ISABELLA CRISTINA TOLÊDO ALVES COSTA

PROTEOMIC PROFILE OF SEMINAL PLASMA AND SPERMATOZOA OF DONKEYS OF THE PÊGA BREED AND ITS RELATIONSHIP WITH ASSISTED REPRODUCTION

Thesis submitted to the Applied Biochemistry Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Maria Cristina Baracat-Pereira

Co-advisers: José Domingos Guimarães
Tiago Antônio de O. Mendes
Camilo José Ramírez López

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Author

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MARIA CRISTINA BARACAT PEREIRA
Data: 23/01/2024 00:22:44-0300
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Maria Cristina Baracat-Pereira
Adviser

With love, I dedicate my Doctoror of Science work to my parents, who always believe in me, even when I don't. You are my strength.

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“For myself, I like a universe that, includes much that is unknown and, at the same time, much that is knowable. A universe in which everything is known would be static and dull, as boring as the heaven of some weak-minded theologians. A universe that is unknowable is no fit place for a thinking being. The ideal universe for us is one very much like the universe we inhabit. And I would guess that this is not really much of a coincidence.”

“Para mim, eu gosto de um universo que inclui muito do que é desconhecido e, ao mesmo tempo, muito do que é conhecido. Um universo em que tudo é conhecido seria estático e monótono, tão chato como o paraíso de alguns teólogos de mente fraca. Um universo em que não se pode conhecer novas coisas não é lugar adequado para um ser pensante. O universo ideal para nós é muito parecido com o universo que habitamos. E eu posso apostar que isso não é uma coincidência”.

(Carl Sagan)

ABSTRACT

Costa, Isabella Cristina Tolêdo Alves, D.Sc., Universidade Federal de Viçosa, September, 2023. **Proteomic profile of seminal plasma and spermatozoa of donkeys of the Pêga breed and its relationship with assisted reproduction**. Adviser: Maria Cristina Baracat-Pereira. Co-advisers: José Domingos Guimarães, Tiago Antônio de Oliveira Mendes and Camilo José Ramírez López.

Donkeys, along with their hybrids, mules and hinnies, resulting from breeding with mares, have a longstanding tradition of being used for labor-intensive tasks. Historically, these animals played pivotal roles in agriculture, warfare, trade, and transportation. However, with the advent of modernization, this dynamic has shifted. In Europe, these animals have become more popular as pets, and some species even faced the brink of extinction. In contrast, Brazil hosts a significant population of undomesticated donkeys, especially in the Northeast. Recently, the demand for donkeys has seen a resurgence, primarily in China, due to the consumption of their milk and meat. Assisted reproduction would be an advantageous approach, as it is more cost-effective and practical than maintaining animals in the field. However, when cryopreserved, donkey semen suffers a drastic decrease in its fertilization capabilities. The first chapter of this thesis delved into discerning the differences between the proteomic profiles of cryopreserved and non-cryopreserved sperm. In cryopreserved samples, proteins associated with infertility were identified, such as SERPINE2 and SERPINC1, both linked to the loss of sperm capacitation, and PLAU, associated with reduced motility. A high concentration of nucleoporins, related to neurodegenerative diseases, was also observed. In contrast, non-cryopreserved sperm displayed a proteomic profile primarily oriented towards energy production and metabolism, reflecting the energetic demands required for maintaining sperm motility and viability. In the second chapter, the proteomic profile of donkey seminal plasma was explored, emphasizing the identification of potential infertility biomarkers. Compared to existing literature, CRISP3 and HSP1 were identified, proteins previously linked to infertility in stallions. Also, CRISP1, PTP an PKA have potencial as biomarkers. The proteomic profile also highlighted proteins connected to energy production, metabolism, and reproduction, suggesting an influential role of seminal plasma proteins in sperm viability during the reproductive process of donkeys.

Keywords: Donkeys. Seminal plasma. Proteomics. Assisted reproduction. Cryopreserved semen.

RESUMO

Costa, Isabella Cristina Tolêdo Alves Costa, D.Sc., Universidade Federal de Viçosa, setembro de 2023. **Perfil proteômico do plasma seminal e dos espermatozoides de jumentos da raça Pêga e sua relação com a reprodução assistida**. Orientadora: Maria Cristina Baracat-Pereira. Coorientadores: José Domingos Guimarães, Tiago Antônio de Oliveira Mendes e Camilo José Ramírez López.

Jumentos, juntamente com seus híbridos burros e mulas resultantes do cruzamento com éguas, têm uma longa tradição de uso em tarefas que exigem força. Historicamente, esses animais desempenharam papéis fundamentais na agricultura, combate armado, comércio e transporte. No entanto, com a onda de modernização, essa dinâmica sofreu alterações. Na Europa, esses animais se tornaram mais comuns como pets, e algumas espécies chegaram à beira da extinção. Em contraste, o Brasil tem uma vasta população de jumentos não domesticados, especialmente no Nordeste. Recentemente, a demanda por jumentos ressurgiu, principalmente na China, devido ao consumo de seu leite e carne. A reprodução assistida seria uma abordagem altamente benéfica, pois é mais econômica e prática do que manter os animais em pasto, contudo, o sêmen de jumento, quando criopreservado, sofre uma drástica diminuição em sua capacidade de fertilização. O primeiro capítulo desta tese se dedicou a discernir as diferenças entre os perfis proteômicos de espermatozoides criopreservados e não criopreservados. Em amostras criopreservadas, identificou-se a presença de proteínas associadas à infertilidade, como SERPINE2, ligada à perda de capacitação espermática, e PLAU, associada à redução da motilidade. Também se observou uma alta concentração de nucleoporinas, relacionadas a doenças neurodegenerativas. Em contraste, os espermatozoides não criopreservados exibiram um perfil proteômico voltado principalmente para a produção de energia e metabolismo, refletindo as demandas energéticas para a manutenção da motilidade e viabilidade espermática. No segundo capítulo, explorou-se o perfil proteômico do plasma seminal de jumentos, com ênfase na identificação de potenciais biomarcadores de infertilidade. Comparando com dados da literatura, identificaram-se CRISP3 e HSP1, proteínas previamente associadas à infertilidade em garanhões. Também, CRISP1, PTP and PKA possuem potencial como biomarcadores. O perfil proteômico também revelou a prevalência de proteínas ligadas à produção de energia, metabolismo e reprodução,

sugerindo um papel influente das proteínas do plasma seminal na viabilidade espermática durante o processo reprodutivo dos jumentos

Palavras-chave: Jumentos. Plasma seminal. Proteômica. Reprodução assistida. Sêmen criopreservado.

SUMMARY

INTRODUCTION

Donkeys

Donkeys (Equus asinus) have always been highly sought-after animals worldwide for agricultural work, transportation, and warfare, as well as their hybrids with mares (Equus caballus), popularly known as mules and hinnies, which have great work and traction capabilities (Gastal *et al*., 1996; McLean *et al*., 2012; Oliveira *et al*., 2016; Canisso *et al*., 2019). For centuries, their physical strength has been utilized for these purposes, a trend that continues today. In Brazil, they are primarily used for herding large cattle flocks (Canisso *et al*., 2019).

Globally, there are about 185 different donkey breeds, and many of them have alarmingly low numbers of individuals (Kugler *et al*., 2008). Industrialization led to the extinction of many European breeds, prompting countries like Spain, Italy, and Portugal to establish conservation and breed rescue programs for endangered donkeys. A significant part of this renewed interest in the species is due to the rediscovery of its milk, which can be used for both food and cosmetic purposes. In contrast, in the northeastern region of Brazil, for instance, there is an overpopulation of wild donkeys, which have no commercial value as they are not domesticated (Canisso *et al*., 2019).

Given that these animals have a high commercial interest and that some breeds are at risk of extinction, the reproduction of donkeys and the preservation of their genetic material are topics of great importance for equine breeders. However, due to the sensitivity of their semen to freezing, breeders face significant challenges in reproducing these animals using artificial insemination from cryopreserved seminal samples. The fertility rate of frozen semen is low in mares (33-53%) (Oliveira *et al*., 2006; Canisso *et al*., 2011) and even lower in female donkeys (0-36%) (Viera *et al.,* 1985; Trimeche *et al*., 1998; Vidament *et al*., 2009). This situation forces producers to always have a live animal in the field for reproduction, which can entail high maintenance costs.

Considering this scenario, there is an urgent need to make the cryopreservation of donkey semen viable without losing the fertility of the sample, bringing savings and convenience to producers of these animals.

Cryopreservation

Seminal cryopreservation is a process of utmost importance for animal reproduction, as it provides convenience and savings, as well as the preservation of genetic material from domesticated species. However, success in this technique is not always observed in all species. Animal reproduction using cryopreserved bull semen can be cited as a positive outcome, but several other mammals such as pigs, sheep, and the subjects of this study, donkeys, do not share these favorable results (Holt, 2000; Rota *et al*., 2012).

This disparity in results among species is due to the physiology and biochemistry of the sperm, the variation in the anatomy and physiology of sperm transport in the female reproductive tract (Holt, 2000), where also significant inflammatory responses of the uterus inseminated by a frozen sample can be generated (Kotlainen et al., 1994), in addition to the quantitative difference in viable sperm necessary for fertilization (Holt, 2000).

Taking into account the factors that differentiate each species in the success of its seminal cryopreservation, it's also necessary to highlight that the viability and longevity of sperm can be compromised (Watson, 1995; Rota *et al*., 2012), either by temperature changes, which can cause damage to the sperm, or by the addition of cryoprotectants that generate osmotic and toxic stress, intracellular ice formation (Blach *et al*., 1989; Watson *et al*., 2000), as well as low protein levels in seminal plasma (Cardozo *et al*., 2006).

Given that the donkey's fertility is heavily affected by the standard cryopreservation method, there are studies suggesting alternative improvements in semen viability by adding donkey colostrum (Álvarez *et al*., 2019) or non-permeable cryoprotectants (Diaz-Jimenez *et al*., 2018) to the sample to counteract its negative effects. However, there are no in-depth studies in the literature on the actual effects

on the protein and metabolic levels of donkey semen that explain this decline in fertility.

In the case of the donkey, conducting proteomic and metabolomic analyses of the seminal plasma and sperm could indicate possible changes that play an important role in maintaining the stability of the sperm cell membrane (Strezek *et al.,* 2002; Vyvial *et al*., 2019), as essential proteins may undergo irreversible changes due to temperature effects and cause harmful effects on in vitro fertilization.

Seminal proteomics

There's a limitation in assessing sperm function solely through clinical form since, in infertility cases, seminal parameters may still fall within the normal range (Ayaz et al., 2018). This emphasizes the need for a more in-depth analysis of the complexity of seminal fluid, which consists of a cellular fraction, accounting for 5% of the semen, and a non-cellular fraction, making up 95% of its entirety, which includes the seminal plasma (Jodar *et al*., 2017).

Seminal proteomics enables the identification of proteins that make up the sperm and the seminal plasma, which can have essential roles in fertility (Mateo *et al*., 2012). Some studies indicate that sperm viability disorders might be linked to low levels of proteins present in the seminal plasma, seasonally (Cardozo *et al.,* 2006), reinforcing that more detailed studies in this area should be undertaken. The advancement in mass spectrometry (MS) techniques has further enhanced the potential for studying and identifying proteins present in sperm (Mateo *et al*., 2012). Proteomics carried out using two-dimensional gels to separate samples can facilitate the identification of unknown forms of a given protein group (Magalhães *et al*., 2016), assisting in exploring the factors causing damage during cryopreservation.

The development of infertility biomarkers for specific treatments for each fertility disorder can be achieved through proteomics. Once the biomarker of interest is validated, there's a higher chance of developing quicker and feasible tests for seminal analysis, like those based on protein microarrays, ELISA, and mass spectrometry (Jodar *et al*., 2017), in addition to possible treatment strategies.

Thus, using proteomics in the seminal analysis of donkeys becomes valuable for determining proteins that could be essential biomarkers for understanding the reduced fertility of sperm following cryopreservation.

Biomarkers of infertility

Seminal vesicles and prostate secretions are present in the non-cellular part of the semen. Among the various contributions they provide, there's the addition of necessary proteins for semen coagulation and liquefaction, such as semenogelin I and II, sourced from the seminal vesicles, and kallikreins (KLK) type 3, from the prostate, in humans (Jodar *et al.,* 2017).

Kallikreins are serine-proteases that have the potential to be biomarkers for disorders in the body, once each of their functions and/or the function of each of the isoforms of this protein is understood, as well as their forms and times of action. In humans, for instance, they are used for the diagnosis of prostate cancer. Each of the 15 forms of kallikrein has an expression profile in specific tissues and putative proteolytic functions (Lawrence *et al*., 2010).

In stallions (*Equus caballus*), a study identified kallikrein-1E2 (KLK2) as one of the negative fertility biomarkers in seminal plasma, along with clusterin and seminal plasma proteins 1 (SP1) and 2 (SP2). On the other hand, cysteine-rich secretory protein 3 (CRISP3) was identified as a positive fertility biomarker (Novak *et al*., 2010).

Since stallions and donkeys belong to the same family, kallikreins have a great potential to serve as a possible biomarker in both species. Therefore, the presence of forms of kallikreins in the cryopreserved semen samples of Pêga breed donkeys will be analyzed, as well as their functionality and proposed involvement in cryopreservation and reproduction.

JUSTIFICATIVE

As donkeys have significant commercial value, their reproduction is highly sought after. When crossbred with mares, they produce hybrid animals renowned for

their remarkable strength, traction, and resilience, making them indispensable for various agricultural tasks. However, breeders face challenges in preserving the donkey's genetic material. After cryopreservation, the donkey's semen experiences a substantial decline in its fertilizing potential, eliminating the possibility of selling this genetic material. Consequently, maintaining a male breeding donkey on farms can increase operational costs. It's noteworthy that the process of cryopreserving mammalian semen impacts the composition and functionality of proteins and other essential low molecular weight molecules in the seminal plasma and spermatic plasma membranes. These elements are pivotal for animal fertility and sperm cell integrity. Hence, it's crucial to understand the cryopreservation's effects on the metabolomic and proteomic profiles of donkey seminal plasma and sperm, as well as the subsequent changes

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Chapter 1

Proteomic profile of spermatozoa with great motility and vigor of Pêga donkeys (*Equus asinus*) and its relationship with assisted reproduction

1. INTRODUCTION

Due to their particular aptitudes for physically demanding tasks requiring strength, donkeys (*Equus asinus*) and their hybrids (*Equus mulus*) with mares (*Equus caballus*) which also display robustness and traction capabilities, have been employed in various contexts, including agriculture, transportation, and armed conflicts, across multiple centuries (Gastal *et al*., 1996; McLean *et al*., 2012; Oliveira *et al*., 2016; Canisso *et al*., 2019).

Other factors contribute to the global interest in these animals, including their role in touristic activities, territorial defense strategies, herding practices, and their function as pets. Furthermore, donkey milk and its by-products have been gaining prominence not only as a nutritional component in food but also as an ingredient in cosmetic products (Canisso *et al*., 2019).

Although there are regions, such as Northeastern Brazil, where an overpopulation of certain yet-to-be-domesticated breeds of these animals is observed, it is crucial to highlight that other breeds, among the 185 cataloged, are in a critical situation of risk of extinction, with a significantly low count of remaining individuals. (Kugler *et al*., 2008; Canisso *et al*., 2019).

In light of the imminent risk of extinction and the growing commercial interest in these animals, seminal cryopreservation emerges as a pivotal technique for advancing assisted reproduction in donkeys. While the use of cryopreserved semen has shown remarkable efficacy in species such as cattle, the same success has not been replicated in all species, as donkeys, for which attempts at assisted reproduction using cryopreserved semen have yet to yield successful outcomes. (Holt, 2000; Rota *et al*., 2012). Fertilization rates resulting from the use of donkeys cryopreserved semen in mares show significant variability, ranging between 33% and 53% (Oliveira *et al*., 2006; Canisso *et al*., 2011). Even more concerning, performance in female donkeys is substantially lower, exhibiting a range between 0% and 36% in terms of reproductive success. (Vieira *et al*., 1985; Trimeche *et al*., 1998; Vidament *et al*., 2009).

Physiological and biochemical factors may modulate the success or failure in assisted reproduction procedures across different species, as discussed by Holt (2000), including temperature fluctuations that can cause damage to sperm cells. Such damage can be attributed to osmotic and toxic stresses, as well as the formation of ice crystals within the sperm cells, causing damage to membranes. (Blach *et al*., 1989; Watson *et al*., 2000).

The utilization of advanced proteomic techniques provides a robust platform for identifying proteins that play fundamental roles in maintaining the fertility of sperm cells. Specifically, mass spectrometry-based approaches (MS) have enabled in-depth investigations by allowing for the identification of previously unknown proteins present in semen. Such insights can be extremely valuable in unraveling the underlying mechanisms that lead to damage in sperm cells subjected to the cryopreservation process (Mateo *et al*., 2012).

In this context, the central objective of this study is to conduct a comprehensive characterization of the proteomic profile of spermatozoa from Pêga breed donkeys, both in fresh conditions and under cryopreservation, using advanced Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) techniques. This research aims not only to elucidate the intrinsic protein compositions of these sperm cells but also to investigate, in a comparative manner, the impacts of the cryopreservation process on the protein integrity of the spermatozoa. The analysis seeks to contribute to a better understanding of how these impacts can influence the reduced fertilization rates observed in mares and female donkeys when inseminated with cryopreserved sperm.

2. MATERIAL AND METHODS

2.1. Ethical Approval

This work was developed as a collaboration between the Laboratory of Proteomics and Protein Biochemistry (LPBP) of the Department of Biochemistry and Molecular Biology at Universidade Federal de Viçosa, the Laboratory of Animal Reproduction (LABRAN) of the Department of Medicine Veterinary at Universidade

Federal de Viçosa, and the Laboratory of Proteomics (LABPROT) at Universidade Federal do Rio de Janeiro.

This study was conducted in strict compliance with the guidelines set forth by the Ethics Committee for Animal Use at the Federal University of Viçosa. The project was submitted and approved and is duly registered under protocol number 28/2020. This study aims to continues the study entitled “Use of different extenders and protocols in the cryopreservation of semen from donkeys of the Pêga breed”, from the Group of Animal Reproduction of the Animal Andrology Laboratory of the Department of Veterinary Medicine at UFV, approved by the Ethics Committee for the Use of Animals at UFV (CEUA) with protocol number 73/2018.

2.2. Materials

All materials used were of the highest grade available. Chemicals and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) or from Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. Materials for electrophoresis procedures were purchased from GE Healthcare (Life Sciences, USA).

2.3. Animals, sample collection and semen analysis

A total of 13 ejaculate were collected from three healthy fertile Pêga breed donkeys (*Equus asinus*) with average age of four-year-old (ranging from three to five) over the course of the breeding season belonging to a stud farm located in the city of Viçosa, Minas Gerais, Brazil (-42.8825 20° 45′ 17″ S, 42° 52′ 57″ W, 663 m). Semen collection was carried out at regular intervals of 8 days using the artificial vagina method previously heated with water at 45 °C. The first animal provided four ejaculates, while the second and third animals contributed five ejaculates each.

All donkeys were kept in similar handling and feeding conditions and submitted a Breeding Soundness Evaluation. At the time of semen collection, the volume (mL), ejaculate appearance (1: aqueous, 2: opalescent, 3: milky, 4: creamy), and the concentration of each ejaculate (using a Neubauer chamber) were evaluated. Sperm motility (percentage scale, from 0 to 100%, indicating no cell to all cells with movement, respectively) and sperm vigor (scale of 0 to 5,

representing no movement to rapid and vigorous movements, respectively) were also evaluated in an aliquot of semen, using conventional optical microscopy (CX31, Olympus, Tokyo, Japan) at 100× magnification, observing at least four microscopic fields for each sample. The results were expressed as the average of the evaluated fields according to the criteria established by the Brazilian College of Animal Reproduction. Immediately after evaluating the quality sperm parameters, 2 mL of semen from each collection were separated, added to a new tube and centrifuged at 150 x g for 10 min to separate the seminal plasma from the sperm. Then, a new centrifugation at 700 x g for 10 min was performed to remove cellular debris. The resulting seminal plasma was filtered using a 0.22 µm cutoff membrane and immediately frozen in liquid nitrogen.

The other semen fraction was diluted in a skimmed milk-based medium (Botu-Sêmen, Botupharma, São Paulo, Brazil) in a ratio of 1:2 (sperm:diluent), divided into aliquots in 15 mL Falcon tubes and centrifuged at 600 x g for 15 min. Immediately, the supernatant was removed and the sperm concentration of the pellets was estimated in a Neubauer chamber. Then, the pellets were resuspended in freezing diluent (Botu-Crio, Botupharma, São Paulo, Brazil) at a final concentration of 100 million spermatozoa per mL and sperm motility and vigor were evaluated after dilution.

The diluted semen was packaged in 0.5 mL straws, duly identified, and refrigerated at 5 °C for 120 min. Subsequently, the straws were placed in nitrogen vapor for 15 min and then immersed in liquid nitrogen. The straws from each ejaculate were thawed at 37 °C for 30 s to evaluate sperm motility, vigor and calculate the percentage of sperm abnormalities after thawing.

2.4. Sample preparation

To compare the effect of cryopreservatives on donkey sperm, each sperm sample collected was evaluated in the presence and absence of cryoprotectants during the freezing process.

2.4.1. Separation of spermatozoa from the seminal plasma

To separate the spermatozoa from the seminal plasma of each sample, the ejaculates were centrifuged at 700 x *g* for 10 min and the precipitate fractions were recovered. Part of the fresh sperm from each donkey were stored in 0.5 mL straws and frozen without adding diluents or cryoprotectants, in liquid nitrogen at -196 °C until processing.

2.4.2. Washing sperm in Botucrio® cryopreservative

To remove the cryoprotectant, the sperm were subjected to three successive washes in phosphate-buffered saline (PBS), pH ~ 7.4. Subsequently, spermatozoa were centrifuged at 17,000 x *g* for 20 min at 4°C. The supernatant was recovered and added to a new tube, and the formed pellet was resuspended in 1 mL of PBS and subjected to a new centrifugation at 10,000 x *g* for 10 min at 4°C. The supernatant was again recovered and added to the same tube containing the supernatant from the first centrifugation. This step was repeated twice.

Both the pellet formed at the end of the centrifugations and the supernatant were lyophilized and subsequently resuspended in 250 µL of solubilization solution (7 M urea, 2 M thiourea, 4% CHAPS (3-3-3-cholamidopropyldimethylammonium-1-propanesulfonate) and 40 mM dithiothreitol (DTT)).

2.4.3. Separation of proteins of the fresh spermatozoa by solubility

Firstly, fresh sperm samples were removed from liquid nitrogen and subjected to freeze-drying, ensuring the complete drying. After freeze-drying, spermatozoa were solubilized in 400 µL of 4 % CHAPS (3-3-cholamidopropyl dimethylammonium-1-propanesulfonate) solution. The samples were then vortexed for 1 min, followed by an ultrasound bath for 30 min at a frequency of 75 MHz. This sequence of procedures was repeated twice more to ensure the effectiveness of the process.

Subsequently, the samples were centrifuged at 8,000 x *g* for 40 min at 4°C. The resulting supernatant was collected and transferred to a new tube. Finally, the remaining pellets were solubilized in 500µL of 10% Sodium Dodecyl Sulfate (SDS) solution. Samples were then vortexed and centrifuged at 5,000 x g for 5 minutes at 4°C. The resulting supernatant was collected and stored in new tubes.

2.5. Protein quantification

Quantification of proteins from sperm samples was performed using the Bi-cinchoninic Acid method (Smith, 1985). Simultaneously, an analytical calibration curve was performed using known concentrations of bovine serum albumin (BSA), to establish the relationship between absorbance and concentration.

2.6. One-dimensional SDS-PAGE gel electrophoresis

The prepared SDS-PAGE gels consisted of two different stages: separation, with 14% T and 3.0% C, and stacking, with 5.1% T and 2.6% C. The runs were performed at 80 V for 2h30 min in the Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA, USA). The amount of soluble proteins used was 15 µg per sample, in the case of *fresh* sperm samples. For sperm in diluent, a volume of 18 µL per sample was used. The separated proteins were compared with Broad Range molecular mass markers (cat.161-0317, Bio-Rad). After running, the gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA), for 2 h and then destained by 10 h (Laemmli, 1970).

2.7. SDS-PAGE by Short-run

Protein pools were formed, each containing 37.5 µg of each protein sample, totaling 150 µg of sample per pool.

To ensure the standardization of the samples contained in each set, the last collection from the second animal was not used, resulting in a total of four collections for each animal. Based on these sets, three subsamples of each were prepared, which are technical replicates, each subsample containing 50µg of proteins. These subsamples were then subjected to migration on two SDS-PAGE gels, each covering approximately 1 cm (Resjö *et al*., 2017).

The regions of the gels that contained the proteins were excised and subsequently subjected to a fixative solution composed of 40% (v/v) methanol and

5% acetic acid for a period of two hours. This procedure was followed by revelation, to locate the proteins in the samples.

This protocol was equally applied to protein samples in 4% CHAPS and 10% SDS from fresh sperm, as well as to samples and the supernatant solution, which would be discarded, from sperm preserved in Botucrio®.

After carrying out the previously mentioned procedures, it was necessary to proceed with the discoloration of the gels. Initially, the fixative solution was removed and 200 µL of a solution containing 50% (v/v) Acetonitrile (ACN) and 25mM Ammonium Bicarbonate (AMBIC) was added to each microtube. These were left to rest for a period of two hours for the first wash.

Once this step was completed, the solution was discarded and 200 µL of the 50% (v/v) ACN/AMBIC 25 mM solution were added again to each microtube. On this occasion, the microtubes were left to rest for an extended period of 24 h at room temperature.

Subsequently, an additional two-hour wash was carried out using the 50% ACN/AMBIC (v/v) 25 mM solution again, as some gels still showed color. In order to dehydrate the gels, two washes were carried out using 200 µL of 100% ACN for five minutes each, followed by discarding the solution.

Finally, to ensure the complete removal of ACN and adequate drying of the gels, they were subjected to a complete drying process in a vacuum concentrator.

2.8. Protein digestion and LC-MS/MS analysis

The proteins contained in the gels of fresh and cryopreservative samples were subjected to an in-gel trypsin digestion process (Shevchenko *et al*., 2006). The peptides were solubilized in 0.1% formic acid, quantified by a QuibtTM fluorometer (Invitrogen) and the concentration adjusted to 0.2 µg/µL. Samples were injected into a nano-LC–MS/MS system consisting of a high-performance, high-pressure nanoLC EASY1000 (Thermo Scientific) coupled to an Orbitrap Q Exactive plus mass

spectrometer (Thermo Scientific). 1 µg of peptides were loaded onto an Acclaim PepMapTM 1000 C18 precolumn, nanoViper 2Pk (100 µm x 2 cm) and an Easy-Spray™ PepMap™ Neo analytical column (75 µm x 250 mm) having internally a C-18 resin ReproSil 2 µm (Thermo Scientific). The applied gradient consisted of phase A (5% ACN/0.1% formic acid) and phase B (95%/0.1% formic acid). The gradient started with 5% solvent B and increased to 25% for 100 min, 25–32% phase B for 5 min, 32–45 for 5 min, and 95% phase B for 10 min.

Mass spectra were acquired in a top 20 positive mode data dependent acquisition (DDA) method. The MS1 scan was acquired on an Orbitrap analyzer set to a range of 350−1800 m/z, resolution of 30,000 (at m/z 400) with a minimum required signal of 5000, isolation width of 2.0. The 20 most intense ions were further fragmented in a High Energy Collisional Dissociation (HCD) cell at 30 NCE and a 60 s dynamic exclusion. Other mass spectrometric conditions were sheath and auxiliary gas flow 0.0, capillary temperature at 235 °C.

2.9. Data Analysis

The raw data files were examined in Xcalibur v.2.1 (Thermo Scientific), the number of MS1 and MS2 spectra formed were checked in RawMeet v.2.0. Proteome Discoverer 2.4 (Thermo Scientific) was used to identify proteins. The Sequest HT algorithm was tested to obtain the PSM for the analyzed peptides against a database of the *Equus asinus* family available at UniProt (https://www.uniprot.org/) acessed in March 2023 02, containing 33,660 proteins. The parameters used were tryptic cleavage with a maximum of two missed cleavages, methionine oxidation (+15,995 Da), cysteine carbamidomethylation (+57,021 Da). The Target Decoy PSM Validator was set to target FDR (Strict) 0.01. Only proteins identified in at least two biological replicates were considered for qualitative and quantitative analysis.

The abundance of the precursors was determined based on ion intensity and the Matching Between Run (MBR) tool. Label-free quantification applying the Precursor Ion Quantifier node of Proteome Discoverer 2.4 was handled by Perseus vs.2.0.9.0 (www.maxquant.net/perseus/ accessed on March 02, 2023) (Tyanova *et al.*, 2016). The area values generated by the software tested to identify the proteins

were loaded, transformed on a logarithmic scale, grouped according to biological replicates, averages and grouped according to treatment (fresh and cryopreservatives), so that each treatment was represented by three biological replicates, with two technical replicates. They were analyzed by principal component analysis (PCA) to evaluate the contrast between conditions. The filters used were: Filter lines based on valid values, configured to consider proteins present in at least two biological replicates in at least one method. After normalizing the data by subtracting the median and statistical evaluation using the t test, the resulting matrix was filtered for valid values so that only proteins with statistical significance remained using the t test (p-value < 0.05).

3. RESULTS

3.1. Semen quality parameters

No significant difference was observed in sperm quality parameters between individuals before freezing and after thawing ($p>0.05$). However, the median value of motility and sperm vigor of cryopresevated semen was lower than that of fresh and diluted semen ($p<0.05$). The sperm motility had a median of 90% fresh and diluted, and a median of 35% after thawing. The vigor had a median of 4 fresh and diluted and a median of 3 after thawing. (Figures 1 and 2).

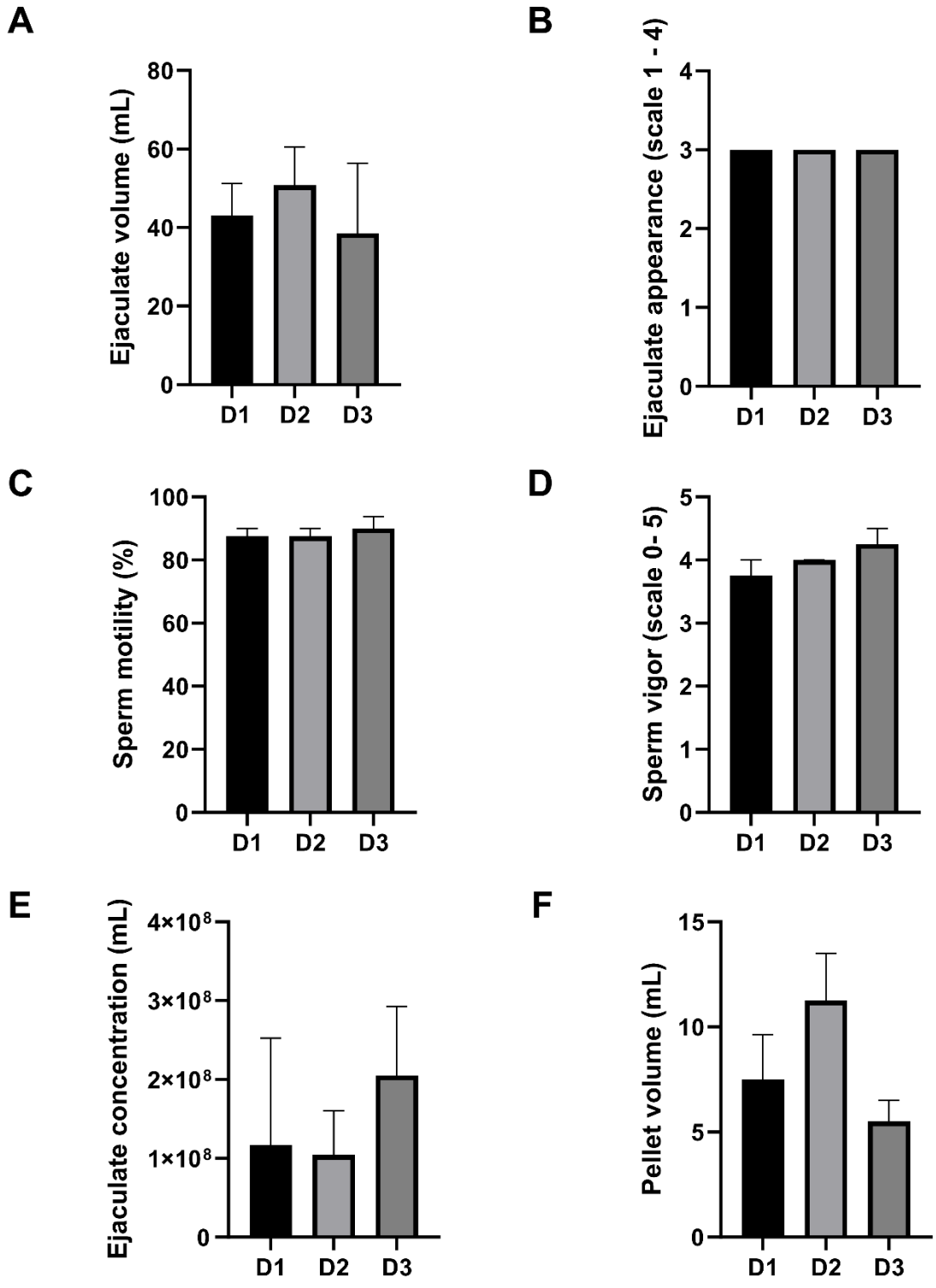

Figure 1. Sperm quality parameters of Pêga donkeys before semen freezing. Those graphics demonstrate the medians of the Pêga breed donkeys regarding ejaculate volume (A), ejaculate appearance (B), sperm motility (C), sperm vigor (D), ejaculate concentration (E), pellet volume (F). Source: Costa, 2023. Authorial.

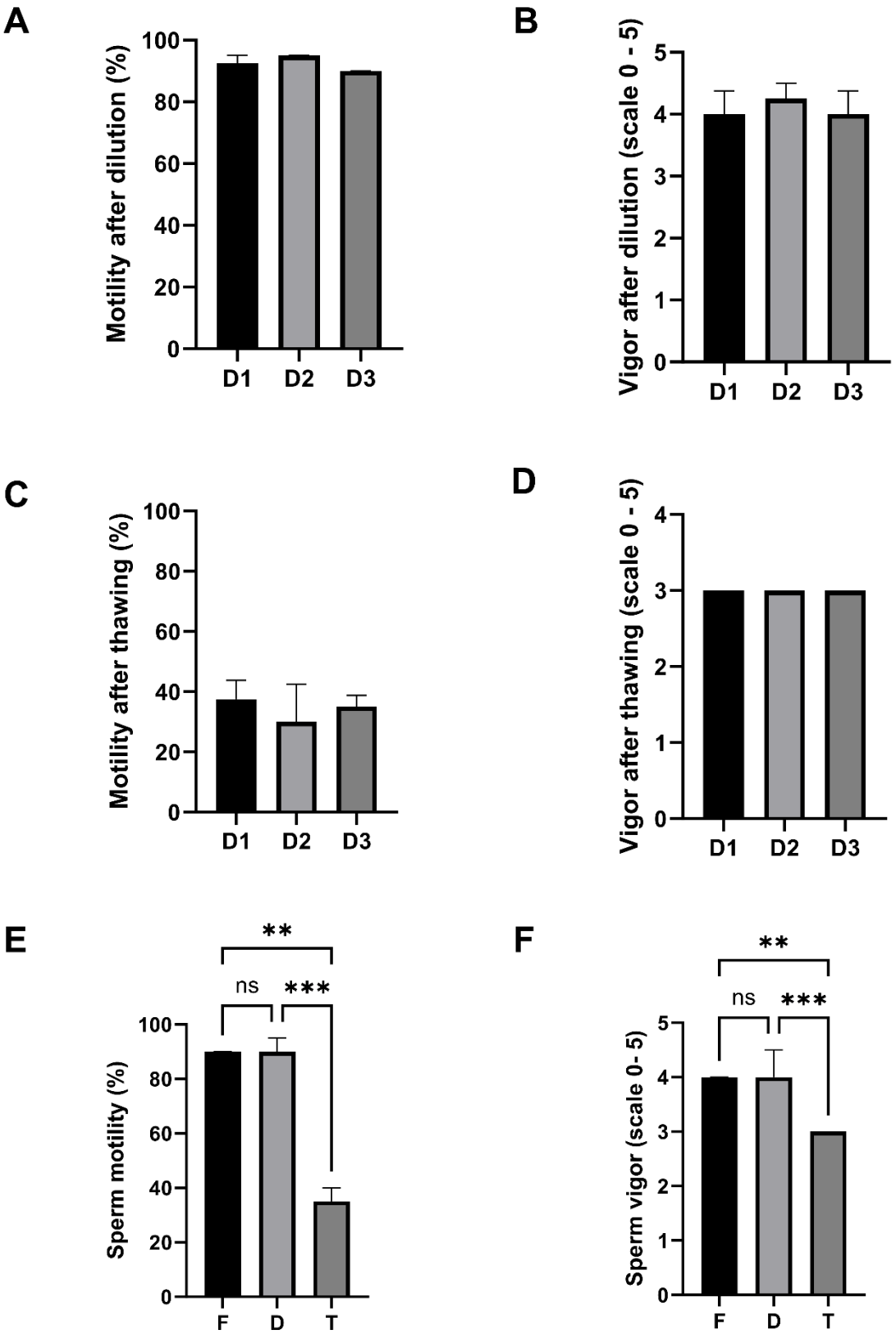

Figure 2. Sperm quality parameters of Pêga donkeys after dilution and after thawing. Those graphics demonstrate the medians of the Pêga breed donkeys parameters for each animal regarding motility after dilution (A), vigor after dilution (B), motility after

thawing (C) vigor after thawing (D), a comparison between the motility of fresh, diluted and thawed semen (E), a comparison between the vigor of fresh, diluted and thawed (F). Source: Costa, 2023. Authorial.

3.2. Protein identification

A total of 499 proteins were identified in the spermatozoa by Proteome Discoverer with the *Equus asinus asinus* database, with 48 contaminants identified, which were not considered, resulting in a total of 451 proteins.

To analyze proteins absent and present in sperm samples added or not of cryoprotectants during the freezing process, Venn diagrams were created using Venny v.2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). First, total proteins that were present in at least two replicates of each treatment were selected. In cryopreservatives (Figure 4), 305 proteins were obtained, whereas fresh (Figure 5) there were 451.

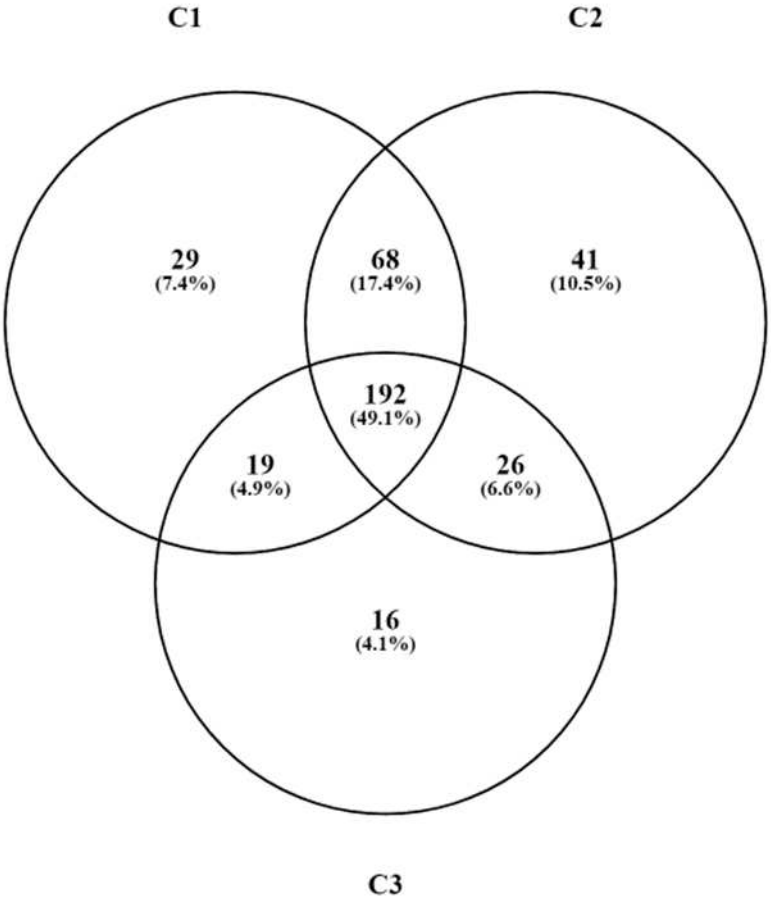

Figure 3. Venn diagram of proteins found in the three replicates of the cryopreservative (C) samples. Only those present in at least two replicates were considered. The total obtained was 305 proteins. Source: Costa, 2023. Authorial.

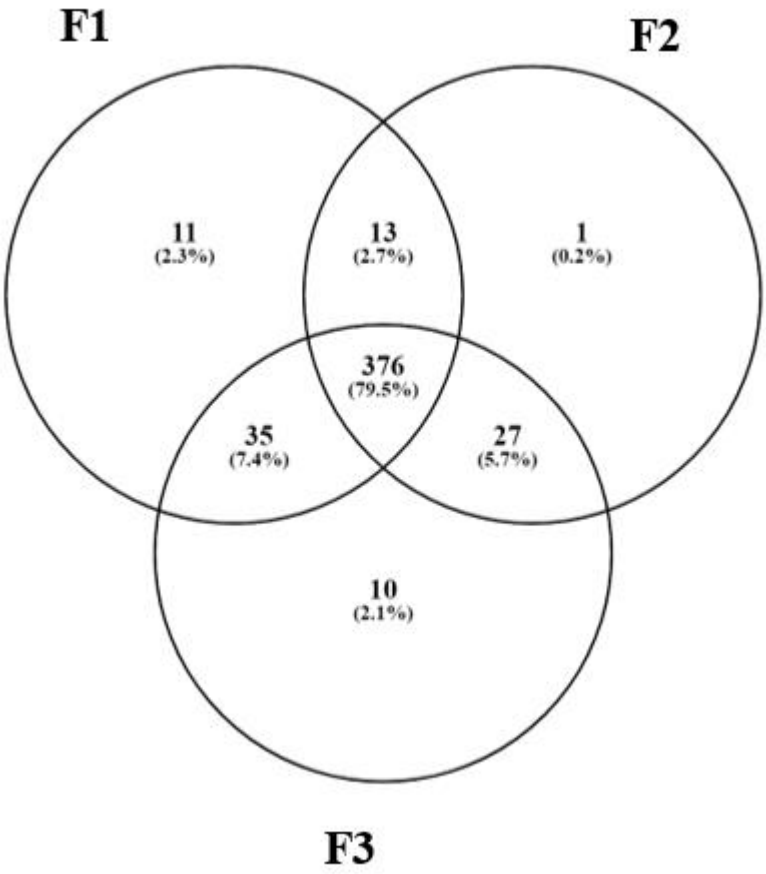

Figure 4. Venn diagram of proteins found in the three replicates of the in fresh (F) samples. Only those present in at least two replicates were considered. The total obtained was 451 proteins. Source: Costa, 2023. Authorial.

Of the 451 total proteins obtained, 13 proteins were exclusively present in cryopreservative samples, and 158, in fresh samples (Figure 5).

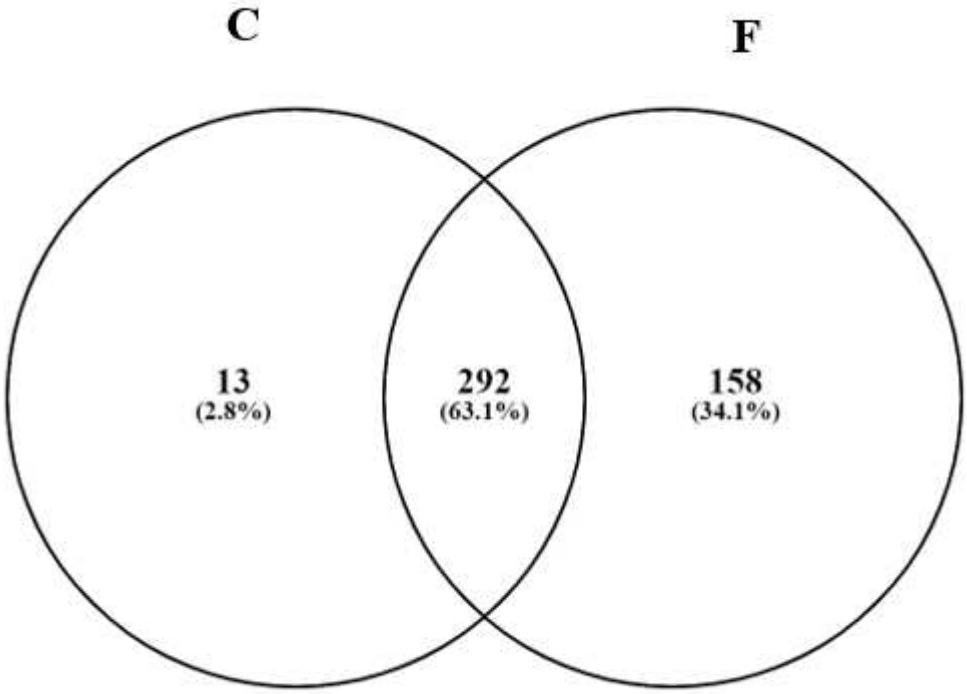

Figure 5. Venn diagram of the 451 proteins found in cryopreservative (C) and natural (F) samples. The total obtained was 305 proteins. Source: Costa, 2023. Authorial

3.3. Functional classification of the spermatozoa proteomic profile

Using the Gene Ontology of STRING DB vs.12.0 program (Szklarczyk et al., 2022), proteins were classified according to biological process (Figure 6), molecular function (Figure 7) and cellular component (Figure 8) for proteins present exclusively in each treatment.

In the comparative results of biological processes, significant differences were observed between the cryopreserved and fresh samples in terms of protein composition. In the cryopreserved spermatozoa (Figure 6A), proteins predominantly associated with the regulation of body fluid levels and RNA localization were identified, with eight proteins in each category. In addition, seven proteins associated with both mRNA transport and blood coagulation were detected. Lastly, five proteins involved in the regulation of blood coagulation were noted.

In contrast, fresh spermatozoa (Figure 6B) exhibited a protein profile in which small molecule metabolic process was the most prominent, with 26 identified proteins. This was followed by the oxidation-reduction process, which had 22 involved proteins. Other notable processes included the generation of precursor metabolites and energy, with 14 proteins; ATP metabolic process, with 12 proteins; and finally, proton transmembrane transport, which eight involved proteins.

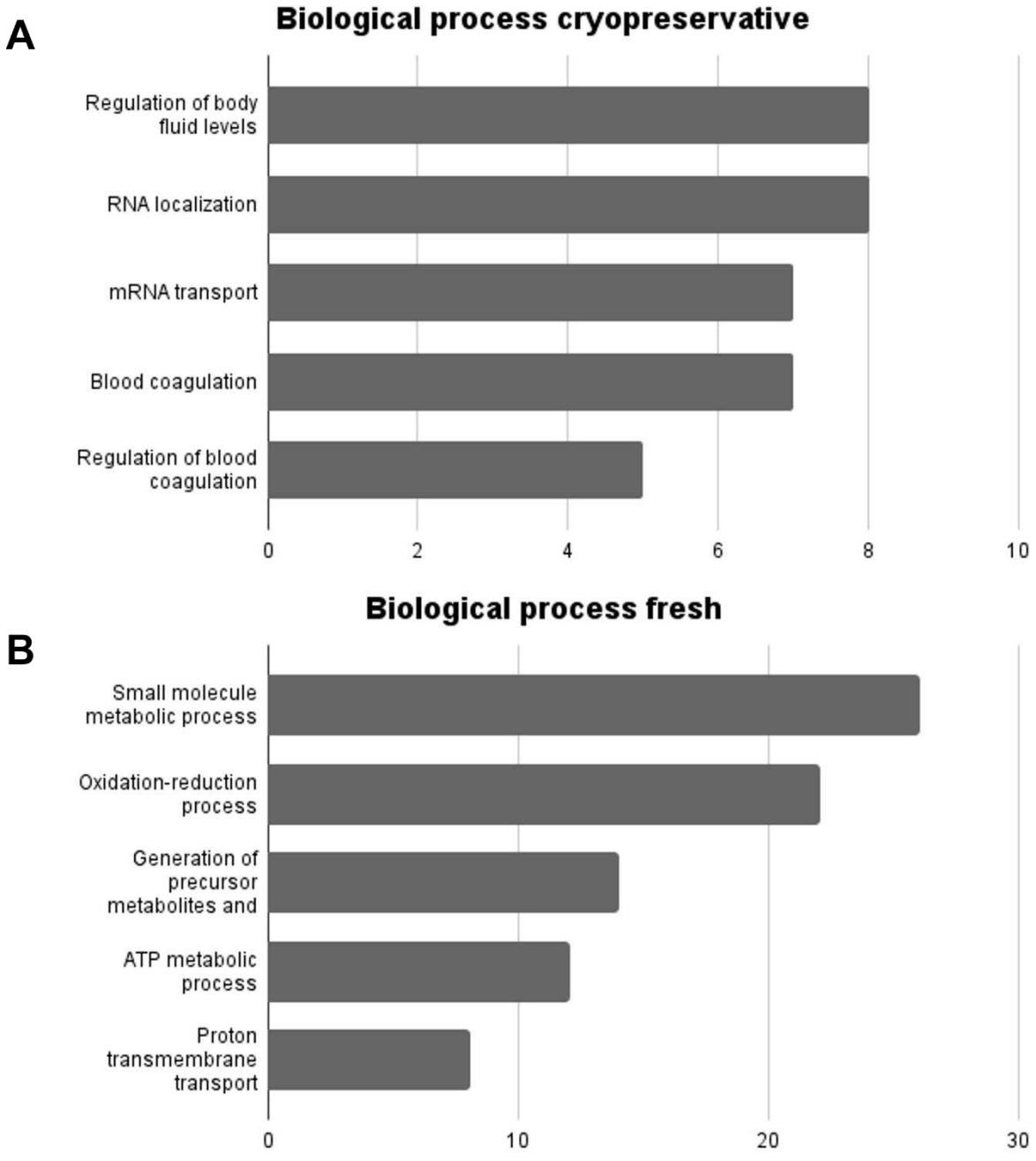

Figure 6. Classification of proteins involved in the biological processes of (A) cryopreservative spermatozoa (B) fresh spermatozoa by STRING DB. Source: Costa, 2023. Authorial.

In the comparative results of molecular functions present in each treatment, no statistically significant results were observed for cryopreserved spermatozoa. However, in the *in natura* spermatozoa samples (Figure 7), a prevalence of proteins associated with catalytic activity was identified, accounting for a total of 65 distinct proteins. This group was followed by proteins involved in the small molecule binding, with 31 proteins, and in nucleotide binding, with 29 proteins. Additional notable categories included oxidoreductase activity, acting on NAD(P)H, comprising nine proteins, and proton transmembrane transporter activity, comprising eight proteins.

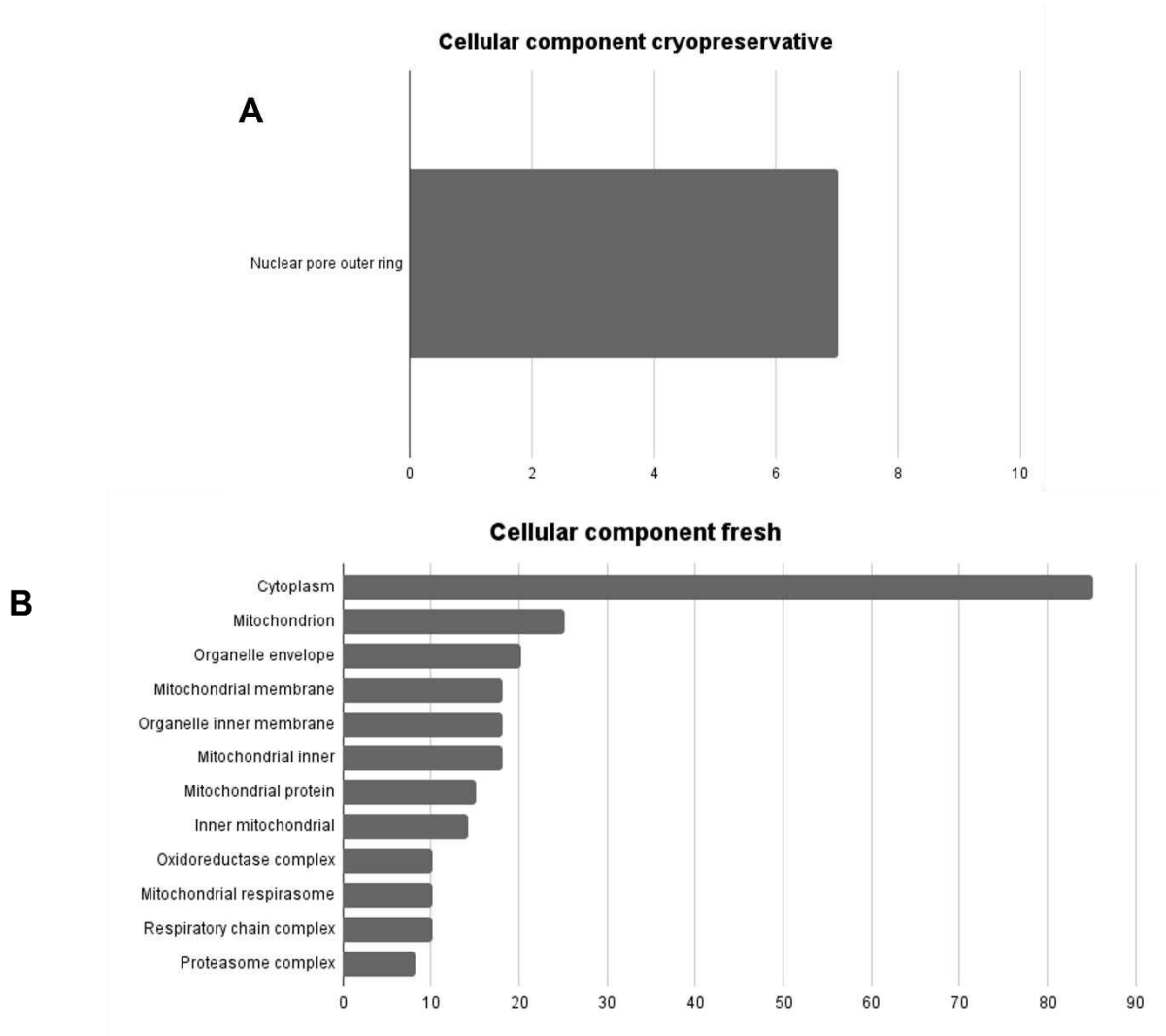

Figure 7. Classification of proteins involved in the cellular components of (A) cryopreservative spermatozoa (B) fresh spermatozoa by STRING DB. Source: Costa, 2023. Authorial.

In the comparative results of molecular functions present in each treatment, no statistically significant results were observed for cryopreserved spermatozoa. However, in the fresh spermatozoa samples (Figure 8), a prevalence of proteins associated with catalytic activity was identified, accounting for a total of 65 distinct proteins. This group was followed by proteins involved in the small molecule binding, with 31 proteins, and in nucleotide binding, with 29 proteins. Additional notable categories included oxidoreductase activity, actying on NAD(P)H, comprising 9 proteins, and proton transmembrane transporter activity, comprising 8 proteins.

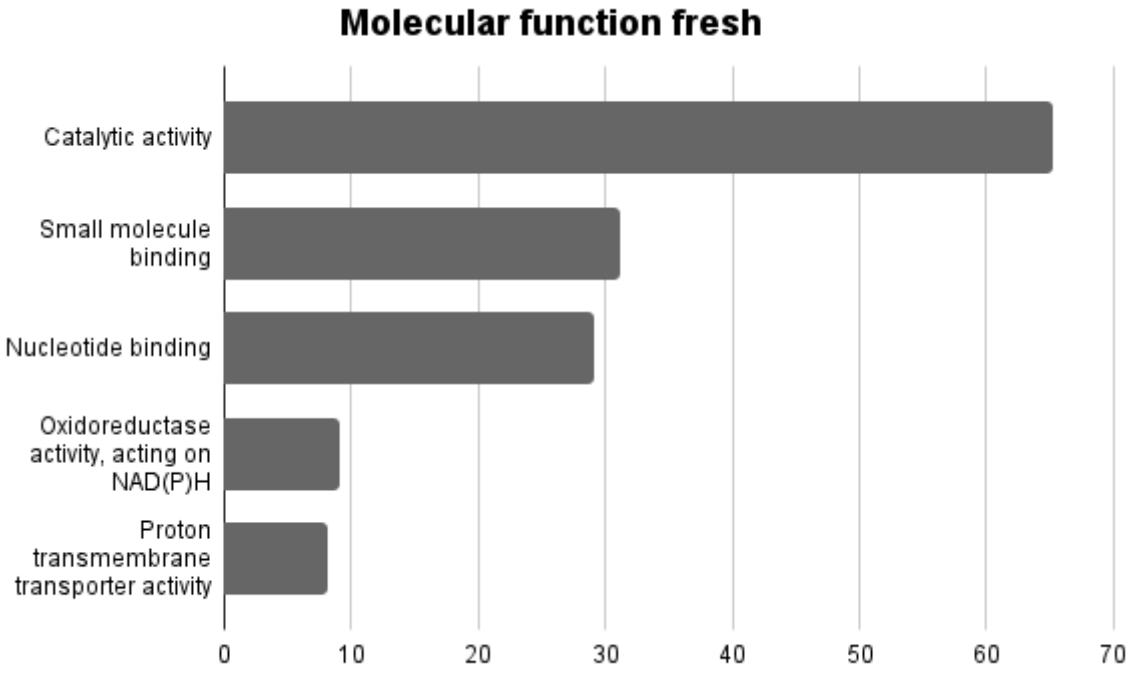

Figure 8. Classification of proteins involved in the cellular components of *fresh* spermatozoa. Source: Costa, 2023. Authorial.

3.4. KEGG Pathways analysis of spermatozoa

A comparative analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, via STRING DB, was conducted for sperm subjected to different treatment approaches (Figure 9).

In the cryopreserved spermatozoa (Figure 9A), there was a predominance of proteins associated with the Amyotrophic Lateral Sclerosis (ALS) pathway, totaling nine proteins. Subsequently, the RNA transport and complement and coagulation cascade pathways emerged, each accounting for seven proteins.

In contrast, for the *in natura* spermatozoa (Figure 9B), the metabolic pathways were the most represented, containing 41 proteins. The prion disease pathway was the second most prevalent, with 22 proteins. The pathways associated with Alzheimer's disease, ALS, Huntington's disease, and Parkinson's disease were equally represented, each with 21 proteins. Other notable pathways included oxidative phosphorylation with 16 proteins, thermogenesis with 15 proteins, non-alcoholic fatty liver disease with 10 proteins, and the proteasome pathway with eight proteins.

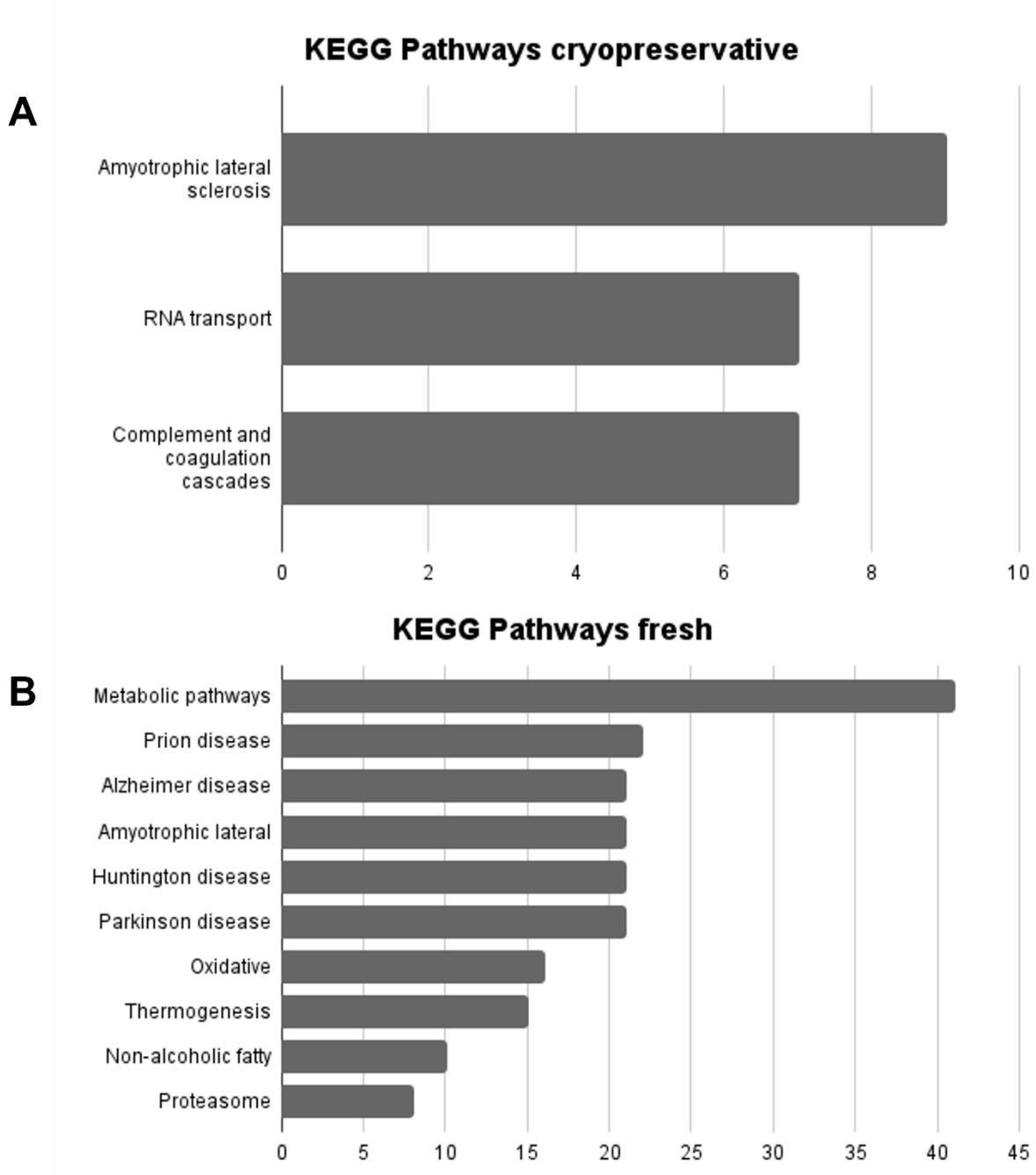

Figure 9. KEEG pathways analysis, by STRING DB, of proteins of (A) cryopreserved (B) fresh spermatozoa. Source: Costa, 2023. Authorial.

3.5. Differentially abundant proteins of spermatozoa

Among the proteins in common between the different sample treatments, 80 of them are differentially abundant when comparing fresh and cryopreservative conditions from a p-value of 0.05 (Figures 10 and 11).

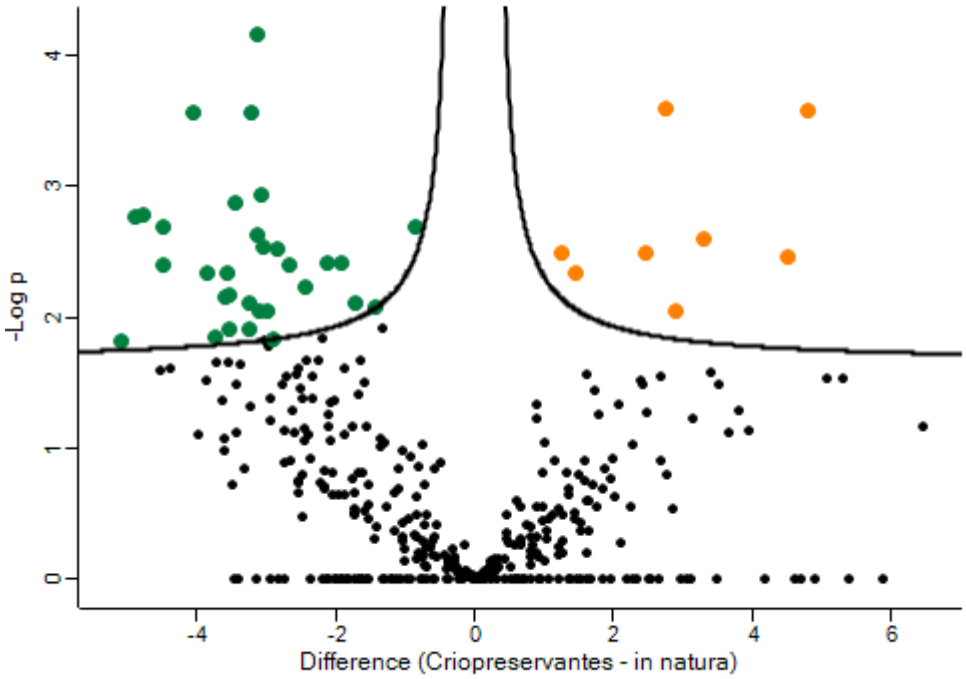

Figure 10. Volcano plot with the 80 differentially abundant proteins when comparing the two conditions fresh (green) and in cryopreservation(orange). Proteins with negative values, on the left of the graph, are up regulated for the fresh condition. Proteins with positive values, on the right of the graph, are up regulated for cryopreservative status, by Perseus software. Source: Costa, 2023. Authorial.

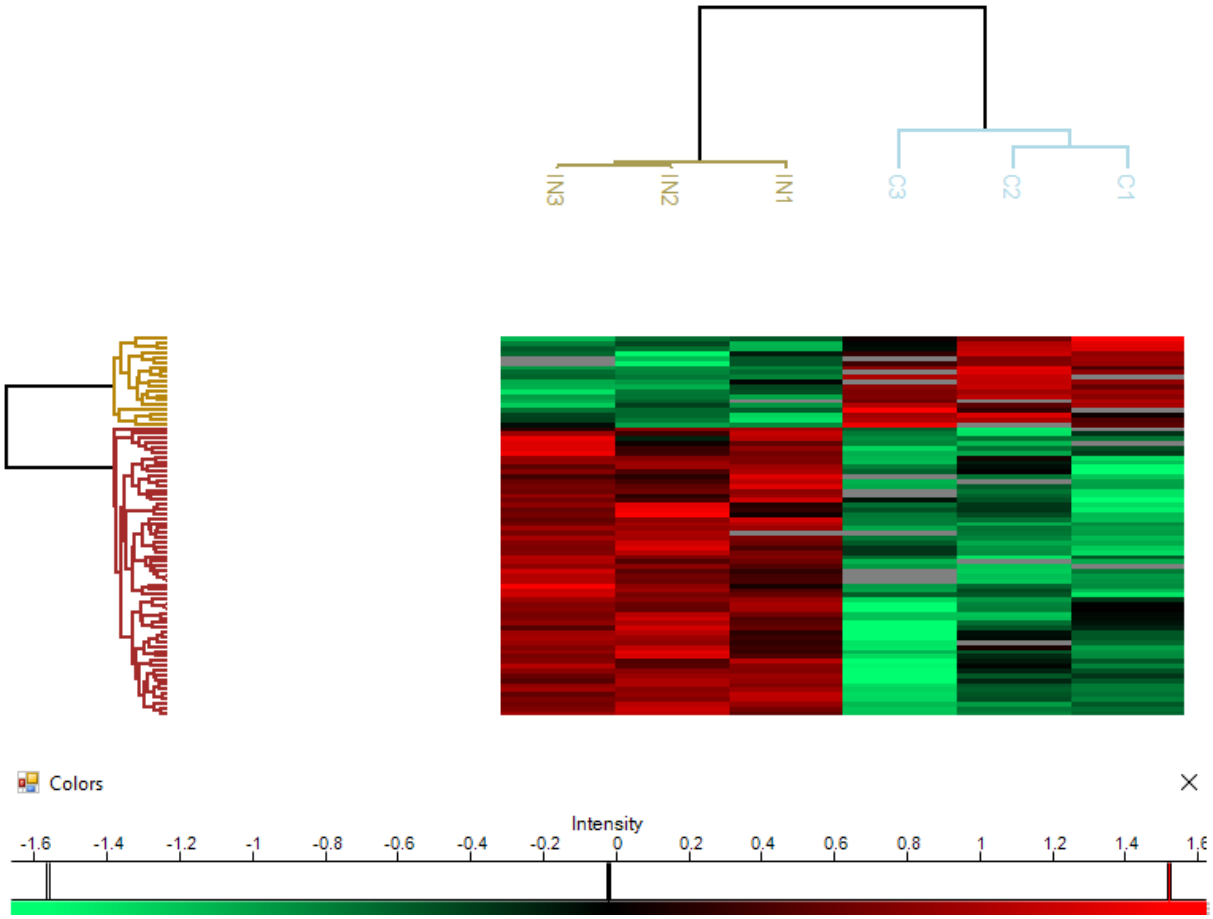

Figure 11. HeatMap showing the 80 differentially abundant proteins when comparing the two conditions *in natura* (IN1 to IN3) and in cryopreservation (C1 to C3). Proteins with negative values are upregulated for the fresh condition. Proteins with positive values are upregulated for cryopreservative status. Source: Costa, 2023. Authorial.

According to the results obtained, 19 proteins were more abundant in cryopreservative samples (Table 1) and 61 proteins were more abundant in fresh samples (Table 2).

Table 1. Differentially abundant proteins in cryopreservative samples found in the HeatMap and Volcano plot. Source: Costa, 2023. Authorial.

ID Protein	Description	Theoretical MW(KDa)	Theoretical pI	Log2FC
A0A8C4PKL7	Ubiquitin carboxyl-terminal hydrolase OS=Equus asinus asinus OX=83772 GN=UCHL1 PE=3 SV=1	24.3	5.10	3,51961
A0A8C4MVN2	ATP-dependent 6-phosphofructokinase OS=Equus asinus asinus OX=83772 GN=PFKP PE=3 SV=1	86.5	6.76	4,78401
A0A8C4MI88	Peptidyl-prolyl cis-trans isomerase OS=Equus asinus asinus OX=83772 PE=3 SV=1	17.9	8.27	1,62546
A0A8C4N110	Cilia and flagella associated protein 52 OS=Equus asinus asinus OX=83772 GN=CFAP52 PE=4 SV=1	68.8	8.37	2,88353
A0A8C4LE68	Sperm associated antigen 6 OS=Equus asinus asinus OX=83772 GN=SPAG6 PE=4 SV=1	55.5	6.37	2,74545
A0A8C4LL60	Argininosuccinate lyase OS=Equus asinus asinus OX=83772 GN=ASL PE=3 SV=1	51.9	5.99	2,07816
A0A8C4M5M5	Beta-actin OS=Equus asinus asinus OX=83772 PE=3 SV=1	48.6	5.37	3,2799
A0A8C4KSJ7	Protein phosphatase 4 regulatory subunit 1 OS=Equus asinus asinus OX=83772 GN=PPP4R1 PE=4 SV=1	104.6	4.68	1,44261
A0A8C4PN73	Tubulin beta chain OS=Equus asinus asinus OX=83772 GN=TUBB2B PE=3 SV=1	49.9	4.78	2,4198
A0A8C4LZI0	Uncharacterized protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	83.1	8.68	2,44344
A0A8C4LSM6	Cytochrome c1 OS=Equus asinus asinus OX=83772 GN=CYC1 PE=3 SV=1	35.3	9.14	0,88367 8
A0A8C4KXW4	Serpin family B member 8 OS=Equus asinus asinus OX=83772 GN=SERPINB8 PE=3 SV=1	42.3	6.43	5,30557
A0A8C4PM27	Cullin 5 OS=Equus asinus asinus OX=83772 GN=CUL5 PE=3 SV=1	83.3	8.92	3,39271
A0A8C4L0I4	ATPase Na+/K+ transporting subunit alpha 2 OS=Equus asinus asinus OX=83772	100.6	5.30	1,72699

	GN=ATP1A2 PE=3 SV=1			
	Enoyl-CoA hydratase, short chain 1 OS=Equus asinus asinus OX=83772 GN=ECHS1 PE=3			
A0A8C4M9D2	SV=1	31.8	9.10	1,23888
	Peptidylglycine alpha-amidating monooxygenase OS=Equus asinus asinus OX=83772			
A0A8C4M879	GN=PAM PE=3 SV=1	109.7	6.22	2,38787
	Tetratricopeptide repeat domain 28 OS=Equus asinus asinus OX=83772 GN=TTC28 PE=4			
A0A8C4PLB6	SV=1	261.7	6.63	4,49541
	1-phosphatidylinositol-3-phosphate 5-kinase OS=Equus asinus asinus OX=83772			
A0A8C4KYG5	GN=PIKFYVE PE=4 SV=1	232.7	6.25	5,08367
A0A8C4MAU5	Protein-arginine deiminase OS=Equus asinus asinus OX=83772 GN=PADI2 PE=3 SV=1	75.7	5.50	2,67301

Table 2. Differentially abundant proteins of fresh samples found in the HeatMap and Volcano Plot. Source: Costa, 2023. Authorial.

ID Protein	Description	Theoretical MW(KDa)	Theoretical pI	Log2FC
A0A8C4LBR3	Ig-like domain-containing protein OS=Equus asinus asinus OX=83772 PE=3 SV=1	39.1	7.05	-4,51547
A0A8C4MCF3	Peroxiredoxin-6 OS=Equus asinus asinus OX=83772 PE=3 SV=1	25.0	5.73	-1,43219
A0A8C4MZA0	Tubulin alpha chain OS=Equus asinus asinus OX=83772 PE=3 SV=1	50.2	4.98	-2,84909
A0A8C4LEM8	Ropporin-1A OS=Equus asinus asinus OX=83772 PE=4 SV=1	23.9	5.06	-3,72555
A0A8C4LUP5	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon OS=Equus asinus asinus OX=83772 GN=YWHAE PE=3 SV=1	28.2	4.79	-3,25211
A0A8C4MU73	T-complex protein 1 subunit gamma OS=Equus asinus asinus OX=83772 GN=CCT3 PE=3 SV=1	60.6	6.42	-2,19709
A0A8C4MAF9	Clusterin OS=Equus asinus asinus OX=83772 GN=CLU PE=3 SV=1	52.1	5.68	-1,67612
A0A8C4PSR3	T-complex protein 1 subunit epsilon OS=Equus asinus asinus OX=83772 GN=CCT5 PE=3 SV=1	59.7	5.57	-2,08376
A0A8C4LMI8	Lipid-binding serum glycoprotein N-terminal domain-containing protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	31.2	8.95	-2,33274
A0A8C4LWV9	Tektin OS=Equus asinus asinus OX=83772 GN=TEKT2 PE=3 SV=1	49.9	5.98	-2,67299
A0A8C4PIQ8	Saccharopine dehydrogenase (putative) OS=Equus asinus asinus OX=83772 GN=SCCPDH PE=4 SV=1	47.7	9.04	-2,14774
A0A8C4MST5	phosphopyruvate hydratase OS=Equus asinus asinus OX=83772 PE=3 SV=1	47.1	6.37	-2,43607
A0A8C4KST7	Glyceraldehyde-3-phosphate dehydrogenase OS=Equus asinus asinus OX=83772 GN=GAPDHS PE=3 SV=1	45.0	8.98	-3,56816
A0A8C4M311	Pyruvate dehydrogenase E1 component subunit alpha OS=Equus asinus asinus OX=83772	43.3	8.36	-3,00992

	GN=PDHA1 PE=4 SV=1			
A0A8C4PI73	ATP synthase subunit b OS=Equus asinus asinus OX=83772 GN=ATP5PB PE=3 SV=1	28.7	9.33	-2,56613
A0A8C4PR02	hexokinase OS=Equus asinus asinus OX=83772 GN=HK1 PE=3 SV=1	102.3	6.36	-3,55929
A0A8C4L5I2	FUN14 domain containing 2 OS=Equus asinus asinus OX=83772 GN=FUNDC2 PE=3 SV=1	20.5	9.55	-1,31465
A0A8C4M974	Tektin OS=Equus asinus asinus OX=83772 GN=TEKT1 PE=3 SV=1	48.2	5.82	-2,48834
A0A8C4M128	L-lactate dehydrogenase OS=Equus asinus asinus OX=83772 PE=3 SV=1	25.9	5.96	-1,74449
A0A8C4MAY9	RAB2A, member RAS oncogene family OS=Equus asinus asinus OX=83772 GN=RAB2A PE=4 SV=1	23.5	6.08	-4,51428
A0A8C4M3Y6	Phospholipase B-like OS=Equus asinus asinus OX=83772 GN=PLBD1 PE=3 SV=1	63.8	9.11	-1,63757
A0A8C4PUM1	IZUMO family member 4 OS=Equus asinus asinus OX=83772 GN=IZUMO4 PE=3 SV=1	28.1	6.69	-5,11001
A0A8C4KZK5	Radial spoke head component 9 OS=Equus asinus asinus OX=83772 GN=RSPH9 PE=4 SV=1	31.2	5.47	-2,93104
A0A8C4L4Y1	Dynein light chain OS=Equus asinus asinus OX=83772 GN=DYNLL1 PE=3 SV=1	10.3	6.89	-4,53458
A0A8C4M9J2	Triosephosphate isomerase OS=Equus asinus asinus OX=83772 GN=TPI1 PE=3 SV=1	30.5	6.25	-4,39392
A0A8C4M1Z8	Cytochrome c oxidase subunit 4 OS=Equus asinus asinus OX=83772 GN=COX4I1 PE=3 SV=1	18.5	8.93	-3,24119
A0A8C4LLX9	Cullin associated and neddylation dissociated 1 OS=Equus asinus asinus OX=83772 GN=CAND1 PE=3 SV=1	136.3	5.52	-2,97924
A0A8C4L9N6	Jacalin-type lectin domain-containing protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	25.9	9.21	-3,54143
A0A8C4MIX0	Acrosin-binding protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	29.5	8.99	-3,45036
A0A8C4PRJ4	Malate dehydrogenase OS=Equus asinus asinus OX=83772 GN=MDH2 PE=3 SV=1	35.5	8.82	-4,80596
A0A8C4MJ90	Phosphatidylethanolamine binding protein 4 OS=Equus asinus asinus OX=83772 GN=PEBP4 PE=3 SV=1	34.5	8.97	-1,58296
A0A8C4MBA0	Fructose-bisphosphate aldolase OS=Equus asinus asinus OX=83772 GN=ALDOA PE=3 SV=1	41.1	8.49	-0,869376
A0A8C4LFZ6	Sperm equatorial segment protein 1 OS=Equus asinus asinus OX=83772 GN=SPESP1 PE=3 SV=1	40.8	5.31	-2,7208
A0A650G432	Cytochrome c oxidase subunit 2 OS=Equus asinus OX=9793 GN=COX2 PE=3 SV=1	26.0	4.63	-3,46534
A0A8C4PM93	Aspartate aminotransferase OS=Equus asinus asinus OX=83772 GN=GOT2 PE=3 SV=1	47.3	9.11	-3,14357
A0A8C4LSL2	LRRC37A/B like protein 1 C-terminal domain-containing protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	14.2	5.50	-3,15147

A0A8C4LE77	Pyruvate kinase OS=Equus asinus asinus OX=83772 GN=PKM PE=3 SV=1	57.9	8.53	-3,75451
A0A8C4MFF0	Zona pellucida binding protein OS=Equus asinus asinus OX=83772 GN=ZPBP PE=3 SV=1	51.5	9.74	-1,87648
A0A8C4KS57	Glutathione hydrolase OS=Equus asinus asinus OX=83772 PE=3 SV=1	60.8	7.24	-2,25652
A0A8C4LIH7	Interleukin 4 induced 1 OS=Equus asinus asinus OX=83772 GN=IL4I1 PE=4 SV=1	64.5	8.48	-3,88221
A0A8C4PMJ4	NPC intracellular cholesterol transporter 2 OS=Equus asinus asinus OX=83772 GN=NPC2 PE=3 SV=1	16.1	8.20	-2,9528
A0A8C4MN76	Synaptophysin like 1 OS=Equus asinus asinus OX=83772 GN=SYPL1 PE=3 SV=1	26.8	9.43	-3,6177
A0A650G410	NADH-ubiquinone oxidoreductase chain 4 OS=Equus asinus OX=9793 GN=ND4 PE=3 SV=1	51.7	9.62	-3,2461
A0A8C4MAQ9	Outer dense fiber of sperm tails 2 OS=Equus asinus asinus OX=83772 GN=ODF2 PE=3 SV=1	73.6	7.53	-2,50477
A0A8C4MMK8	Actin related protein T3 OS=Equus asinus asinus OX=83772 GN=ACTRT3 PE=3 SV=1	41.1	5.76	-3,12794
A0A8C4MFF3	alpha-mannosidase OS=Equus asinus asinus OX=83772 GN=MAN2C1 PE=3 SV=1	111.3	5.91	-3,86082
A0A8C4MCL1	Solute carrier family 2, facilitated glucose transporter member 3 OS=Equus asinus asinus OX=83772 PE=3 SV=1	55.3	5.55	-3,0532
A0A8C4MHA1	ADP/ATP translocase OS=Equus asinus asinus OX=83772 GN=SLC25A31 PE=3 SV=1	35.8	9.65	-3,02402
A0A8C4KU09	Pyruvate dehydrogenase E1 component subunit beta OS=Equus asinus asinus OX=83772 GN=PDHB PE=4 SV=1	39.1	6.21	-3,56263
A0A8C4L9K2	Sperm acrosome associated 1 OS=Equus asinus asinus OX=83772 GN=SPACA1 PE=4 SV=1	26.5	4.92	-4,90177
A0A8C4PNG7	Lactate/malate dehydrogenase C-terminal domain-containing protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	6.2	4.81	-3,09347
A0A8C4KZH9	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial OS=Equus asinus asinus OX=83772 GN=NDUFV2 PE=3 SV=1	25.5	8.82	-2,76679
A0A8C4LHN4	Lysozyme like 4 OS=Equus asinus asinus OX=83772 GN=LYZL4 PE=3 SV=1	16.3	8.94	-2,52883
A0A8C4L0V3	Ig-like domain-containing protein OS=Equus asinus asinus OX=83772 PE=4 SV=1	15.4	6.17	-3,38622
A0A8C4LXH9	4-aminobutyrate aminotransferase, mitochondrial OS=Equus asinus asinus OX=83772 GN=ABAT PE=3 SV=1	68.3	8.74	-4,05982
A0A8C4PRP5	A-kinase anchoring protein 3 OS=Equus asinus asinus OX=83772 GN=AKAP3 PE=3 SV=1	94.1	5.83	-3,26674
A0A8C4PM07	Sperm acrosome associated 3 OS=Equus asinus asinus OX=83772 GN=SPACA3 PE=3 SV=1	18.3	6.27	-3,63083

A0A8C4LPP1	Ubiquinol-cytochrome c reductase core protein 1 OS=Equus asinus asinus OX=83772 GN=UQCRC1 PE=4 SV=1	57.4	6.22	-2,33571
A0A8C4PFC6	Ubiquitin-40S ribosomal protein S27a OS=Equus asinus asinus OX=83772 GN=RPS27A PE=3 SV=1	17.9	9.68	-2,02079
A0A8C4PN40	Proteasome subunit beta OS=Equus asinus asinus OX=83772 GN=PSMB2 PE=3 SV=1	22.8	6.59	-2,46649
A0A8C4LC29	Citrate synthase OS=Equus asinus asinus OX=83772 GN=CS PE=3 SV=1	44.7	7.70	-1,93811

3.6. Protein interaction networks of spermatozoa

Specific clusters of proteins were identified in the spermatozoa of each different treatment modalities, based on the resulting protein interaction networks. In the case of cryopreserved spermatozoa, five local clusters emerged (Figure 12). Among these, one cluster contained proteins implicated in the complement and coagulation cascades, as well as in the complement activation of lectin pathway (Figure 12A). Another was focused on complement and coagulation cascades (Figure 12A), a third was a mixed cluster involving processes of hemostasis and regulation of protein activation cascade (Figure 12B), and the remaining two were associated with the nuclear pore outer ring (Figure 12C and 12D).

Conversely, for the fresh spermatozoa, seven local clusters were identified (Figure 13). These included: one cluster with proteins associated with the proteasome regulatory particle and the proteasome core complex of the alpha-subunit complex; a second focused on the proteasome accessory complex and the proteasomal ubiquitin-independent protein catabolic process; a third involving the NADH dehydrogenase complex and Complex III of the respiratory chain; a fourth associated with the proteasome complex; a fifth related to the respirasome; a sixth focused on the respirasome and the assembly of the NADH dehydrogenase complex; and a seventh dedicated to oxidative phosphorylation and the assembly of the mitochondrial respiratory chain complex.

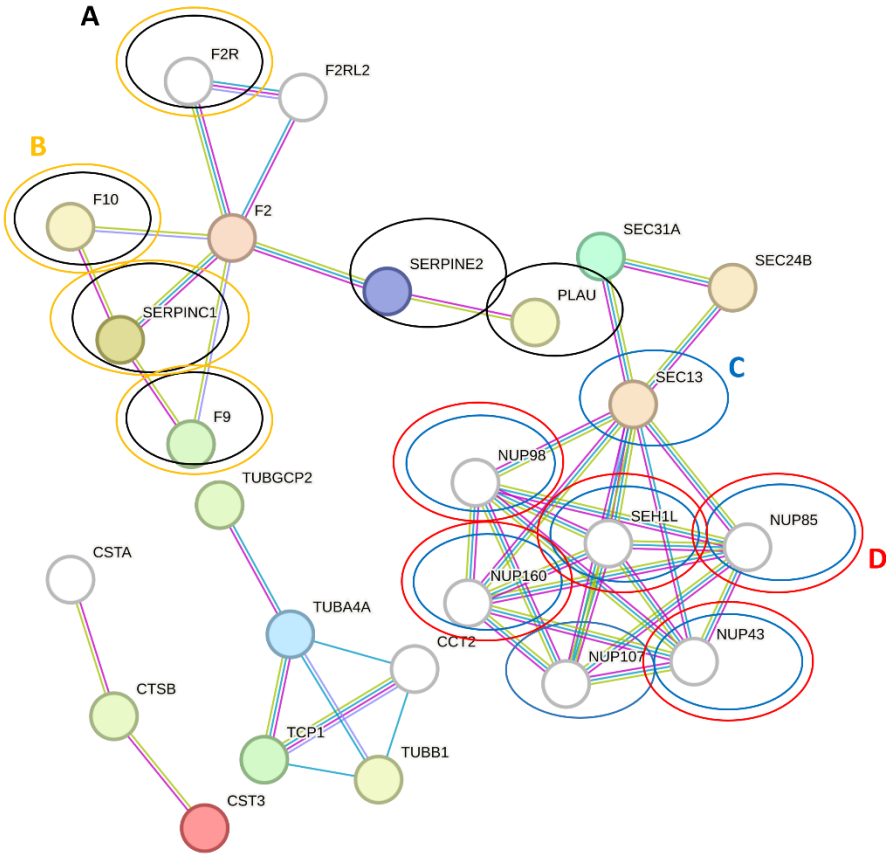

Figure 12. Network of spermatozoa proteins interactions in cryopreservative, with evidence in the networks of local clusters found, in which those circled in black (A) contained proteins implicated in the complement and coagulation cascades, the complement activation of lectin pathway, those circled in yellow (B) is a mixed cluster involving processes of hemostasis and regulation of protein activation cascade and those circled in blue (C) and red (D) are related with the nuclear pore outer ring. Source: Costa, 2023. Authorial.

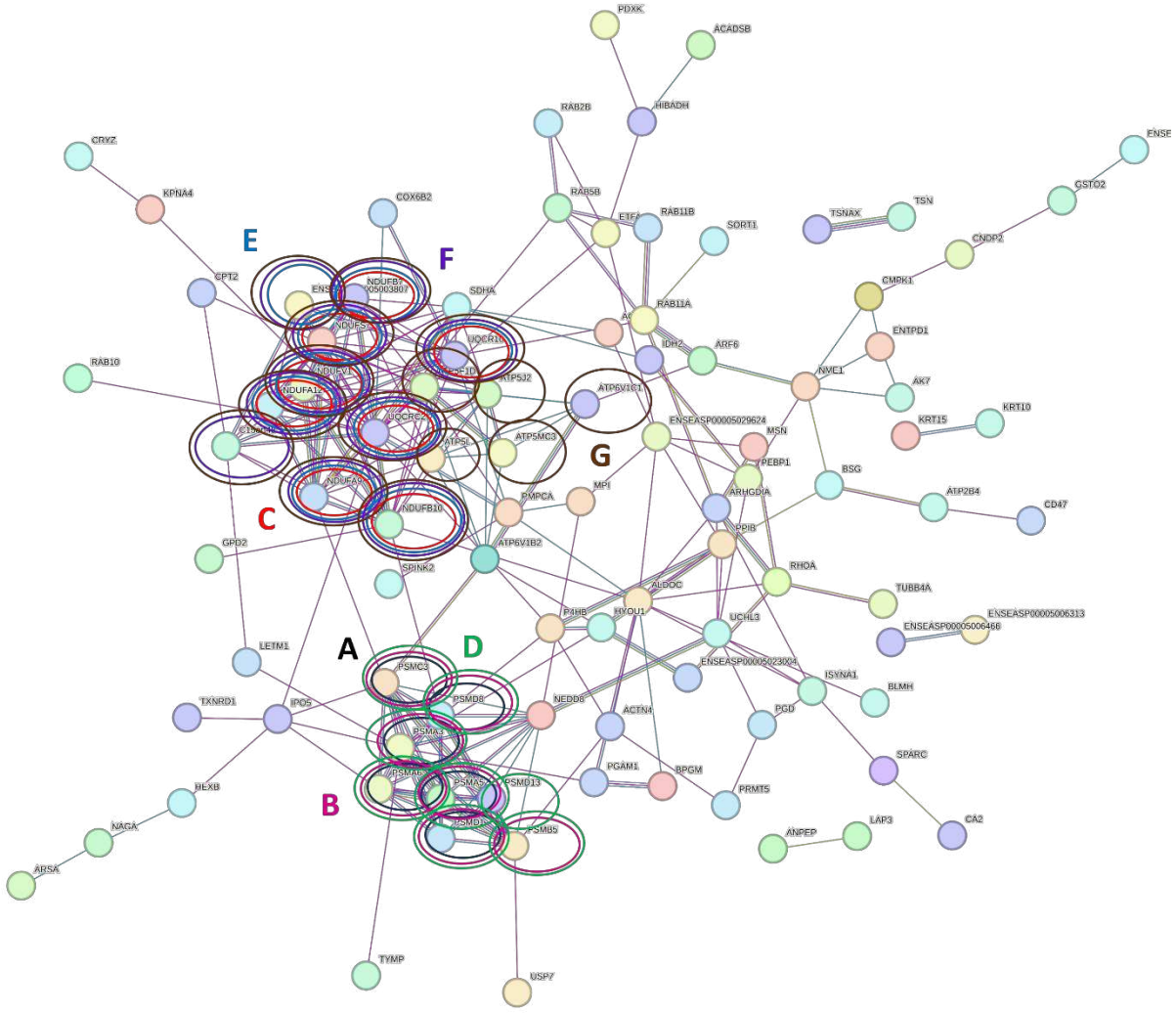

Figure 13. Network of fresh spermatozoa proteins interactions , with evidence in the networks of local clusters found, in which those circled in black (A) contained proteins implicated in the proteasome regulatory particle and the proteasome core complex of the alpha-subunit complex, those circled in pink (B) are focused on the proteasome accessory complex and the proteasomal ubiquitin-independent protein catabolic process, those circled in red (C) are involving the NADH dehydrogenase complex and Complex III of the respiratory chain, those circled in green (D) are associated with the proteasome complex, those circled in blue (E) are related to the respirasome, those circled in purple (F) are focused on the respirasome and the assembly of the NADH dehydrogenase complex and those circled in brown (G) are dedicated to oxidative phosphorylation and the assembly of the mitochondrial respiratory chain complex. Source: Costa, 2023. Authorial

Table 3. Proteins involved in each cluster of spermatozoa in cryopreservative (Figure 12). Source: Costa, 2023. Authorial.

Local network cluster	Proteins
Complement and coagulation cascades, and complement activation, lectin pathway	F2R - Coagulation factor II thrombin receptor F10 - Coagulation factor X SERPINC1 -Serpin family C member 1 SERPINE2 - Serpin family E member 2 F9 - Coagulation factor IX PLAU - Plasminogen activator, urokinase
Complement and coagulation cascades	F2R - Coagulation factor II thrombin receptor F10 - Coagulation factor X SERPINC1 -Serpin family C member 1 SERPINE2 - Serpin family E member 2 F9 - Coagulation factor IX PLAU - Plasminogen activator, urokinase
Mixed, incl. Hemostasis, and regulation of protein activation cascade	F2R - Coagulation factor II thrombin receptor F10 - Coagulation factor X SERPINC1 -Serpin family C member 1 F9 - Coagulation factor IX
Nuclear pore outer ring	SEC13 - SEC13 homolog, nuclear pore and COPII coat complex component NUP 98 - Nucleoporin 98 SEH1L - SEH1 like nucleoporin NUP85 - Nucleoporin 85 NUP160 - Nucleoporin 160

	NUP107 - Nucleoporin 107
	NUP43 - Nucleoporin 43
Nuclear pore outer ring	NUP 98 - Nucleoporin 98
	SEH1L - SEH1 like nucleoporin
	NUP85 - Nucleoporin 85
	NUP160 - Nucleoporin 160
	NUP43 - Nucleoporin 43

Table 4. Proteins involved in each cluster of spermatozoa fresh (Figure 13). Source: Costa, 2023. Authorial.

Local network cluster	Proteins
Proteasome regulatory particle, and proteasome core complex, alpha-submplexunit complex	PSMC3 - Proteasome 26S subunit, ATPase 3 PSMA3 - Proteasome 20S subunit alpha 3 PSMA6 - Proteasome 20S subunit alpha 6 PSMA5 - Proteasome 20S subunit alpha 5 PSMD8 - Proteasome 26S subunit, non-ATPase 8 PSMD1 - Proteasome 26S subunit, non-ATPase 1
Proteasome accessory complex, and proteasomal ubiquitin-independent protein catabolic process	PSMC3 - Proteasome 26S subunit, ATPase 3 PSMA3 - Proteasome 20S subunit alpha 3 PSMA6 - Proteasome 20S subunit alpha 6 PSMA5 - Proteasome 20S subunit alpha 5 PSMD8 - Proteasome 26S subunit, non-ATPase 8 PSMD1 - Proteasome 26S subunit, non-ATPase 1 PSMB5 - Proteasome 20S subunit beta 5
NADH dehydrogenase complex, and respiratory chain complex III	NDUFB7 - NADH:ubiquinone oxidoreductase subunit B7 NDUFS7 - NADH:ubiquinone oxidoreductase core subunit S7 NDUFV1 - NADH:ubiquinone oxidoreductase core subunit V1 NDUFA12 - NADH:ubiquinone oxidoreductase subunit A12 UQCR10 - Ubiquinol-cytochrome c reductase, complex III UQCRC2 - Ubiquinol-cytochrome c reductase core protein 2 NDUFA9 - NADH:ubiquinone oxidoreductase subunit A9 NDUFB10 - NADH:ubiquinone oxidoreductase subunit B10
Proteasome complex	PSMC3 - Proteasome 26S subunit, ATPase 3 PSMA3 - Proteasome 20S subunit alpha 3 PSMA6 - Proteasome 20S subunit alpha 6

	PSMA5 - Proteasome 20S subunit alpha 5
	PSMD8 - Proteasome 26S subunit, non-ATPase 8
	PSMD1 - Proteasome 26S subunit, non-ATPase 1
	PSMB5 - Proteasome 20S subunit beta 5
	PSMD13 - Proteasome 26S subunit, non-ATPase 13
Respirasome	NDUFB7 - NADH:ubiquinone oxidoreductase subunit B7
	NDUFS7 - NADH:ubiquinone oxidoreductase core subunit S7
	NDUFV1 - NADH:ubiquinone oxidoreductase core subunit V1
	NDUFA12 - NADH:ubiquinone oxidoreductase subunit A12
	UQCR10 - Ubiquinol-cytochrome c reductase, complex III
	UQCRC2 - Ubiquinol-cytochrome c reductase core protein 2
	NDUFA9 - NADH:ubiquinone oxidoreductase subunit A9
	NDUFB10 - NADH:ubiquinone oxidoreductase subunit B10
	ENSEASP00005003807 - Cytochrome c oxidase subunit 6C
Respirasome, and NADH dehydrogenase complex assembly	NDUFB7 - NADH:ubiquinone oxidoreductase subunit B7
	NDUFS7 - NADH:ubiquinone oxidoreductase core subunit S7
	NDUFV1 - NADH:ubiquinone oxidoreductase core subunit V1
	NDUFA12 - NADH:ubiquinone oxidoreductase subunit A12
	UQCR10 - Ubiquinol-cytochrome c reductase, complex III
	UQCRC2 - Ubiquinol-cytochrome c reductase core protein 2
	NDUFA9 - NADH:ubiquinone oxidoreductase subunit A9
	NDUFB10 - NADH:ubiquinone oxidoreductase subunit B10
	ENSEASP00005003807 - Cytochrome c oxidase subunit 6C
	C15orf48 - Chromosome 15 open reading frame 48
Oxidative phosphorylation, and mitochondrial respiratory chain complex assembly	NDUFB7 - NADH:ubiquinone oxidoreductase subunit B7
	NDUFS7 - NADH:ubiquinone oxidoreductase core subunit S7
	NDUFV1 - NADH:ubiquinone oxidoreductase core subunit V1
	NDUFA12 - NADH:ubiquinone oxidoreductase subunit A12
	UQCR10 - Ubiquinol-cytochrome c reductase, complex III

UQCRC2 - Ubiquinol-cytochrome c reductase core protein 2

NDUFA9 - NADH:ubiquinone oxidoreductase subunit A9

NDUFB10 - NADH:ubiquinone oxidoreductase subunit B10

ENSEASP00005003807 - Cytochrome c oxidase subunit 6C

C15orf48 - Chromosome 15 open reading frame 48

ATP5F1D - ATP synthase F1 subunit delta

ATP5J2 - ATP synthase, H+ transporting, mitochondrial Fo complex subunit F2

ATP5L - ATP synthase, H+ transporting, mitochondrial Fo complex subunit G

ATP5MC3 - ATP synthase membrane subunit c locus 3

ATP6V1C1 - ATPase H+ transporting V1 subunit C1

4. DISCUSSION

Profiling the proteomes of specifical proteins in each treatment, through the use of LC-MS/MS, enabled the comparison of the main biological functions in each treatment.

In cryopreserved sperm, it is observed that the main biological processes are involved with coagulation regulation and with RNA localization and transport. Regarding coagulation regulation processes, SERPINE2 (Serpin Family E Member 2), SERPINC1 and PLAU (Plasminogen activator, urokinase) proteins stand out as potential infertility indicators.

As reported in the literature, mouse sperm supplemented with purified SERPINE2 showed suppression of sperm capacitation and blocking of sperm-oocyte interactions, as well as inhibiting cholesterol efflux from sperm by interfering with signal transduction related to capacitation. The authors also reported thatSERPINE2 was found on the surface of the sperm, bound to the plasma membrane of the acrosome. However, it was detected in greater abundance in uncapacitated sperm, suggesting that SERPINE2 is lost after capacitation (Lu et al., 2011).

In the case of PLAU, in sperm function tests of mice subcutaneously immunized with this protein, a decrease in motility, concentration, and sperm viability was observed, as well as the in vitro fertilization rate for epididymal tail sperm compared to controls (Ying *et al.*, 2015).

In processes involving mRNA transport and RNA localization, the presence of nucleoporins was detected, proteins that make part of the nuclear pores. In young, non-stressed animals, nucleoporins are part of sperm development, hence their higher level of expression (Thomas *et al.*, 2023). These proteins also are in accordance with the cellular component found.

In fresh sperm, it is observed that the main biological processes are intrinsically linked to energy production and cellular respiration. Proteins actively

participating in the electron transport chain stand out in this context. This finding is consistent with expectations, as an increase in energy production is crucial for enhancing sperm motility during the fertilization process. These processes also are related to their cellular componentes occorence, mithocondria and cytoplasm.

In the analysis of KEGG metabolic pathways for cryopreserved sperm, we observe that the second and third most representative pathways involve proteins related to RNA transport and coagulation and complement cascades. But, the pathway that stands out with the highest number of proteins is associated with Amyotrophic Lateral Sclerosis (ALS). As reviewed in the literature, nucleoporins are generally present in the sperm of young and unstressed animals. However, in older animals or those exposed to stressful conditions, certain nucleoporins, such as Nup98, which are positioned in cytoplasmic foci, have the potential to condense and form small protein aggregates. These condensed protein aggregates may serve as triggers in pathways related to certain neurodegenerative diseases, such as ALS (Chandra; Lusk, 2022; Thomas *et al.*, 2023).

In the KEGG metabolic pathway analyses for fresh sperm cells, the most representative pathways are related to metabolism, which aligns with the findings on energy production observed in the main biological processes of the samples analyzed. There are also reports in the literature that the inhibition of glycolysis and oxidative phosphorylation can lead to the non-occurrence of sperm capacitation and an increase in the spontaneous acrosome reaction (Dahan; Breitbart, 2022). Also, pathways associated with neurodegenerative diseases and the ubiquitin-proteasome system are also prominent.

According to literature, the ubiquitin-proteasome pathway plays a crucial role in maintaining protein homeostasis. Factors such as aging can lead to alterations in the expression and activity of 26S proteasome subunit, resulting in the accumulation of these proteins. This phenomenon of protein accumulation is directly related to the development of various neurodegenerative diseases, including ALS, Prion, Huntington's, Alzheimer's, and Parkinson's (Fernández-Cruz; Reynaud, 2022).

Regarding the differentially abundant proteins found only in cryopreserved spermatozoa, most are related to metabolic pathways, such as ATP-dependent 6-phosphofructokinase (PFKP), a key enzyme in the glycolytic pathway, which is crucial for energy generation. We also found some that are involved in the structure of the spermatozoon, like the Cilia and flagella associated protein 52 (CFAP52). Lastly, we also identified proteins relationed to the acrosomal reaction, such as the Serpin.

In the differentially abundant proteins of fresh spermatozoa, there are also many proteins involved in metabolism and energy production, primarily located in the mitochondria, such as pyruvate dehydrogenase, and in glycolysis, like pyruvate kinase. These are crucial for providing energy to the cell. Additionally, there are proteins related to the structure of the sperm, acrosomal reaction, substance transport, and antioxidant proteins, which can prevent oxidative stress in the cells.

It can be observed that the protein interaction networks in cryopreserved sperm are organized into five distinct clusters. These clusters predominantly consist of proteins involved in the coagulation cascade and in the membranes of nuclear pores, factors that may be directly correlated with infertility cases.

In contrast, the interaction networks fresh sperm contain seven clusters. These clusters are primarily composed of proteins focused on metabolism and energy production, crucial elements for maintaining sperm motility.

5. CONCLUSION

The protein profile of sperm, both in cryopreserved and fresh states, shows significant variations in terms of composition and function, which aligns with biological observations and data reported in scientific literature.

In the case of natural sperm, the predominant proteins are those associated with metabolism and energy production. This protein composition is essential for providing the sperm cell with the necessary energy to maintain its viability and fertilization potential.

On the other hand, cryopreserved sperm exhibit a high concentration of proteins related to coagulation processes and transmembrane transport, but also linked to conditions resulting in infertility. Notably, the proteins SERPINE2, SERPINC1, and other serpins PLAU, and NUP98 have potential as infertility biomarkers in Pêga breed donkeys. In vitro and in vivo research focused on these proteins could provide valuable insights into their mechanisms of action, contributing to the development of strategies to mitigate their adverse effects on frozen sperm.

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Chapter 2

Proteomic profile of seminal plasma of Pêga donkeys (*Equus asinus*) with great spermatozoa motility and vigor and its relationship with assisted reproduction

1. INTRODUCTION

Traditionally valued for labor and transportation, donkeys (*Equus asinus*) have been widely used for their physical strength and extreme resistance to heavy loads. Their role has significantly aided trade, agriculture, and various other domains. However, as modernization took over, their primary applications transformed (GASTAL et al., 1996; MCLEAN et al., 2012; OLIVEIRA et al., 2015; CANISSO et al., 2019; WANG et al., 2022).

In recent times, there's been a renewed interest in these animals, spurred by the consumption of their milk and meat in certain regions (CANISSO et al., 2019; WANG et al., 2022). However, in Brazil the Pêga donkeys are mostly created in the southwest region and used to produce quality mules (*Equus mulus*) for work in rural areas and in morphology and gait competitions (IVIS, 2010). This breed of donkeys is characterized, in addition to their docile temperament, by their ambling gait, which has encouraged marching competitions of these animals at annual national exhibitions organized by breeders. Additionally, an elite book can be found containing a record of the best breeding males and females of the breed. This makes Pêga breed donkeys highly sought after in the competition arena, which usually attracts significant investments (ABCJPEGA, 2024).

These situations demand the adoption of reproductive strategies that allow us to accelerate genetic progress. In this context, artificial insemination and semen cryopreservation are the most economical and convenient alternatives. However, success rates for frozen donkey semen are remarkably low, reaching 0-36% with female donkeys (TRIMECHE et al., 1998; OLIVEIRA et al., 2015; VIDAMENT et al., 2009) and 33-53 % with mares. when it is intended to produce hybrid animals (VIEIRA et al., 1985; CANISSO et al., 2011).

Fertility biomarkers can help with possible treatment strategies. It is known that the main proteins described as involved in reproduction are SPs, semenogelins, kallikreins (JODAR et al., 2017), albumins and CRISP3 (NOVAK et al., 2010). Previous studies with Dezhou breed donkeys, a Chinese breed, showed that the antioxidant proteins QS0X1, PRDX1 and GPX5, enzymes GAPDH and LDHB, the heat shock proteins HSPA5 and HSPA1L, and the proteins CRISP3 and NPC2 are more abundant in the seminal plasma of optimal freezability, great motility and great vigor characteristics, which may suggest that those proteins can be positive biomarkers in

those animals. Then, those results can be compared with different breeds of donkeys and have a great potential of being biomarkers for that species (YU et al., 2021).

Given that damages from freezing, as consequence of the formation intracellular ice crystals and osmotic and toxic stresses caused by cryoprotectants (BLACH et al., 1989; WATSON, 2000), can be compounded by seminal plasma's effects on sperm integrity, this study sets out to detail the proteomic patterns of the Pêga donkey breed. Advanced Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) techniques, which has an important role in investigation of seminal proteins (DE MATEO et al., 2013), will be employed to probe the proteins that seem to be important to reproduction, understanding their implications for the assisted reproduction and to investigate proteins with a potential as biomarkers of infertility that are already known in other species and in a different donkey breed.

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2. MATERIAL METHODS

2.1. Ethical Approval

This study was conducted in strict compliance with the guidelines set forth by the Ethics Committee for Animal Use at the Federal University of Viçosa. The project was submitted and approved and is duly registered under protocol number 28/2020. This study aims to continues the study entitled “Use of different extenders and protocols in the cryopreservation of semen from donkeys of the Pêga breed”, from the Group of Animal Reproduction of the Animal Andrology Laboratory of the Department of Veterinary Medicine at UFV, approved by the Ethics Committee for the Use of Animals at UFV (CEUA) with protocol number 73/2018.

2.2. Materials

All materials used were of the highest grade available. Chemicals and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) or from Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. Materials for electrophoresis procedures were purchased from GE Healthcare (Life Sciences, USA).

2.3. Animals, sample collection and semen analysis

A total of 14 ejaculate were collected from three healthy fertile Pêga breed donkeys (*Equus asinus*) with average age of four-year-old (ranging from three to five) over the course of the breeding season belonging to a stud farm located in the city of Viçosa, Minas Gerais, Brazil (-42.8825 20° 45′ 17″ S, 42° 52′ 57″ W, 663 m). Semen collection was carried out at regular intervals of 8 days using the artificial vagina method previously heated with water at 45 °C. The first animal provided four ejaculates, while the second and third animals contributed five ejaculates each.

All donkeys were kept in similar handling and feeding conditions and submitted a Breeding Soundness Evaluation. At the time of semen collection, the volume (mL), ejaculate appearance (1: aqueous, 2: opalescent, 3: milky, 4: creamy), and the concentration of each ejaculate (using a Neubauer chamber) were evaluated. Sperm motility (percentage scale, from 0 to 100%, indicating no cell to all cells with movement, respectively) and sperm vigor (scale of 0 to 5, representing no movement to rapid and vigorous movements, respectively) were also evaluated in an aliquot of semen, using conventional optical microscopy (CX31, Olympus, Tokyo, Japan) at 100× magnification, observing at least four microscopic fields for each sample. The results were expressed as the average of the evaluated fields according to the criteria established by the Brazilian College of Animal Reproduction. Immediately after evaluating the quality sperm parameters, 2 mL of semen from each collection were separated, added to a new tube and centrifuged at 150 x g for 10 min to separate the seminal plasma from the sperm. Then, a new centrifugation at 700 x g for 10 min was performed to remove cellular debris. The resulting seminal plasma was filtered using a 0.22 µm cutoff membrane and immediately frozen in liquid nitrogen.

The other semen fraction was diluted in a skimmed milk-based medium (Botu-Sêmen, Botupharma, São Paulo, Brazil) in a ratio of 1:2 (sperm:diluent), divided into aliquots in 15 mL Falcon tubes and centrifuged at 600 x g for 15 min. Immediately, the supernatant was removed and the sperm concentration of the pellets was estimated in a Neubauer chamber. Then, the pellets were resuspended in freezing diluent (Botu-Crio, Botupharma, São Paulo, Brazil) at a final concentration of 100 million spermatozoa per mL and sperm motility and vigor were evaluated after dilution.

The diluted semen was packaged in 0.5 mL straws, duly identified, and refrigerated at 5 °C for 120 min. Subsequently, the straws were placed in nitrogen vapor

for 15 min and then immersed in liquid nitrogen. The straws from each ejaculate were thawed at 37 °C for 30 s to evaluate sperm motility, vigor and calculate the percentage of sperm abnormalities after thawing.

2.4. Precipitation and determination of soluble protein concentration

For the process of precipitating soluble proteins, 100 µL of seminal plasma samples were used along with 600 µL of a solution containing cold acetone, 10% trichloroacetic acid (TCA), and 1 mmol L^{-1} dithiothreitol (DTT). The precipitated proteins were then resuspended in 250 µL of a solubilization solution consisting of 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 40 mmol L^{-1} DTT.

For the quantification of soluble proteins, an analytical calibration curve was constructed using standard solutions of bovine serum albumin (BSA) with known concentrations. Subsequently, the seminal plasma samples were combined with 150 µL of 1X Bradford reagent and topped off with milli-Q water to reach a final volume of 200 µL. The protein concentration in the samples was estimated based on the linear relationship established by the calibration curve (BRADFORD, 1976).

2.5. One-dimensional denaturing gel electrophoresis (SDS-PAGE)

Gels were prepared to verify the quality of protein extraction and quantification processes. SDS-PAGE was performed by running the gel in two stages (splitting: 14% T and 3% C, stacking: 5.1% T and 2.6% C) at 80 V for 2.5 h using the mini-gel apparatus (Mini-PROTEAN® Tetra Cell, Bio-Rad, Hercules, CA, USA). A total of 25 µg of each sample was loaded, and samples were compared to the Broad Range molecular weight marker (Bio-Rad, Hercules, CA, USA). Gels were subsequently stained with Coomassie Blue R-250 for 2 h and de-stained for about 10 h (LAEMMLI, 1970).

2.6. SDS-PAGE by short run

Protein pools were prepared, each containing 37.5 µg from individual samples, totaling 150 µg per pool, using four semen collections from each of the three animals. Each sub-sample was allocated to have three replicates on the gel, each loaded with 50 µg of proteins. These were run on SDS-PAGE gels until they reached a distance of 1-cm (RESJÖ et al, 2017). Regions of the gel containing the proteins from each sample were excised and fixed in a solution of 50% vol/vol methanol and 5% vol/vol acetic acid for 2 h, followed by staining to localize the proteins within the samples.

Subsequently, the gels were de-stained by removing the fixing solution and adding 200 µL of a 50% vol/vol acetonitrile (ACN) and 25 mmol L^{-1} ammonium bicarbonate (AMBIC) solution, which was left in place for 2 h. After discarding this solution, another 200 µL was added to each sample and left to stand overnight at room temperature. Given that the gels remained stained after this wash, an additional washing step was conducted for another 2 h. Following this, two washes were carried out with 200 µL of 100% vol/vol ACN for 5 min each to dehydrate the gels. Upon complete removal of the ACN, the samples were thoroughly dried in a vacuum concentrator.

2.7. In-gel digestion

The gels were initially reduced at 56 °C for 30 min in a solution containing 65 mmol L^{-1} dithiothreitol (DTT) and 100 mmol L^{-1} AMBIC, to initiate the in-gel digestion (SHEVCHENKO et al., 2006). Subsequently, the gels underwent cycles of washing, hydrating, and dehydrating using AMBIC and ACN, before being completely dried in a vacuum concentrator. Rehydration was performed in an ice bath, using 25 ng μL^{-1} of porcine pancreatic trypsin (Promega, Madson, WI, USA) in an activation solution comprised of 40 mmol L^{-1} AMBIC and 10% vol/vol ACN, over a period of 45 min. An additional 100 µL of the activation solution was then added to each sample.

The gels were next incubated at 37 °C for 22 h, followed by sonication for 10 min and subjected to the vortex for 20 s. The supernatants were transferred to new microtubes. A solution of 5% vol/vol formic acid and 50% vol/vol ACN was added to the gels, which were then vortexed for 20 s, incubated at room temperature for 15 min, and sonicated for 2 min. The supernatant was again collected. The samples

containing peptides were subsequently concentrated via vacuum centrifugation. Finally, 10 µL of a solution containing 0.1% (v/v) trifluoroacetic acid (TFA) in ultrapure water was added to each concentrated sample. The trypsinized peptides were then desalted using C18 reverse-phase microcolumns ZipTip® (ZTC18S096, Millipore, Burlington, NJ, USA) and eluted with 50% vol/vol ACN acidified with 0.1% vol/vol TFA.

2.8. Protein identification by LC-MS/MS analysis

To solubilize the peptides from the short-run replicates, 20 µL of an aqueous solution of 0.1% vol/vol formic acid (LC-MS grade) were added, followed by storage in designated tubes for subsequent analysis using the nano LC-MS/MS system. For the nano-analysis, 1 µL of the prepared solution was utilized on an UPLC nanoAcquity system (Waters, Milford, MA, USA). The trap column used was a nanoAcquity UPLC® 2GV/MTrap with 5 µm Symmetry® C18, measuring 180 µm x 20 mm, at a flow rate of 7 µL min^{-1} for a duration of 3 min. Peptide separation was carried out using a nanoAcquity UPLC® column with 1.7 µm BEH130 and dimensions of 100 µm x 10 mm, operating at a flow rate of 0.3 µL min^{-1}. Mobile phase solvent A consisted of water acidified with 0.1% vol/vol formic acid, while solvent B comprised ACN acidified with 0.1% vol/vol formic acid. The chromatographic separation was programmed as follows (% vol/vol): an initial 2% solvent B for 1 min; a gradient from 2% to 30% solvent B over 299 min; a steep increase from 30% to 85% solvent B within 5 min; a hold at 85% solvent B for 5 min; a quick decrease from 85% to 2% solvent B within 5 min; and a final hold at 2% solvent B for 5 min, totaling a 320 min analytical run.

Peptides were automatically injected into the MAXIS 3G mass spectrometer (Bruker Daltonics, Germany) in online mode, utilizing a CaptiveSpray ionization source. Peptide analysis was performed using the most appropriate method (IE_GCF_01-02-2017), with a drying gas flow rate of 3 L min^{-1}, an ionization source temperature of 150ºC, and a transmission voltage of 2 kV. The raw data were saved in Mascot Generic Format (.mgf) for mass list storage.

2.9. Data Analysis

Data files were scrutinized using the Xcalibur software version 2.1 (Thermo Scientific, Waltham, MA, USA). RawMeet 2.0 was employed to verify the number of MS1 and MS2 spectra generated. Protein identification was executed via Proteome Discoverer 2.4 (Thermo Scientific, Waltham, MA, USA). The algorithm Sequest HT was utilized to correlate Peptide-Spectrum Matches (PSM) for the peptides studied against an *Equus asinus asinus* database from UniProt database (https://www.uniprot.org/), updated as of August 2023 and featuring 33,664 proteins. The search parameters were a maximum of two missed cleavage and semi tryptic peptides; carbamidomethylation (C) was chosen as fixed modification and oxidation (M) and acetylation (protein N-terminal) as variable modifications; a precursor mass tolerance of 10 ppm and 0.05 Da of ion fragment mass tolerance. Match between runs was used with a maximum retention time shift of 5 min. The False Discovery Rate (FDR) was < 1% protein level, peptide and PSM level. Proteins were grouped in master proteins using the maximum parsimony principle (BOUTET et al., 2016). Protein quantification was made up of the Extracted Ion Chromatogram (XIC) performing a relative quantification according to the peak intensity of the three most abundant unique peptides of each protein in the workflow node Precursor Ion Area Detector in PD2.4.

Protein interaction networks were made using STRING DB vs 12.0 (https://string-db.org/ accessed on 02 september 2023). The searches were carried out using the FASTA sequences of the proteins, obtained from the UniProtKB database, and compared with the *Equus asinus asinus* database. The type of network selected was a physical network, with interaction edges per occurrence, active interaction sources textming, experiments and databases, with a high confidence level (0.700). Nodes with no connection were hidden. The other settings were left at default.

3. RESULTS

3.1. Semen quality parameters

No significant difference was observed in sperm quality parameters between individuals before freezing and after thawing ($p>0.05$). However, the median value of motility and sperm vigor of cryopresevated semen was lower than that of fresh and diluted semen ($p<0.05$). The sperm motility had a median of 90% fresh and diluted, and a median of 35% after thawing. The vigor had a median of 4 fresh and diluted and a median of 3 after thawing. (Figures 1 and 2).

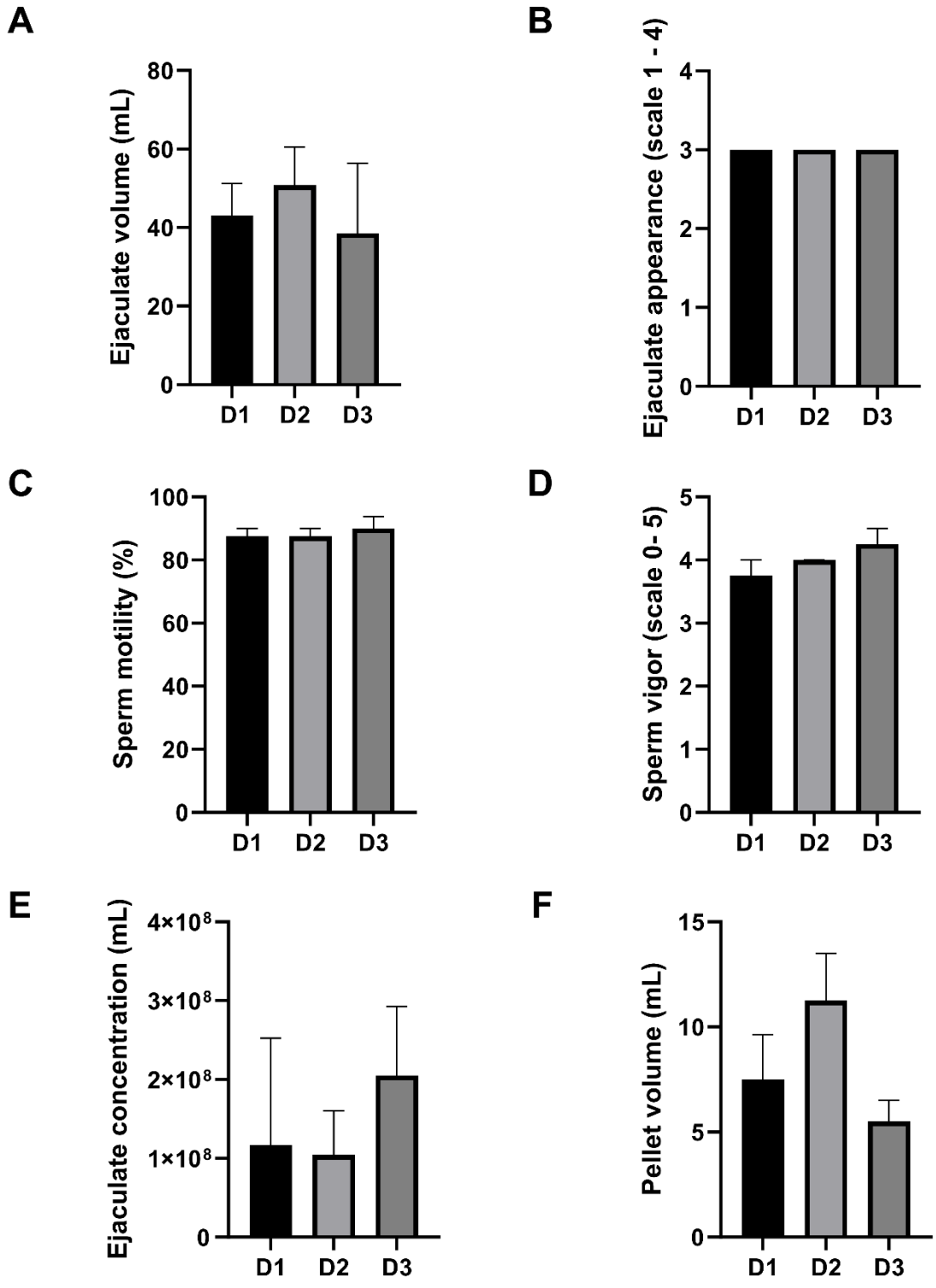


Figure 1. Sperm quality parameters of Pêga donkeys before semen freezing. Those graphics demonstrate the medians of the Pêga breed donkeys regarding ejaculate volume (A), ejaculate appearance (B), sperm motility (C), sperm vigor (D), ejaculate concentration (E), pellet volume (F). Source: Costa, 2023. Authorial.

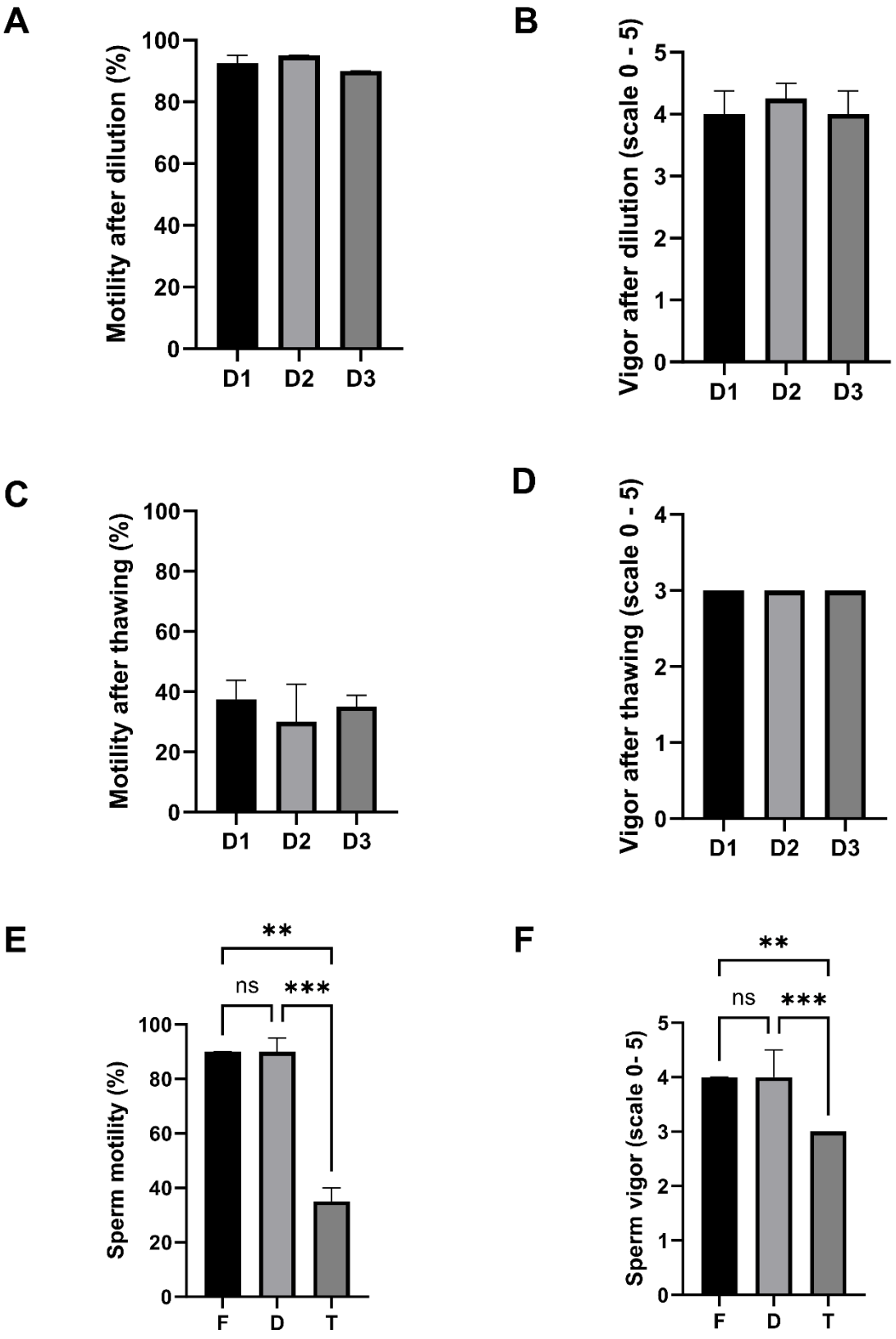


Figure 2. Sperm quality parameters of Pêga donkeys after dilution and after thawing. Those graphics demonstrate the medians of the Pêga breed donkeys parameters for

each animal regarding motility after dilution (A), vigor after dilution (B), motility after thawing (C) vigor after thawing (D), a comparison between the motility of fresh, diluted and thawed semen (E), a comparison between the vigor of fresh, diluted and thawed (F). Source: Costa, 2023. Authorial.

3.2. Protein identification

When compared to the *Equus asinus asinus* database, a total of 557 proteins were identified in the seminal plasma using the Proteome Discoverer software. Out of this number, 35 proteins were discarded after being recognized as contaminants, resulting in a total of 522 proteins identified in the analyzed samples. Of these 522, 441 were of high confidence, with an FDR<0.1, and 81 were of medium confidence, with an FDR between 0.1 and 0.3. (Supplementary Table 1).

3.3. Functional classification of seminal plasma profile

Proteins were categorized using the Gene Ontology tool from STRING DB vs 12.0 (SZKLARCZYK et al., 2023). This allowed their classification based on their biological processes, molecular functions, and cellular components.

Regarding the primary biological processes (Figure 3), it was observed that proteins are predominantly involved in metabolic processes (associated to transformation of cellular energy), represented by 260 proteins (49.8% of the total 522 proteins); small molecule metabolic processes with 104 proteins (19.9%); catabolic processes with 101 proteins (19,3%); organic substance catabolic processes with 90 proteins (17.2%); and reproduction, which included 76 proteins (14.6%). However, while some processes had fewer proteins, there was a significant number of processes directly associated with energy and reproduction (Supplementary Table 4).

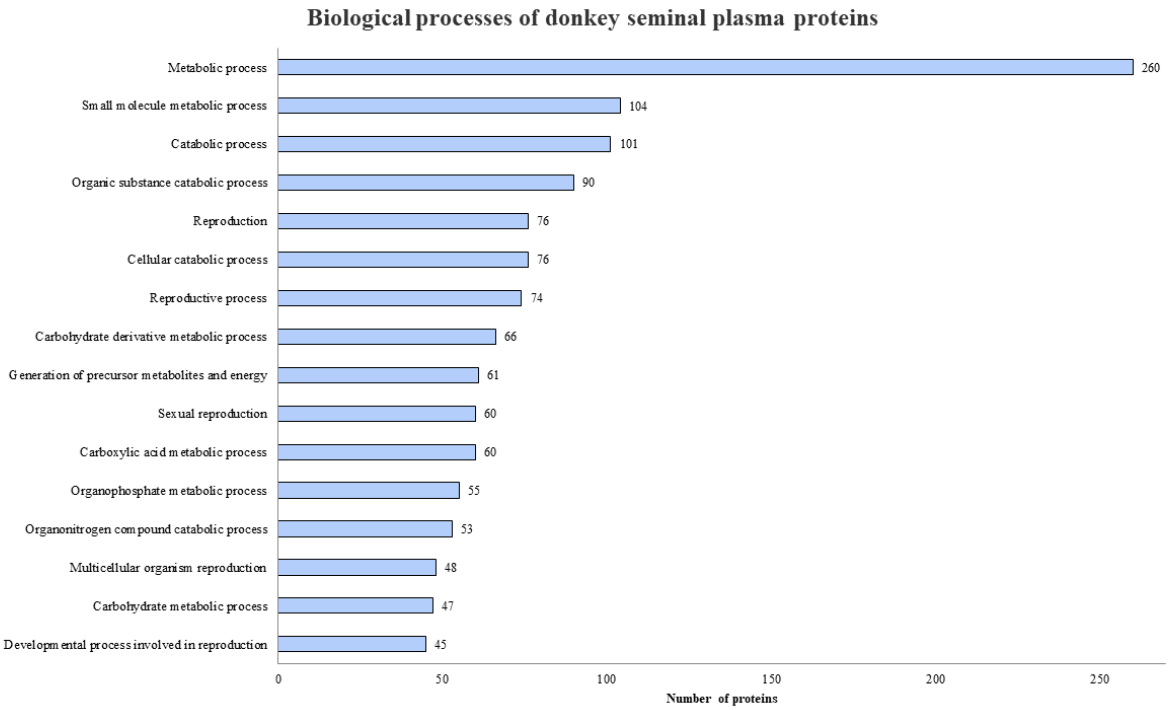


Figure 3. Classification of proteins involved in the 16 main biological processes of seminal plasma of Pêga donkeys by STRING DB. Source: Costa, 2023. Authorial.

In the findings related to molecular functions (Figure 4), the categories with the most associated proteins are catalytic activity with 260 proteins (49.8% of the total 522 proteins), ion binding with 198 proteins (37.9%), small molecule binding with 131 proteins (25.1%), hydrolase activity with 120 proteins (23,0%), and anion binding with 109 proteins (20.9%). Most of these functions are related to diverse molecular interactions (Supplementary Table 5).

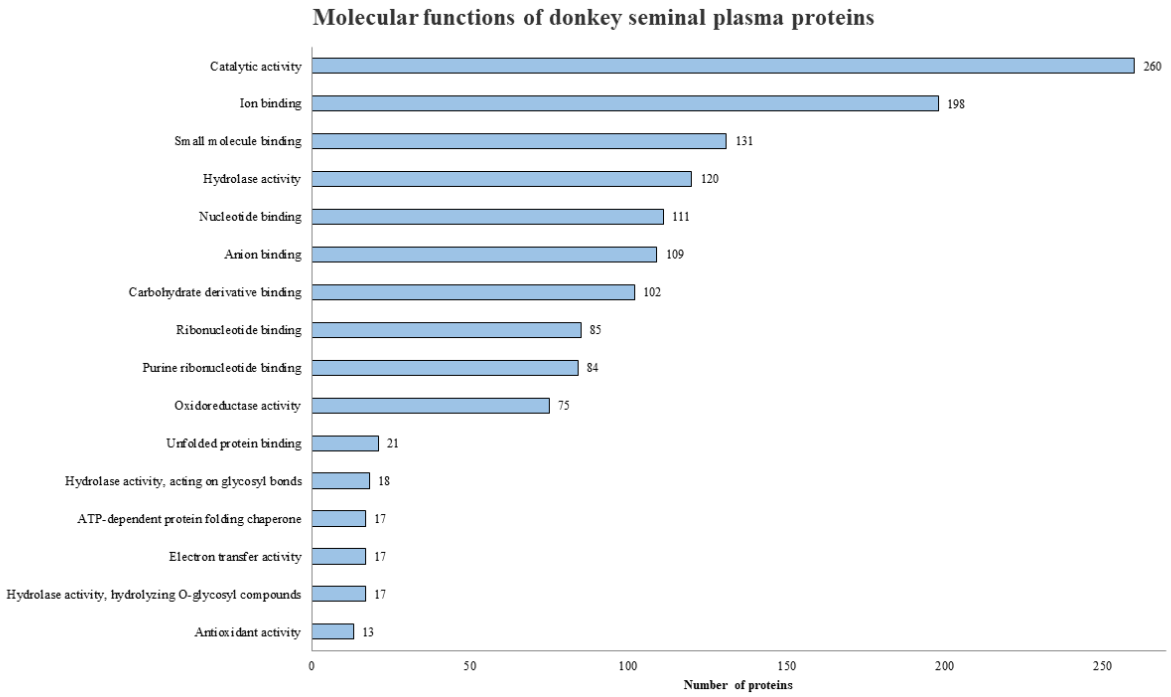


Figure 4. Classification of proteins involved in the 16 main molecular functions of seminal plasma of Pêga donkeys by STRING DB. Source: Costa, 2023. Authorial.

In the results pertaining to cellular components (Figure 5), the categories with the most proteins involved include the cellular anatomical entity, with 510 proteins (97.7% of the total 522 proteins); the intracellular anatomical structure, with 409 proteins (78.4%); the cytoplasm, with 380 proteins (72.8%); the extracellular region, with 142 proteins (27.2%); and the organelle membrane, with 109 proteins (20.9%). Even though some categories have fewer proteins, a significant number are associated with the mitochondria, cilium, and secretory vesicles (Supplementary Table 6).

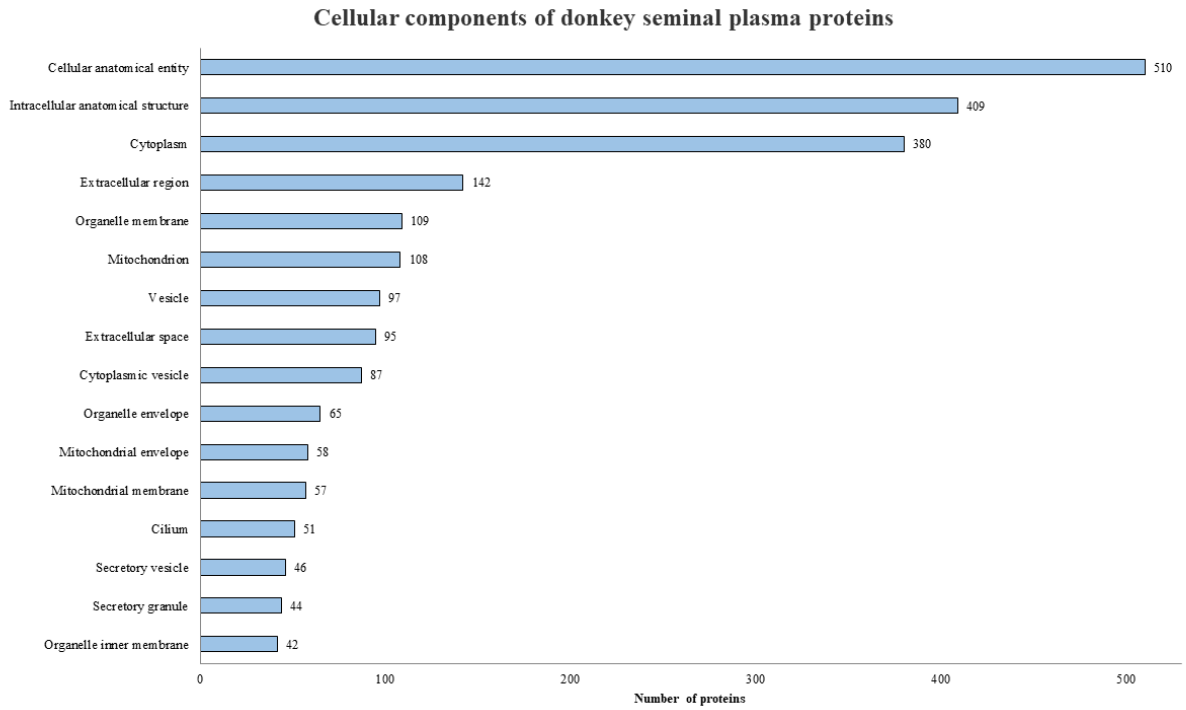


Figure 5. Classification of proteins involved in the 16 main cellular components of seminal plasma of Pêga donkeys by STRING DB. Source: Costa, 2023. Authorial.

3.4. KEGG Pathways analysis of seminal plasma

An analysis of pathways was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database through the STRING DB software. The pathways (Figure 6) with the most associated proteins were metabolic pathways with 109 proteins (20.9%), Prion disease and Huntington's disease, each with 44 proteins (8.4% each), Amyotrophic Lateral Sclerosis (ALS) with 43 proteins (8.2% each), and both Parkinson's and Alzheimer's diseases with 41 proteins each (7.9% each) (Supplementary Table 7).

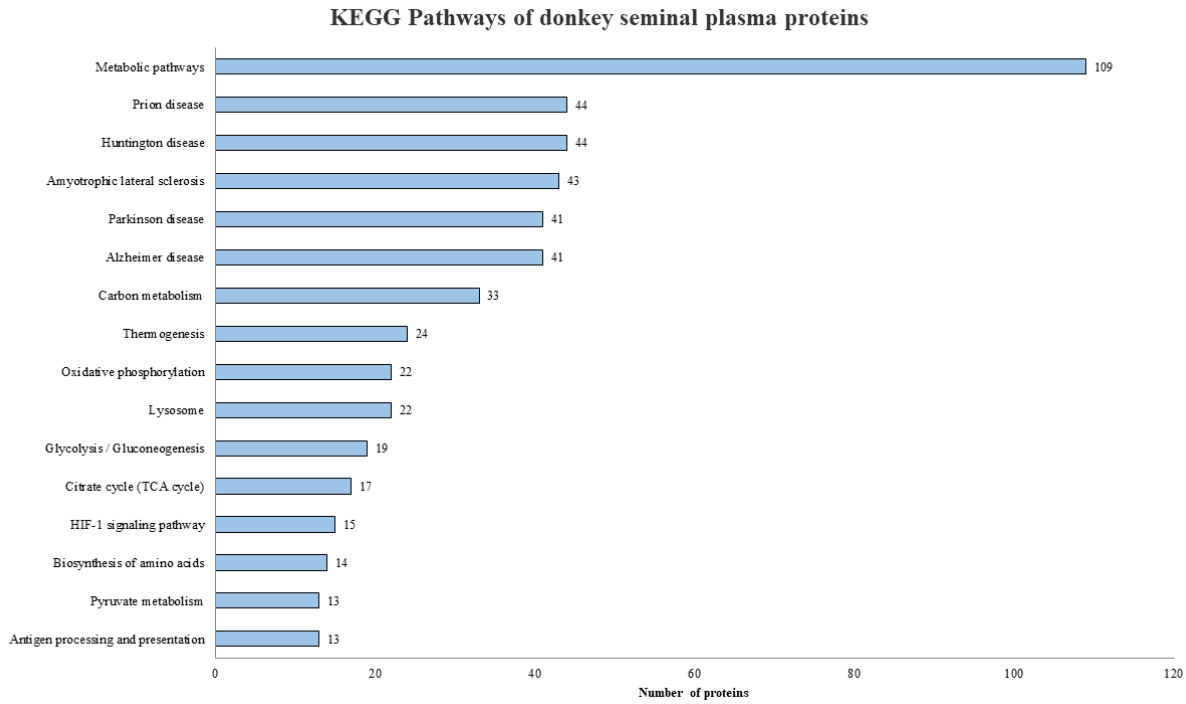
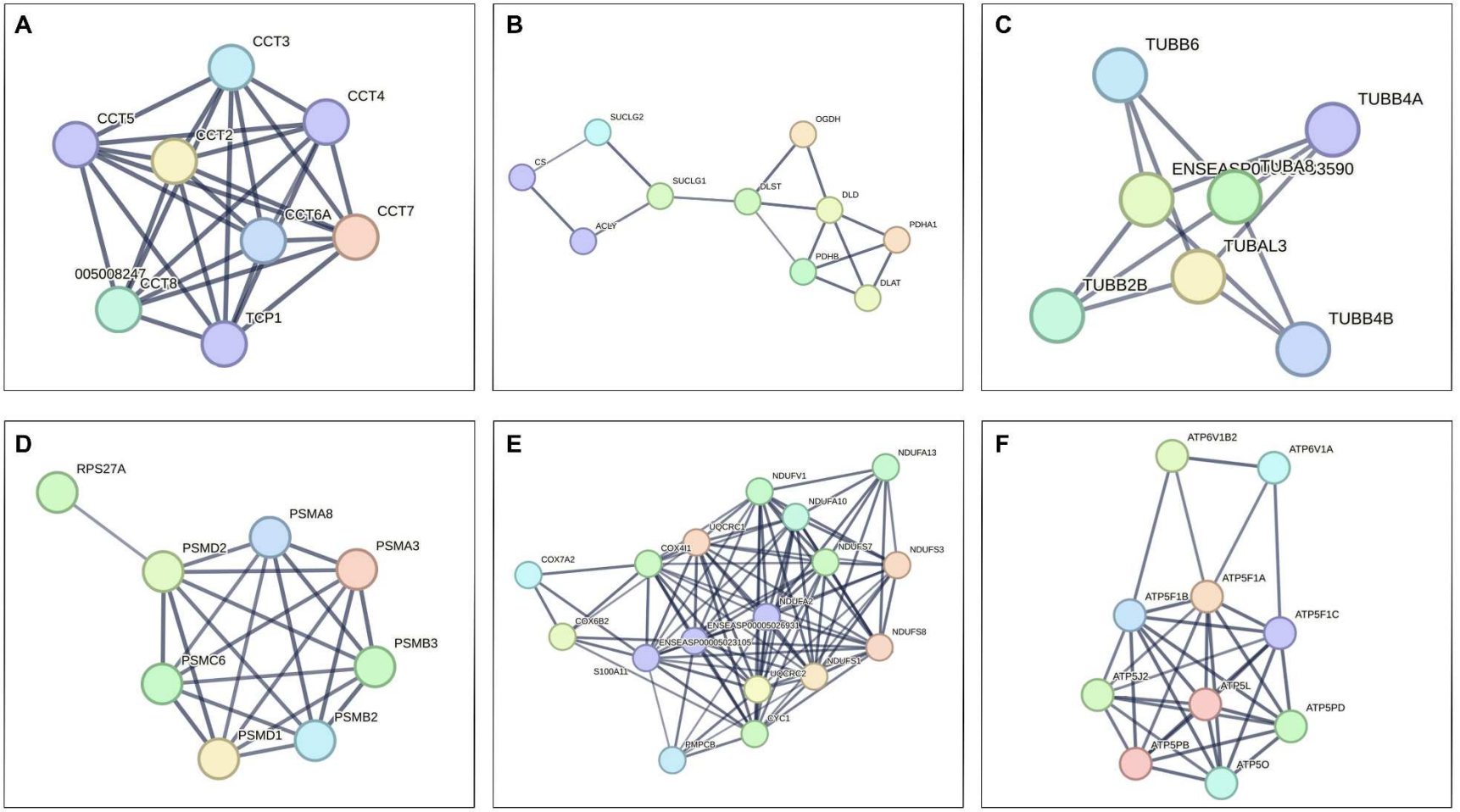


Figure 6. Classification of proteins involved in the 16 main KEGG pathways of seminal plasma of Pêga donkeys by STRING DB. Source: Costa, 2023. Authorial.

3.5. Protein interaction networks of seminal plasma

Several local protein clusters were discerned from the protein analyses using STRING. In total, 53 clusters emerged from the interaction network. They are involved in Metabolism e energy, Reproduction, Cellular Structure and components, Oxidative Stress, Fatty Acid and Lipid Metabolism and Mixed Processes.

When examining the interaction network (Figure 7) from STRING DB, six main clusters stand out. These can be distinguished based on the functions of the proteins within them. In the, (Fig. 3A) proteins are involved with protein folding, (Figure 7B), (Figure 7E) and (Figure 7F) contain respectively proteins implicated in the metabolism and cellular respiration, (Figure 7C) are focused on cellular structure and (Figure 7D) are associated with intracellular protein degradation.


A
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CCT4
CCT5
CCT2
CCT6A
CCT7
005008247
CCT8
TCP1
B
SUCLG2
OGDH
CS
SUCLG1
DLST
DLD
ACLY
PDHA1
PDHB
DLAT
C
TUBB6
TUBB4A
TUBA8
TUBAL3
TUBB2B
TUBB4B
D
RPS27A
PSMA8
PSMD2
PSMA3
PSMB3
PSMC6
PSMB2
PSMD1
E
NDUFA13
NDUFV1
NDUFA10
UQCRC1
COX4I1
NDUFS7
NDUFS3
COX7A2
NDUFA2
COX6B2
NDUFS8
S100A11
NDUFS1
UQCRC2
CYC1
PMPCB
F
ATP6V1B2
ATP6V1A
ATP5F1A
ATP5F1B
ATP5F1C
ATP5J2
ATP5L
ATP5PD
ATP5PB
ATP5O

Figure 7. Most relevant proteins involved in the mainly clusters in the network of seminal plasma proteins interactions, in which (A) are involved with protein folding, (B), (E) and (F) contain respectively proteins implicated in the metabolism and cellular respiration, (C) are focused on cellular structure and (D) are associated with intracellular protein degradation. Source: Costa, 2023. Authorial.

4. DISCUSSION

LC-MS/MS spectrometry allowed us to detail the proteomic profile of donkey seminal plasma. When analyzing the proteins based on their biological processes, the proteins involved in metabolic activity emerge as the predominant function of them in the samples. As described in previous studies, the metabolic pathway might play a crucial role in reproduction by preventing the occurrence of spontaneous acrosome reaction (sAR). This reaction is marked by enzymes that start to degrade the zona pellucida even before interaction with the ovum. Research has shown that by inhibiting glycolytic routes and oxidative phosphorylation, there's an increase in sAR, also there is an inhibition of protein kinase A (PKA), which is essential for sperm capacitation. Furthermore, there is a partial or total inhibition of tyrosine protein phosphorylation (PTP), a key event in sperm capacitation, when those one or both pathways are suppressed (JIN et al., 2011; HINO et al., 2016; DAHAN & BREITBART, 2022). Given this context, it is understandable that proteins involved in metabolic pathways hold significant relevance in the biological processes under study. It is also understandable that the metabolic pathways appear so prominently in the protein interaction networks and in the KEGG Pathways. The higher role of proteins in metabolic pathways can be related to the results found in differential abundance of some proteins in samples of Dezhou donkeys with great freezability. Two specific enzymes of the glycolytic via and oxidative phosphorylation, respectively, were related positively with reproduction: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase B (LDHB). Those pathways provide adenosine triphosphate (ATP) to the spermatozoa and the higher the ATP concentration, the better is the motility. Furthermore, lower ATP levels are found in the post-thawing period, which may suggest why these cells may exhibit lower motility (PIOMBONI et al., 2012; JIANG et al., 2015; YU et al., 2021).

Still in relation to enzymes, catalytic activity stands out among molecular functions. Given that sperm capacitation relies on the acrosomal reaction for successful ovum fertilization, there is a destabilization of the sperm cell membrane during this process. Hence, it makes sense for this reaction to be so pronounced, because there is a need of enzymes working on the membrane to a successful fertilization (FERNÁNDEZ & HEEB, 2007).

There was no appearance of kallikreins in our results, but rather the appearance of the angiotensin-converting enzyme (ACE), which is also involved in the kallikrein-kinin system, as opposed to kallikrein. ACE has a testicular tissue isoform that acts in sperm capacitation, the acrosome reaction, and binding to the zona pellucida, meaning it also has an important role in reproduction (FUCHS et al., 2005; OJAGHI et al., 2017; OJAGHI et al., 2022).

In the comparative study of seminal plasma of Dezhou donkeys with different levels of freezability, it was also found differential abundance of some proteins in samples of animals with great freezability that are involved with the reproduction (YU et al., 2021). It is worth highlighting the appearance of these proteins in our qualitative analysis of this study. One of those was the cysteine-rich secretory protein 3 (CRISP3). That protein is part of known potential molecular biomarkers for fertility in stallions and is related to a positive marker of good freezability (NOVAK et al., 2010). Other proteins found were the heat shock proteins A5 and the heat shock 70 kDa protein-like (HSPA5 and HSPA1L), which are from the same family of heat shock proteins with 70 kDa. The abundance of that family of proteins is indicated for association with good freezability (REGO et al., 2016; YU et al., 2021). HSPA5 is one protein found in the surface of human spermatozoa that is involved in apoptosis (NAABY-HANSEN & HERR, 2010; YU et al., 2021). Then, the Niemann-Pick disease type 2 (NPC2) was also found, a protein that can be related to a higher affinity to cholesterol and helps to enhance their binding with the sperm membrane (VALENCIA et al., 2017; YU et al., 2021).

Finally, there were more proteins found in this study that are according to the Dezhou donkey's seminal plasma profile with great freezability. Those are the antioxidant proteins involved in oxidation-reduction. These proteins are important to control the production of reactive oxygen species (ROS) that can cause sperm dysfunction due to oxidative stress (OS) caused by cryopreservation (ARMSTRONG et al., 1999; PEÑA et al., 2015; YU et al., 2021). As an example of antioxidant proteins, we can

cite the evidentiate by the study of Denzhou donkey the sulfhydryl oxidase (QSOX1), peroxiredoxin-1 (PRDX1), and glutathione peroxidase (GPX5), which were also found in our study (NOVAK et al., 2010).

5. CONCLUSION

Our work corresponds to an important comprehensive proteomic analysis of seminal plasma of Pêga breed donkeys, which provides a profile that can be related to the assisted reproduction and their effects in the spermatozoa. These results showed that proteins related to metabolic activity emerge as a predominant factor in the seminal plasma samples of donkeys, evidencing that there is a large need for energy aiming reproduction events. In a broader view, certain proteins emerged as potential positive biomarkers of fertility in Pêga breed donkeys, when in abundance. This finding is supported by research with a different breed of donkeys, the Dezhou breed donkey, and other species that are close relatives of donkeys, such as stallions. Among these proteins, CRISP3, NPC2, HSPA5, HSPA1L can be highlighted, such as antioxidants proteins, like QSOX1, PRDX1 AND GPX5, which were already reported positively related to good freezability, great motility and great vigor. Also, an abundance of proteins involved in the glycolytic pathway and oxidative phosphorylation, such as GAPDH and LDHB, can suggest a higher spermatozoa capability of fecundation. Comparative studies in this breed can provide more information about these potential proteins as biomarkers. In conclusion, seminal plasma plays a key role in correctly promoting sperm function. Thus, understanding its composition and function is essential to devise strategies that counteract the degradation of sperm fertilizing capacity by cryopreservation.

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ATTACHMENT A: Supplemental material Chapter 1

Supplemental File 1 Source: Costa, 2023. Authorial.

(https://docs.google.com/spreadsheets/d/1CpnEnzhN403iuc6DAIlyi_z9m4UQsDwWn8TYp2qWbvI/edit#gid=0)

ATTACHMENT B: Supplemental material Chapter 2

Supplemental File 1 Source: Costa, 2023. Authorial.

(https://docs.google.com/spreadsheets/d/1y8otISufpHuV8ir75NZMjEkPnRyc5Vle/edit#gid=550806402)