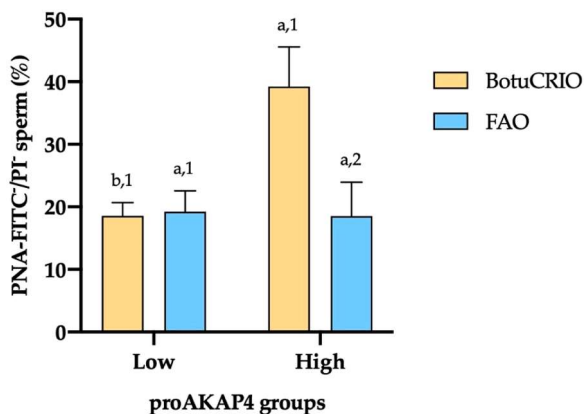


Fig. 9. Mean ± SEM of the percentage of sperm with an intact acrosome membrane (PNA-FITC⁻/PI⁻) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each extender type. (1,2) Different numbers indicate significant differences ($P \leq 0.05$) between extender types within each group.

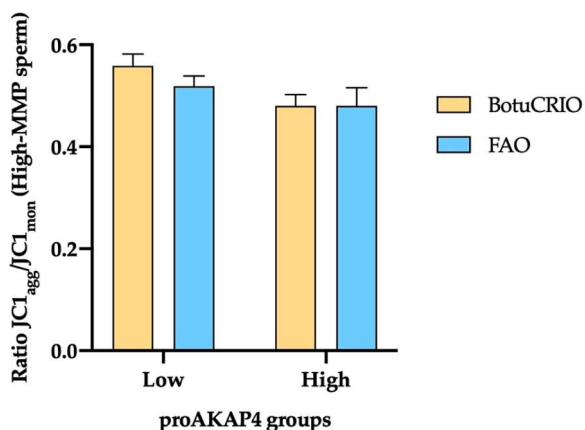


Fig. 10. Mean ± SEM of the percentage of sperm with high-MMP (Ratio JC1_{agg}/JC1_{mon}) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders.

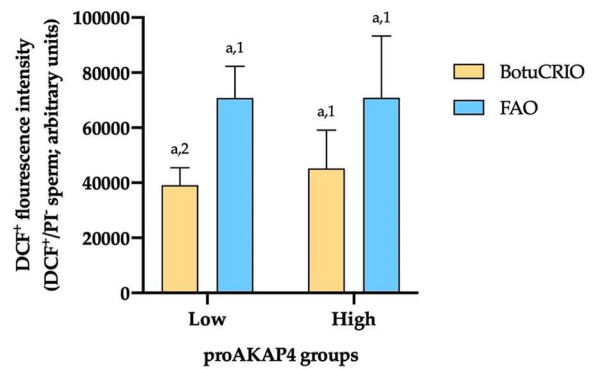


Fig. 11. Mean ± SEM of the percentage of sperm with high intracellular total ROS levels (DCF⁺/PI⁻) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders.

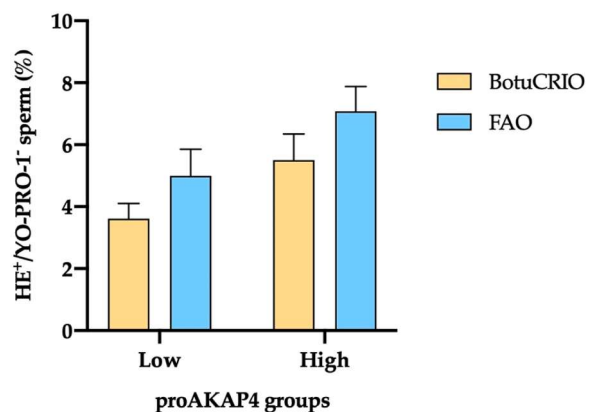


Fig. 12. Mean ± SEM of the percentage of sperm with high O₂ levels (HE⁺/YO-PRO-1⁻) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders.

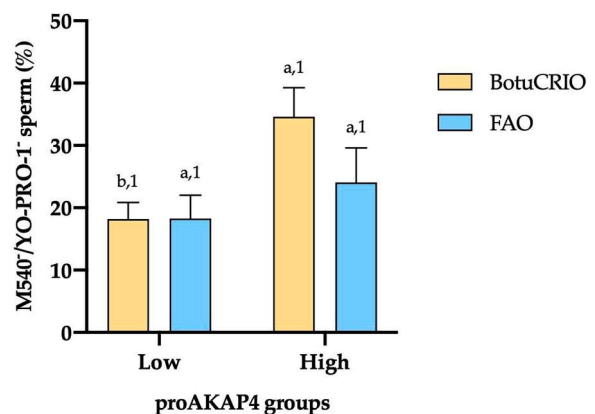


Fig. 13. Mean ± SEM of the percentage of sperm with low plasma membrane lipid disorder (M540⁻/YO-PRO-1⁻) and with low and high proAKAP4 concentrations of Asturcon ponies' ejaculates frozen with BotuCrio and FAO extenders. (a,b) Different letters indicate significant differences ($P \leq 0.05$) between groups within each extender type. No significant differences ($P > 0.05$) between extender types within each group.

JC-1 forms aggregates that emit orange fluorescence. In contrast, low mitochondrial membrane potential maintains JC-1 molecules in the monomeric form, emitting green fluorescence. Four populations were determined: (i) viable sperm with high MMP; (ii) viable sperm with low MMP; (iii) non-viable sperm with high MMP; and (iv) non-viable sperm

with low MMP. Mitochondrial membrane potential was expressed as the ratio between the intensity of fluorescence of JC-1_{agg} and JC-1_{mon} in viable sperm.

2.5. ProAKAP4 ELISA assays

The ProAKAP4 assay was performed by quantitative ELISA. This analysis was performed at ONIRIS Laboratory in Nantes (France) using the Horse 4MID kit (SPQI, Lille, France). This kit is a quantitative ELISA sandwich assay for specifically detecting and quantifying the proAKAP4 in equine semen samples. The reference curve is prepared from a provided seven-point standard solution and determines the concentration of proAKAP4 present in each stallion after thawing the semen sample. Every sample was prepared following the manufacturer's instructions for cryopreserved stallion semen.

The samples used for the analysis were taken from the different experimental groups described in Section 2.1. A 40 µL aliquot of diluted semen from each group was diluted with 160 µL of the specific lysis buffer provided in the kit. Once the dilution was complete, a vigorous vortex for 1 minute was performed, then 200 µL of dilution buffer was added and rapidly mixed into the 96-well coated plate. The next step was incubation for 2 hours under gentle agitation on an ELISA plate horizontal shaker. After three successive washing steps, the horseradish-conjugated detection antibody was added to each well and incubated for 1 hour. After adding the TMB substrate, the reaction was allowed to proceed for 10 min, during which the colour developed. The intensity of the colour reaction was quantitatively proportional to the amount of proAKAP4 present in each sample. The colour reaction was stopped by a stop solution added to each well, and colour intensities were acquired using a spectrophotometer equipped with a 450 nm filter. Optical densities were used to determine proAKAP4 concentrations using the manufacturer's calculation sheet (4BioDx, Lille, France). ProAKAP4 concentrations were expressed in ng per 10 million spermatozoa. Concentrations were calculated using the Horse 4MID kit calculation sheet according to the following formula: $\text{proAKAP4 (ng/10 million spermatozoa)} = (\text{proAKAP4 in ng/mL} / \text{spermatozoa concentration in M/mL}) \times 10 \times 16 \times (2/3)$. The factor 10 accounted for the post-thaw dilution applied during sample preparation, while the 2/3 factor corresponded to the dilution volume loaded into each well of the 96-well plate used for the Horse 4MID assay.

2.6. Motility and proAKAP4 consumption by freezing-thawing process

ProAKAP4 consumption during freezing was calculated as the percentage difference between the mean proAKAP4 concentration in thawed straws prepared with the two extenders and the proAKAP4 concentration in fresh ejaculates. The same calculation was applied to total and progressive sperm motility.

2.7. Statistical analysis

The statistical package R (V 4.0.3, R Core Team; Vienna, Austria) and GraphPad Prism software (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA) were used for data analysis and graphing, respectively. The Shapiro-Wilk and Levene tests were used to check for data normality and homoscedasticity of variances, respectively. The arcsin \sqrt{x} function was applied to transform the data to obtain a normal distribution only when necessary. Fresh and thawed ejaculates from Asturcon ponies, as well as ejaculates frozen with BotuCRIO and FAO extender, were classified into two groups based on proAKAP4 concentration (low or high) using complete linkage hierarchical clustering based on Euclidean distances. The effect of freezing (fresh vs. thawed) and the effect of the diluent used in the freezing process (BotuCRIO vs. FAO) in each group (low or high proAKAP4 concentration) on proAKAP4 concentration, motility parameters, and sperm quality parameters were compared using a two-way ANOVA. The Bonferroni post-hoc test was used in all analyses to perform

pairwise comparisons. Correlation coefficients (r) between ProAKAP4 concentration and sperm quality parameters of frozen Asturcon ponies' sperm were obtained using Pearson's correlation. A hierarchical cluster analysis was performed to classify frozen-thawed horse ejaculates into two groups based on proAKAP4 concentration (low or high) to establish a threshold (cutoff limit) for achieving good freezing results and a quality threshold once the straws were thawed [24]. Differences were considered statistically significant when $P \leq 0.05$. Results are expressed as means \pm standard error of the mean (SEM).

3. Results

3.1. Motility and proAKAP4 consumption by freezing-thawing process

As shown in Table 1, ProAKAP4 concentration was lower ($P < 0.05$) in post-thaw semen in both extenders (FAO and BotuCRIO) compared with fresh ejaculates. Total and progressive motility were also reduced ($P < 0.05$; Table 1) from fresh to thawed semen for both extenders. No significant differences were observed between the two extenders for proAKAP4 concentration or for total and progressive motility (Table 1).

3.2. Classification of fresh and thawed Asturcon ponies' ejaculates according to proAKAP4 concentration

Hierarchical cluster analysis ($P < 0.001$) based on proAKAP4 concentration grouped fresh and thawed Asturcon ponies' ejaculates into two categories: low (23.04 ± 2.73 ng/10M spz) and high (123.647 ± 11.40 ng/10M spz) proAKAP4 concentration (Fig. 1).

3.3. Relationship between proAKAP4 groups and the effect of freezing Asturcon ponies' ejaculates on sperm motility and kinematic parameters

In the low proAKAP4 group, total (Fig. 2a) and progressive motility (Fig. 2b) were lower ($P < 0.05$) in fresh and thawed ejaculates compared with the high proAKAP4 group. While total motility was similar ($P > 0.05$) in fresh and thawed semen within each proAKAP4 group, progressive motility was higher ($P < 0.05$) in fresh ejaculates than in thawed ejaculates within the high proAKAP4 group.

Curvilinear velocity (VCL, Fig. 3a) was significantly lower ($P < 0.05$) in fresh and thawed ejaculates with low proAKAP4 concentrations compared to those with high concentrations. On the other hand, VCL was higher ($P < 0.05$) in fresh ejaculates than in thawed ejaculates within the low proAKAP4 group.

No significant differences were observed among the groups in straight-line velocity (VSL, Fig. 3b) or beat-cross frequency (BCF, Fig. 3h).

Average path velocity (VAP, Fig. 3c) and amplitude of lateral head displacement (ALH, Fig. 3g) were higher ($P < 0.05$) in fresh ejaculates with high proAKAP4 concentrations than in those with low concentrations. No significant differences in VAP or ALH were observed when comparing fresh and thawed ejaculates within each group.

The linearity coefficient (LIN, Fig. 3d), straightness coefficient (STR, Fig. 3e), and wobble coefficient (WOB, Fig. 3f) were significantly higher ($P < 0.05$) in thawed ejaculates with low proAKAP4 concentration compared to fresh ejaculates. No significant differences were observed between the high- and low- proAKAP4 concentration groups in either fresh or thawed ejaculates.

3.4. Relationship between proAKAP4 groups and the effect of freezing Asturcon ponies' ejaculates on plasma membrane integrity

Sperm with intact plasma membrane (SYBR14⁺/PI⁻; Fig. 4) were significantly lower ($P < 0.05$) in both fresh and thawed ejaculates with low proAKAP4. Within the low proAKAP4 group, the percentage of sperm with intact plasma membrane was higher ($P < 0.05$) in fresh than in frozen samples.

3.5. Classification of Asturcon ponies' ejaculates frozen with BotuCRIO and FAO extender according to proAKAP4 concentration

Hierarchical cluster analysis ($P < 0.001$) based on the proAKAP4 concentration of ejaculates frozen with BotuCRIO and FAO revealed two groups: low (11.56 ± 1.60 ng/10M spz) and high (62.95 ± 3.62 ng/10M spz) proAKAP4 concentration (Fig. 5).

3.6. Relationship between proAKAP4 groups and the effect of BotuCRIO and FAO extenders for freezing of Asturcon pony's ejaculates on sperm motility and kinematic parameters

Total (Fig. 6a) and progressive (Fig. 6b) sperm motility were significantly lower in ejaculates with low proAKAP4 concentrations frozen with BotuCRIO and FAO extenders compared to those from the high proAKAP4 group. In contrast, there was no significant difference in the percentage of total and progressive sperm motility within the proAKAP4 group, regardless of the freezing extender.

Curvilinear velocity (VCL, Fig. 7a) was significantly lower ($P < 0.05$) in ejaculates with low proAKAP4 concentrations frozen with BotuCRIO and FAO extenders compared to those with high proAKAP4 concentrations. No significant differences attributable to the extenders (BotuCRIO and FAO) used for freezing were observed within each group.

Straight line velocity (VSL, Fig. 7b), average path velocity (VAP, Fig. 7c), and beat-cross frequency (BCF, Fig. 7h) were higher in ejaculates frozen with BotuCRIO extender from the high proAKAP4 group than those from the low proAKAP4 group. No significant differences in VSL, VAP, or BCF were observed across extender types (BotuCRIO or FAO) within each proAKAP4 group.

The linearity coefficient (LIN, Fig. 7d) and straightness coefficient (STR, Fig. 7e) were higher ($P < 0.05$) in ejaculates with low proAKAP4 concentration frozen with FAO extender than in those frozen with BotuCRIO extender. No significant differences in LIN or STR were observed between the high and low proAKAP4 groups frozen with the BotuCRIO and FAO extenders.

Finally, no significant differences were observed in wobble coefficient (WOB, Fig. 7f) and amplitude of lateral head displacement (ALH, Fig. 7g).

3.7. Relationship between proAKAP4 groups and the effect of BotuCRIO and FAO extenders for freezing of Asturcon ponies' ejaculates on sperm quality

3.7.1. Plasma membrane integrity

The percentage of sperm with intact plasma membrane (SYBR14⁺/PI⁻; Fig. 8) was significantly lower ($P < 0.05$) in ejaculates frozen with BotuCRIO extender in the low proAKAP4 group than in the high proAKAP4 group. There were no significant differences in intact plasma membrane percentages between extender types (BotuCRIO or FAO) within each proAKAP4 group.

3.7.2. Acrosome membrane integrity

The percentage of sperm with an intact acrosome membrane (PNA-FITC⁻/PI⁻; Fig. 9) was significantly lower ($P < 0.05$) in ejaculates with low proAKAP4 concentrations frozen with BotuCRIO extender compared to those with high proAKAP4 concentrations. Sperm acrosome integrity was higher ($P < 0.05$) in ejaculates with high proAKAP4 frozen with BotuCRIO extender than with FAO extender.

3.7.3. Mitochondrial membrane potential (MPP)

No significant differences in the percentage of sperm with high-MMP (Ratio JC1_{agg}/JC1_{mon}; Fig. 10) were observed between low- and high-proAKAP4 concentration ejaculates frozen with BotuCRIO or FAO extenders.

3.7.4. Intracellular total reactive oxygen species (ROS) levels

The fluorescence intensity of viable sperm with high intracellular total ROS levels (DCF⁺/PI⁻; Fig. 11) was significantly higher in ejaculates with low proAKAP4 concentration frozen with FAO extender than in those frozen with BotuCRIO extender. No significant differences were observed between high and low proAKAP4 concentration groups, regardless of the extender used.

3.7.5. Intracellular superoxides levels

No significant differences in the percentage of sperm with high O₂⁻ levels (HE⁺/YO-PRO-1⁻; Fig. 12) were observed between low- and high-proAKAP4 concentration ejaculates frozen with BotuCRIO or FAO extenders.

3.7.6. Plasma membrane lipid disorder

The percentage of sperm with low plasma membrane lipid disorder (M540⁻/YO-PRO-1⁻; Fig. 13) was significantly higher ($P < 0.05$) in sperm from ejaculates frozen with BotuCRIO extender with high proAKAP4 concentrations compared to those with low concentrations. No significant difference in plasma membrane lipid disorder was observed across freezing extenders.

3.8. Sperm freezability depending on proAKAP4 levels concentration

Finally, in assessing sperm freezability, once straws were thawed, quality clusters revealed a threshold of 42.10 ng proAKAP4/10M of spz, below which all the ejaculates were considered as low-quality semen. In fresh semen, the mean concentration in the low-quality group was 40.90 ± 5.75 ng proAKAP4/10M of spz.

4. Discussion

Cryopreservation procedures are known to reduce the survival, fertilising capacity, and motility of mammalian sperm [7,32,33]. Recently, cryopreservation has also been associated with alterations in proAKAP4 levels, a key protein involved in the structural and functional organization of the sperm flagellum, which may contribute to the impaired motility observed after thawing [22]. In our study, a significant reduction in proAKAP4 concentration and total and progressive sperm motility was observed in semen frozen/thawed with BotuCRIO and FAO extenders compared to fresh semen, highlighting the adverse effects of cryopreservation on sperm quality and survival. The proportional loss of proAKAP4 closely aligns with the decrease in motility, emphasizing that proAKAP4 is a good biomarker of sperm motility in stallions [22,24,34], as in other mammals [20], including humans [35].

Upon thawing, a concentration of 42.10 ng of proAKAP4/10M spermatozoa was established as the threshold for good frozen/thawed semen. While Bloommaert et al. [23] initially proposed a threshold of 60 ng/10M spermatozoa, subsequent studies confirmed a value close to 40 ng/10M, consistent with the manufacturer's commercial information [36]. This value is also consistent with the 37.77 ng/10M previously identified by our group [24]. Similarly, in cattle, ejaculates with proAKAP4 concentrations above 40 ng/10M spermatozoa exhibit superior semen quality [37].

On the other hand, proAKAP4 concentration in fresh semen may serve as a key indicator of post-thaw semen quality. A threshold of 70.33 ng proAKAP4 per 10 million spermatozoa was identified as a predictor of freezability. Fresh ejaculates above this threshold showed satisfactory post-thaw quality and were classified as having good or acceptable freezability, whereas those below the threshold exhibited poorer post-thaw outcomes and were classified as having low freezability.

A more detailed analysis of CASA-derived motility descriptors showed that VCL and VAP were significantly affected by the freeze-thaw procedure in ejaculates with low proAKAP4 concentration, whereas no significant changes were observed in ejaculates with high proAKAP4 concentration. These findings support the potential role of proAKAP4 as

an indicator of sperm freezability, since cryopreservation is known to reduce total and progressive motility as well as other kinematic parameters such as VCL, VSL, and VAP [38].

Our experimental observations did not reveal significant differences in sperm motility patterns between the BotuCRIO and FAO extenders under our specific conditions. However, other authors have reported differences when using different extenders. In horses, four hours after thawing, the Sperlin extender showed superior motility parameters and higher proAKAP4 concentrations compared to INRA Freeze [23]. Kowalsky et al. [39] reported that the incorporation of Holothuroidea extract into the extender formulation increased proAKAP4 concentrations. However, Sergeant et al. [40] reported that proAKAP4 levels after thawing vary depending on the cryoprotectant used. Further comparative studies evaluating different extenders and cryoprotectants (glycerol, egg yolk, LDL, dimethylformamide, etc.) are needed to determine whether they exert differential effects on proAKAP4 levels. Cryoprotectants are designed to enhance cell survival by protecting sperm cells from cryodamage [33]. However, cryoprotectants are known to affect proAKAP4 levels [20], and their presence may also influence semen quality after thawing [41–43]. A previous study from our group in stallion semen [22] reported significant changes in sperm motility patterns associated with cryoprotectant exposure, together with alterations in proAKAP4 concentration.

Flow cytometry enabled a deeper assessment of the relationship between sperm condition and proAKAP4 levels. The positive correlation between proAKAP4 concentration and the proportion of sperm with intact acrosomal membranes suggests that higher proAKAP4 levels are associated with improved acrosome integrity, indicating a potential role of proAKAP4 in maintaining sperm structural integrity. Additionally, acrosome integrity was higher in straws frozen with the BotuCRIO extender, indicating the extender's effect on this parameter.

Furthermore, plasma membrane integrity was lower in ejaculates with low proAKAP4 concentration. These findings are consistent with those previously described in bull semen, where a strong positive correlation between proAKAP4 levels and both plasma and acrosomal membrane integrity has been reported [44]. In agreement with this, our data showed that ejaculates frozen with the BotuCRIO extender and with low proAKAP4 concentration exhibited reduced sperm membrane integrity.

On the other hand, sperm damage is known to increase intracellular oxidative stress [45]. In this regard, total intracellular levels of ROS were significantly higher when semen was frozen with the FAO extender compared to BotuCRIO, suggesting a higher degree of cellular damage. In line with this, ejaculates with high proAKAP4 concentration frozen with BotuCRIO showed lower sperm membrane lipid disorder compared to those with low proAKAP4 concentration.

Together, these results indicate that BotuCRIO is the more suitable freezing extender. It induces lower sperm damage, as shown by higher acrosomal integrity in the high proAKAP4 group and lower total intracellular ROS levels in the low proAKAP4 group.

Regarding mitochondrial membrane potential analysis, and consistent with two of our previous publications [22,25], mitochondrial activity was not associated with proAKAP4 concentration. This finding suggests that sperm energy expenditure is not directly related to the quality of sperm movement as reflected by proAKAP4 levels. Similarly, ROS production was not associated with proAKAP4 concentration, further supporting our results, as mitochondrial membrane potential is a major source of ROS in sperm. Mitochondrial ROS production contributes significantly to oxidative stress, which can impair sperm quality and function [46].

5. Conclusion

ProAKAP4 has been reported as a biomarker of semen quality in farm animals [20,39,40,46] and of fertility [40,46]. In the present study, our results suggest that proAKAP4 may also serve as a reliable marker of

freezability, extender performance, and preservation of sperm structure and function following the freezing and thawing. ProAKAP4 concentration could potentially be used to identify individuals with semen better suited for cryopreservation within groups or breeds of equids presenting relatively low sperm quality. This approach may be a valuable strategy for conserving genetic material in endangered breeds.

Author contributions

All authors have read and agreed to the published version of the manuscript.

Ethical statement

Equine Reproduction Center of Universitat Autònoma de Barcelona is an authorized center to produce equine genetic material (semen) ES09018. Semen samples were obtained by CEE rules and there aren't experimental clinical veterinary practices.

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CRediT authorship contribution statement

M. Dordas-Perpinyà: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **J. Catalán:** Writing – review & editing, Methodology, Formal analysis, Data curation. **N. Sergeant:** Methodology, Investigation. **I. Yáñez-Ortiz:** Validation, Software, Formal analysis, Data curation. **J. Martínez-Hernández:** Methodology. **C. Tamargo:** Resources, Investigation, Funding acquisition. **C. Olegario Hidalgo:** Resources, Investigation, Funding acquisition. **L. Briand-Amirat:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **J. Miró:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

Nicolas Sergeant, coauthor of the manuscript, is cofounder of Spqi SAS.

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