UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

La proteómica como identificador de biomarcadores de calidad seminal en caballos

Proteomics for the identification of biomarkers of seminal quality in horses

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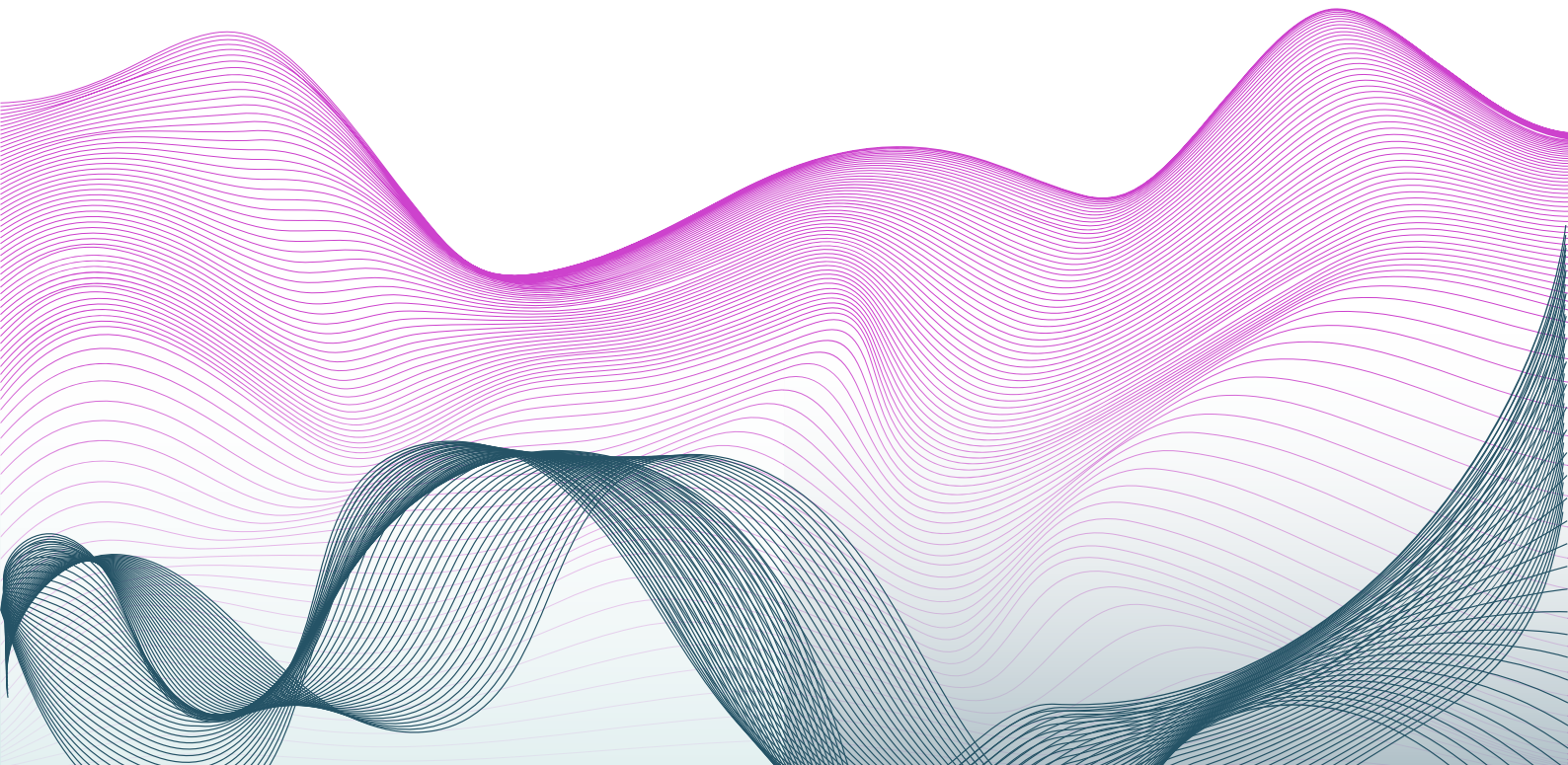
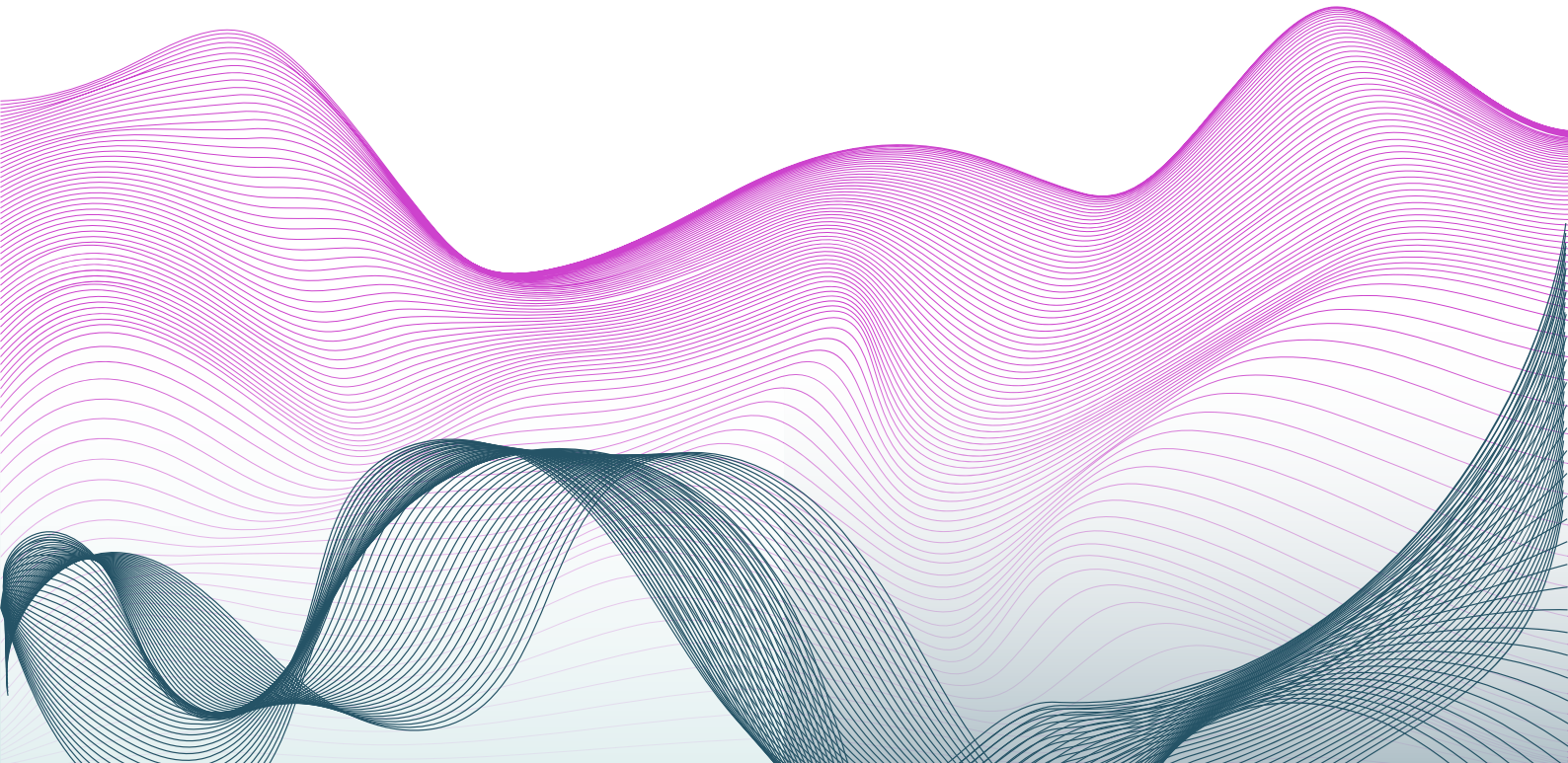
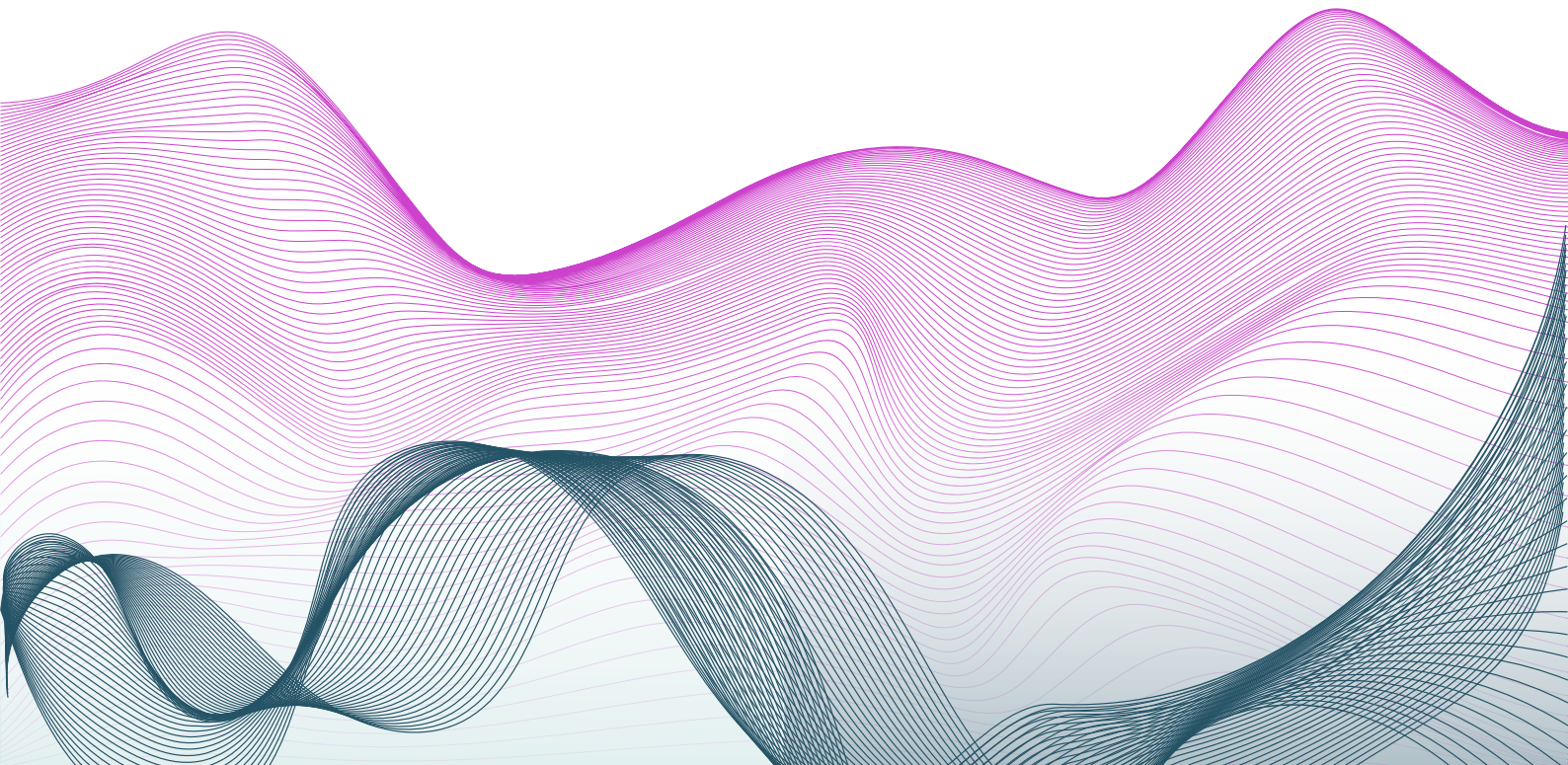


R E S U M E N



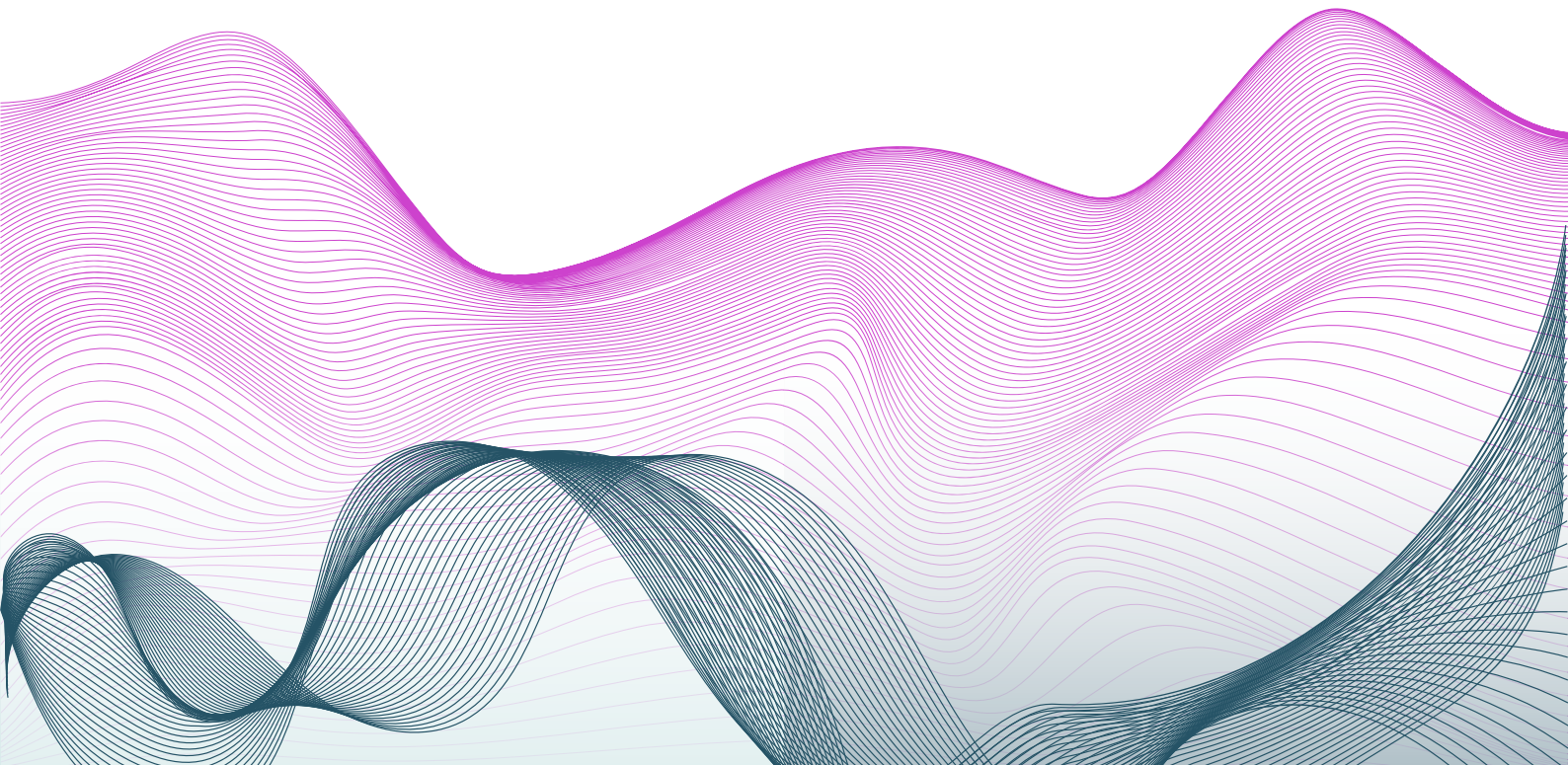
El sector equino, y concretamente la industria encargada de proveer servicios para la cría de esta especie, tiene una gran importancia económica a nivel global. En los últimos años han habido avances sustanciales en el conocimiento de la biología del esperma y las biotecnologías reproductivas. A este respecto, los equinos difieren de otras especies de producción, ya que su selección no se ha centrado en la fertilidad, sino en otras características, como la morfología o la capacidad atlética. Esto ha llevado a una situación en la que existe una amplia variabilidad en la fertilidad de los sementales y, a pesar de los recientes avances en el campo de la reproducción equina y los mecanismos moleculares que afectan a los espermatozoides, existe una falta de comprensión básica sobre las causas de la infertilidad y la intolerancia de las tecnologías reproductivas en algunos individuos. Hasta la fecha, la conservación del semen en el semental se realiza principalmente a través de la refrigeración y la congelación; ambas estrategias que ralentizan el metabolismo espermático. Esto permite la extensión de la vida útil de los espermatozoides in vitro con reservas de energía suficientes para permitir una fertilización exitosa una vez depositados en el tracto reproductivo de la yegua. Para comprender completamente los mecanismos responsables de la variabilidad individual y de la diferente respuesta a estas tecnologías, es necesaria una comprensión más profunda de la biología y el metabolismo de los espermatozoides. La estructura del espermatozoide y el hecho de que es una célula traduccional y transcripcionalmente silenciosa la convierten en el sujeto ideal para el estudio mediante proteómica. Hasta la fecha, la proteómica ha sido responsable de proporcionar cantidades significativas de nueva información para aumentar nuestra comprensión de estas células altamente especializadas. Como resultado, es probable que en el futuro cercano se produzcan modificaciones de los protocolos actuales utilizados para la conservación y manipulación de espermatozoides in vitro, asegurando la regulación de la homeostasis espermática y la modulación de la producción excesiva de especies reactivas de oxígeno, culminando en resultados de fertilidad más exitosos. Además, los estudios proteómicos también están contribuyendo a la identificación de biomarcadores que pueden usarse para predecir el comportamiento de los espermatozoides frente a ciertas tecnologías reproductivas y, en el futuro, esto sin duda conducirá a una reducción de los costes y a la personalización de las formulaciones de medios utilizadas para la conservación del semen, las temperaturas para el almacenamiento a corto plazo y otros aspectos de los protocolos de tecnología reproductiva, lo que conducirá a una conservación más exitosa del material genético del semental y, posteriormente, a las tasas de fertilidad.

S U M M A R Y



The equine breeding industry is of high economic value and in recent years there have been substantial advances in knowledge of sperm biology and biotechnologies. Equines are somewhat different to other production species as human selection has not focused on fertility, but on other characteristics, such as morphology or athletic ability. This has led to a situation where there is a wide variability in fertility of stallions and, despite recent advances in the field of equine reproduction and molecular mechanisms affecting sperm, there is a lack of basic understanding as to the causes of infertility and intolerance of artificial reproductive technologies in some individuals. To date semen conservation in the stallion is principally through refrigeration and freezing; both strategies which slow spermatic metabolism. This enables extension of sperm lifespan in vitro with sufficient energy reserves to allow for successful fertilisation once deposited in the mare reproductive tract. To fully understand the mechanisms behind individual variability and why individuals respond differently to these technologies, a deeper understanding of sperm biology and metabolism is necessary. The structure of the spermatozoa and the fact that it is a translationally and transcriptionally silent cell makes it the ideal subject for study using proteomics. To date proteomics has been responsible for providing significant amounts of new information to augment our understanding of these highly specialised cells. As a result, it is likely that the near future will see modifications of current protocols used for sperm conservation and manipulation in vitro, ensuring regulation of spermatic homeostasis and modulation of excessive production of reactive oxygen species, resulting in more successful fertility outcomes. In addition, proteomic studies are also contributing to the identification of biomarkers which can be used to predict the behaviour of spermatozoa in the face of certain reproductive technologies and in the future this will no doubt lead to a reduction in costs and the customisation of extender formulations used for semen conservation, temperatures for short-term storage and other aspects of artificial reproductive technology protocols, leading to more successful conservation of stallion genetic material, and subsequently fertility rates.

A B B R E V I A T I O N S



2-DG: 2-deoxyglucose

4-HNE: 4-hydroxynonenal

AGE: advanced glycation end products

AI: artificial insemination

ART: artificial reproductive technology

ADP: adenosine diphosphate

ATP: adenosine triphosphate

CASA: computer assisted sperm analysis

CO_2: carbon dioxide

$CO_3^{-\bullet}$: carbonate radical

CoA: coenzyme A

CRISPs: cysteine-rich secretory proteins

DNA: deoxyribonucleic acid

ETC: electron transport chain

$FADH_2$: flavin adenine dinucleotide

FeS: iron sulphide

GLUT: facilitated diffusion glucose transporters

GTP: guanosine triphosphate

H^+: hydrogen cation

H_2O_2: hydrogen peroxide

HSP-1: horse seminal plasma protein 1

HSP-90: heat shock protein 90

KEGG: Kyoto Encyclopaedia of Genes and Genomes

mRNA: messenger ribonucleic acid

NAD^+: oxidised nicotinamide adenine dinucleotide

NAD: nicotinamide adenine dinucleotide

NADH: reduced nicotinamide adenine dinucleotide

$NADP^+$: oxidised nicotinamide adenine dinucleotide phosphate

NADPH: reduced nicotinamide adenine dinucleotide phosphate

$^{\bullet}NO$: nitric oxide

NO_2: nitrogen dioxide

$NO_2^{\bullet}$: nitric dioxide

O_2: oxygen

$O_2^{\bullet-}$: superoxide anion

$^{\bullet}OH$: hydroxyl radical

$ONOO^-$: peryoxynitrite

OXPHOS: oxidative phosphorylation

PI3K: phosphoinositide 3-kinase

PPP: pentose phosphate pathway

rRNA: ribosomal ribonucleic acid

RNA: ribonucleic acid

ROS: reactive oxygen species

SOD1: superoxide dismutase

ST3GAL1: ST3 beta-galactoside alpha-2,3-sialyltransferase 1

TCA cycle: tricarboxylic acid cycle

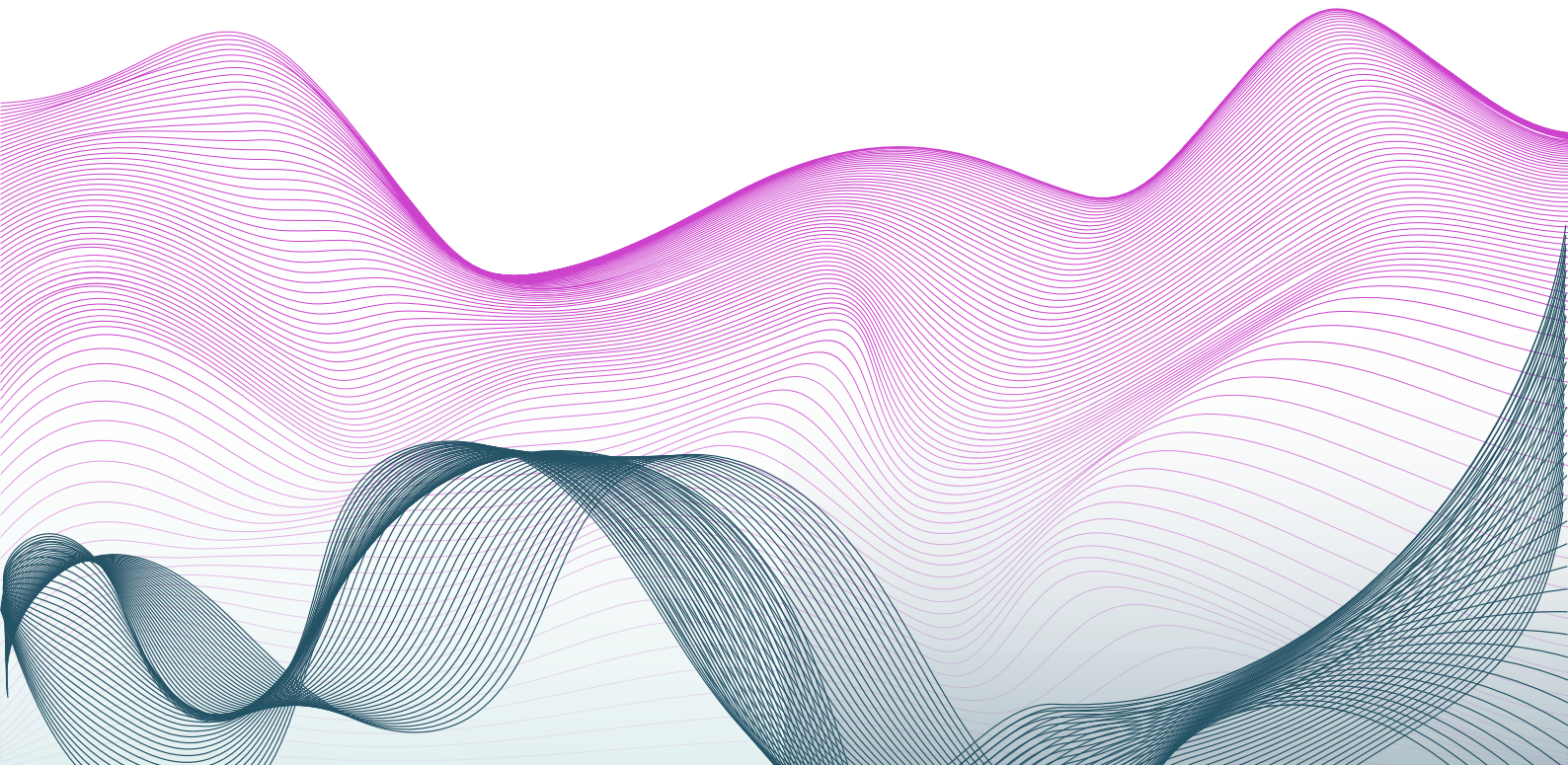
UHPLC-MS/MS: ultra-high performance liquid chromatography tandem mass spectrometry

VAP: average path velocity

VCL: curvilinear velocity

VSL: straight line velocity

INTRODUCTION



Background

Recent years have seen a significant leap in scientific knowledge of sperm biology, in a large part due to substantial advances in sperm biotechnologies allowing a more in-depth comprehension of male infertility (1,2). Techniques which have allowed for these advances to take place include flow cytometry (3–5), and the additional "omics" techniques which have subsequently been implemented in the field to aid further research (6–16). In particular, the use of proteomics in the area of equine reproduction has led to a better understanding of mechanisms behind the function and metabolism of equine spermatozoa (16).

Horses have not been bred primarily for fertility, with other factors such as performance or morphology being favoured as selection criteria. In addition to this there have been restrictions on the use of certain technologies by stud books, the organisations responsible for regulation of certain horse breeds, which has led to a delay in the study and development of techniques when compared to other production species. This means that there are large variations in fertility between different stallions and differences in sperm quality are common (17). Equines are also unique as they usually become more valuable with age and when they have proven sporting or morphological achievements, however, decreases in sperm quality and fertility are also linked to advanced age (18–20).

In addition to disparities in sperm quality, significant variability is also seen in how well individual ejaculates tolerate the application of reproductive technologies and how successfully initial quality can be conserved (3,4). This individual variability is of special clinical relevance because of the now extensive use of artificial insemination (AI) and the increased demand for conservation of ejaculates; either refrigerated for short-term storage or cryopreserved and stored in liquid nitrogen for longer term storage. Cryopreservation is of particular interest with regards to sperm quality, due to the less than optimal outcomes from current protocols and convenience for providing means for storage of sperm from young horses while they develop their sporting career, or valuable, already proven stallions after death.

Proteomic studies have made enormous headway in furthering current understanding of sperm biology and have led to the identification of specific proteins with potential for use as biomarkers to predict sperm quality and tolerance of artificial reproductive technologies, which are likely to have economic benefits for the sector. Seminal plasma has also been shown to contain potential biomarkers, which could be useful to predict responses to different conservation techniques, as well as improve and individualise protocols in order to optimise sperm quality in different stallions (23). The relevance does not stop at biomarkers for quality and conservation, the spermatozoa is a transcriptionally silent cell but contains proteins responsible for post translational modifications (24–27) with effects on early embryo development.

Spermatozoa

Equine, like other mammalian spermatozoa are highly specialised haploid cells, whose sole purpose is to fertilise the oocyte, thus transferring paternal DNA and leading to production of a zygote. They only contain essential structures, and most of their cytoplasm is eliminated during spermiogenesis, along with extensive DNA compaction (28). This compacted morphology means that sperm are unable to transcribe DNA into mRNA or translate mRNA into peptides and are described as being translationally and transcriptionally silent. Mature spermatozoa are formed of three parts: head (containing the

nucleus and acrosome), neck (containing the proximal centriole) and tail or flagellum. The flagellum is also divided into three parts, the midpiece, the principal piece and the end piece, with mitochondria only found in the midpiece. Each equine spermatozoa contains between 40 to 50 mitochondria which are organised end to end in a spiral structure around the midpiece of the flagellum. Abnormalities, particularly those related to mitochondria have been shown to have numerous effects on normal spermatozoa function, including fertilisation rates (29–34). Mitochondria are central to energy production in the spermatozoa, as well as having numerous other functions, such as redox regulation, calcium homeostasis, being involved in signalling pathways and apoptosis (35–37). These organelles have a dynamic, highly differentiated structure which responds rapidly to the energetic needs of cells (38).

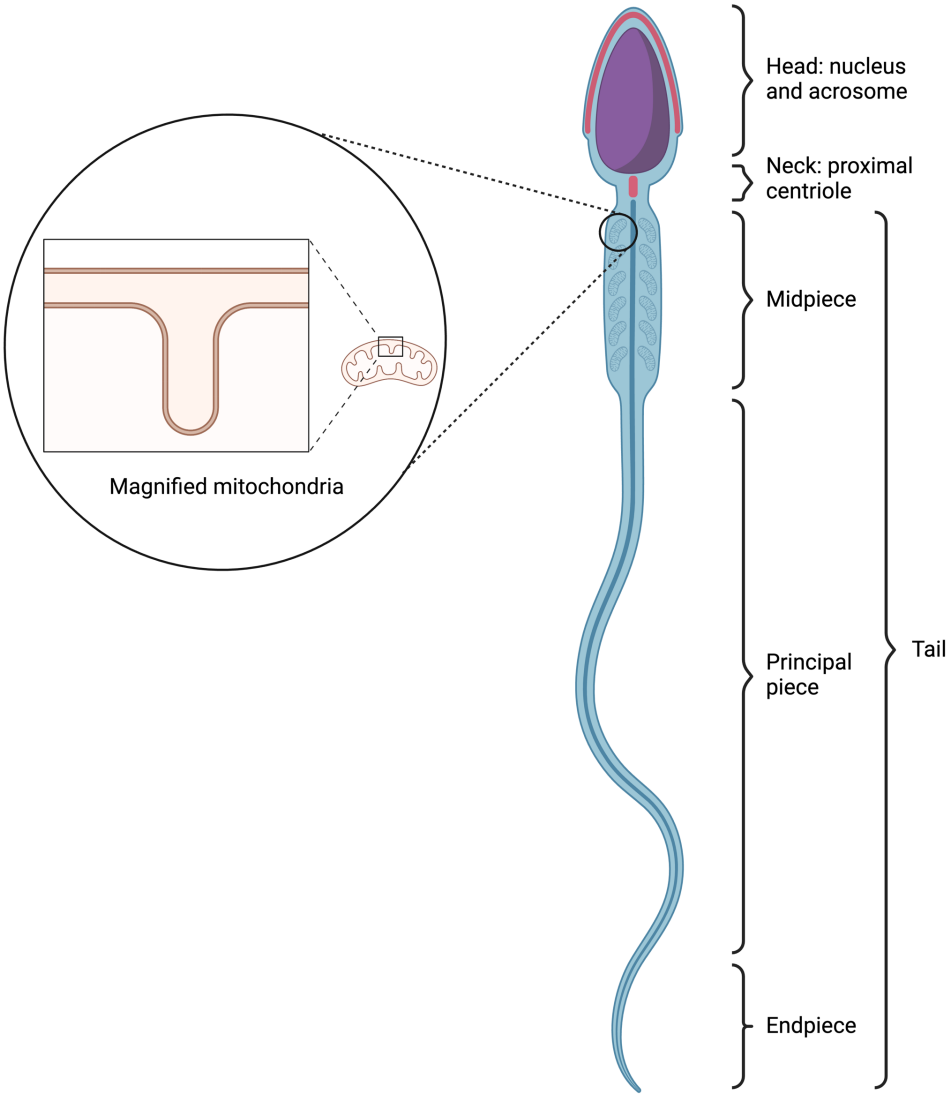


Figure 1. Diagram of sperm anatomy, showing magnified mitochondria.

Implementation of the “omics” techniques to perform proteomic analysis of these cells has recently shown that spermatozoa undergo loss and addition of proteins and RNA whilst travelling through the epididymis as a result of the effects and actions of membrane bound extracellular vesicles which are released from the epididymal epithelium (39–41). During their passage through the epididymis spermatozoa attain new proteins via interactions with epididymosomes, small vesicles encapsulated by a membrane, which are unique to specific regions of the epididymis (39) and act as transporters. Some of these proteins simply coat the spermatozoa and attach to the sperm membrane during post-testicular maturation (42,43) and others are incorporated into the intracellular structures of spermatozoa (44) contributing to post-translational modifications, which are of particular importance in mature spermatozoa, regulating maturation, motility, and other functions (40,45). These post-translational modifications include phosphorylation, glycosylation, and methylation of proteins amongst others. Proteins which are known to interact with the sperm surface in ungulates and to influence functionality are of three main classes (46), including the spermadhesins (47), cysteine-rich secretory proteins (CRISPs) (48) and fibronectin II (Fn-2) type proteins (49,50). The presence of these proteins is a reflection of the secretory activity of the male reproductive tract and as a consequence its functionality (51,52). In addition, research has more recently led to the proposal that epididymosomes may also be part of a protein removal mechanism, with levels of some proteins being reduced during sperm maturation and transit through the epididymis (53). Functional transformation of spermatozoa is completely dependent on protein composition, making them the ideal subject for study using proteomics (10). Despite our knowledge of the importance of this process, the exact mechanism used to transfer proteins to spermatozoa by epididymosomes is still not fully understood (44). In vitro the obvious lack of extracellular vesicle containing substances means that spermatozoa are completely reliant on their environment and the extenders used to store them for antioxidants (28), sources of energy substrates (54), salts and other elements which they require for survival.

Traditionally, sperm quality in stallions and other species (55–61) has been and is still used in a clinical setting to predict fertility based on sperm characteristics such as total and progressive motility (62,63), morphology (64) and DNA quality (65,66), despite the fact that the relationship between these characteristics and fertility is still unclear (67). Possibly in a large part due to the lack of research using objective methodologies and relevant, comparable fertility endpoints (68). Nevertheless, even though evaluation across species of sperm quality using parameters such sperm concentration, total and progressive (linear) motility and sperm morphology is the most widely used and best predictive test currently available, this is no guarantee of fertility even when parameters fall within normal reference ranges. Vice versa, values outside these ranges do not necessarily signify infertility (69–72). In addition, female and numerous other factors contribute to overall fertility outcomes (73–76).

Seminal plasma

Until relatively recently the importance of the role of seminal plasma and its components in modulating spermatic function and interactions with the female reproductive tract has been largely underestimated (77–81). Seminal plasma is a fluid composed of a variety of secretions from the testis, epididymis, and accessory sex glands, with composition varying significantly between species. This, in addition to spermatozoa forms the ejaculate. In the stallion, seminal plasma secretions are produced in large volumes predominantly by the epididymis and accessory glands, in particular the prostate and its roles are varied, including immunosuppressive and immunoregulatory functions (82,83) as well as

possessing profound antioxidant properties (84). It contains a large number of microvesicles, principally prostasomes (85). Contact of spermatozoa with the various different fluids which constitute seminal plasma is important for development of spermatozoa after they leave the testicle, transforming them from immotile cells to extremely active cells capable of fertilisation. Functions of seminal plasma range from regulation of spermatic viability to nourishment and acting as a vehicle (86–90) for RNA's and proteins with a role in early embryo development, as well as signalling and interacting with the endometrium (83,90–92). There are several hundred proteins involved in epididymal maturation (11,12) of spermatozoa. Some of these have been previously documented but the use of the omics techniques, which have significantly contributed to knowledge of sperm biology (93–97), and proteomics, in particular, has allowed for significant advances in their identification. Despite the importance of proteomics there has, until recently been limited literature describing use of the technique in equines, specifically focused on seminal plasma.

Even though seminal plasma has multiple, significant roles, including support of sperm metabolism and redox regulation of spermatozoa, as well as being rich in antioxidants (51,52,98–100), it is widely recognised that, particularly in a natural setting, the presence of seminal plasma is not necessarily beneficial. This is especially relevant when trying to conserve semen for prolonged periods of time, where it can in fact be damaging to spermatozoa in some stallions (101,102). These effects can be seen when assessing sperm quality in certain stallions using motility and viability tests. This means that semen from these stallions needs processing using centrifugation to remove seminal plasma prior to refrigeration to optimise sperm quality and subsequent pregnancy outcomes. To achieve successful cryopreservation of spermatozoa in the stallion, most of the seminal plasma must be removed in all cases. Under natural conditions seminal plasma is not intended as a storage medium, and although its components which are present in finite amounts do promote the normal function of spermatozoa (103), these cells would usually be exposed to it for a very short amount of time, whilst ejaculation is taking place, until coming into contact with the mare's reproductive tract and endometrial secretions.

In this short period of time several fundamental changes take place in spermatozoa, including possible absorption of antioxidants (104), changes which facilitate capacitation (105) and the ability to respond to progesterone and essential membrane modifications, all of which are necessary for spermatozoa to be able to fertilise the oocyte. Proteins in seminal plasma responsible for changes such as capacitation and spermptosis (101,106,107), of which several have been identified such as clusterin (108) and horse seminal plasma protein 1 (HSP-1) (109) are clearly not beneficial in an in vitro setting where the aim is to maintain sperm quality and viability over prolonged periods of time. This together with numerous studies has led to a wide acceptance that removal of seminal plasma is necessary to maximise longevity of refrigerated sperm (101,106,110). This in combination with appropriately formulated extenders which can help to sequester some of these proteins which are undesirable for longer term cooled storage (111–114) is a technique used to optimise results. Despite this, individual variations in composition and inter-individual variability present in this species mean that the possibility of a negative reaction to certain cooling extenders can still be seen in some individuals (115).

Sperm metabolism

Spermatic metabolism is central to survival of spermatozoa and consists of a sophisticated network of biochemical reactions which are capable of converting nutrients into metabolic substrates. Metabolism in spermatozoa varies between species (116,117), favouring different metabolic pathways for energy

production, ultimately responsible for phosphorylating adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (118,119). In the stallion spermatozoa both glycolytic and oxidative phosphorylation (OXPHOS) pathways are responsible for ATP production. OXPHOS however, is favoured and is the main mechanism for ATP production (120,121) as it results in more efficient production of energy (121) to sustain both motility and membrane integrity (121–127). The OXPHOS pathway occurs in the mitochondria, which acts as a control centre and is an organelle essential for the correct functioning of the spermatozoa in numerous different ways. Mitochondrial activity is indistinguishably linked to fertilising capacity in the stallion (128), in contrast to other species where the glycolysis pathway is favoured (129).

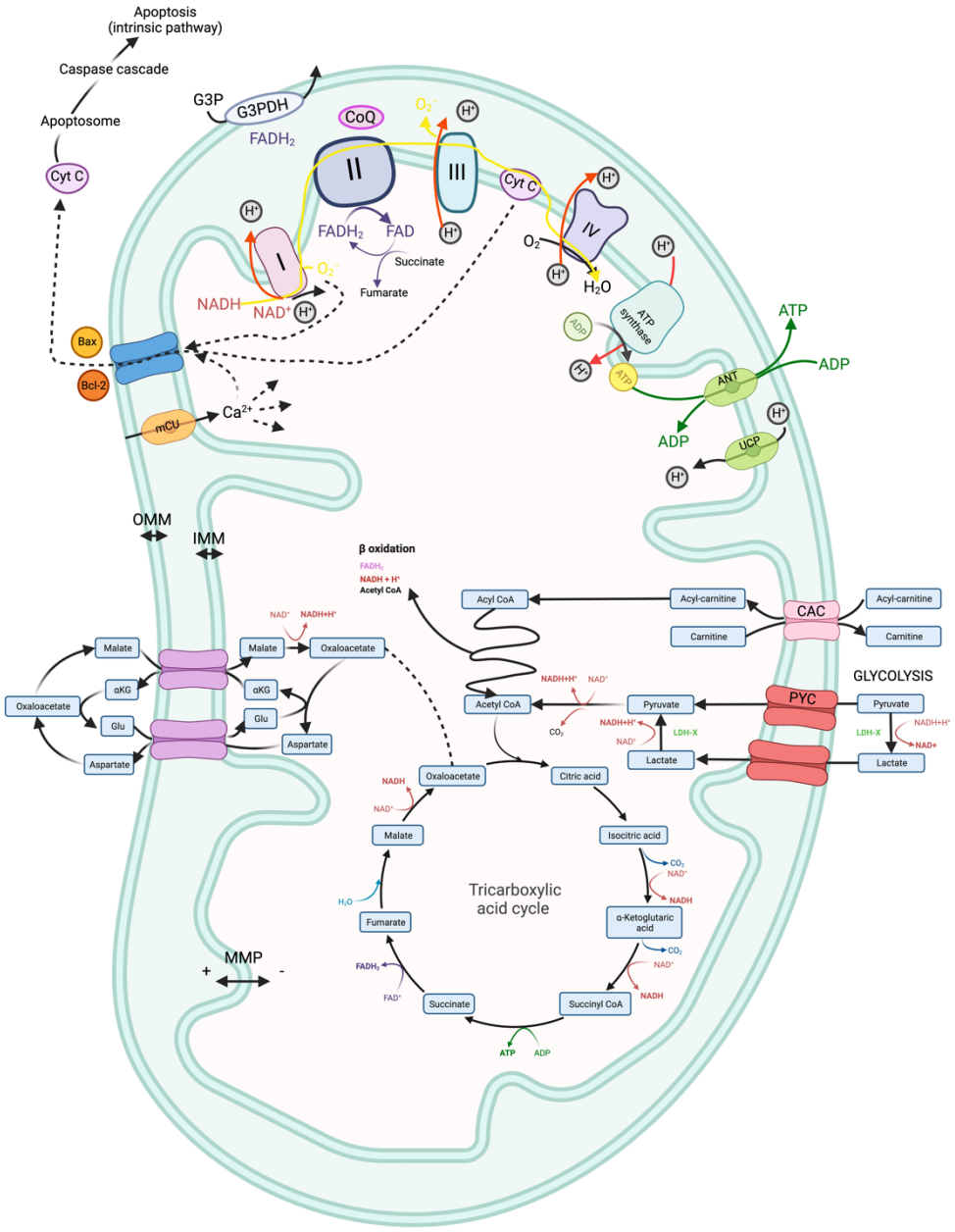


Figure 2. Diagrammatic summary of mitochondrial metabolic pathways and locations where they take place.

In summary, the glycolysis pathway converts glucose into pyruvate. Exogenous hexoses are interiorised by spermatozoa using facilitated diffusion glucose transporters (GLUT) (54). Glucose then undergoes phosphorylation to glucose-6-phosphate in the spermatozoa's cytoplasm after which a number of pathways can be used, including glycolysis to produce pyruvate, glycogen synthesis (130) and the pentose phosphate pathway (PPP). Following this further oxidation of pyruvate, by pyruvate dehydrogenase to acetyl CoA occurs, forming $NADH^+$ in the process. Although traditionally pyruvate was thought to be the main substrate interiorised into the mitochondria to feed the tricarboxylic acid cycle, under aerobic conditions pyruvate is also reduced to form lactate. Lactate has recently been shown to be more efficient than pyruvate at sustaining motility, and can also be oxidised intra-mitochondrially to form pyruvate, essential for energy production in the mitochondria, and key to effectively supporting motility in the equine spermatozoa (125,131,132). Unlike in equines both humans and pigs use glycolysis as the main metabolic pathway to obtain ATP in the spermatozoa (133,134). Although in stallion spermatozoa OXPHOS is favoured, it is not the only source of ATP as certain parts of the spermatozoa have been found to rely on glycolysis, for example, the flagella (120,135).

As with all metabolic pathways glycolysis can also lead to the production of intermediate metabolites which are undesirable and potentially responsible for sperm toxicity, in particular the oxoaldehydes, glyoxal and methyl glyoxal (3,136,137). These metabolites are strong electrophiles due to their carbonyl groups and are therefore able to oxidise lipids, proteins, and DNA in addition to contributing to generation of advanced glycation end products, which cause further damage to spermatozoa.

OXPHOS which is comparatively more efficient, producing 30 ATP molecules per electron transport chain cycle, is thought to be advantageous in a natural context, where speed is of the essence when stallions and mares are free to cohabit in a natural environment (138). OXPHOS is a metabolic pathway formed of a cascade of oxidation-reduction reactions, organised in protein complexes (I-V), known as the electron transport chain (ETC), which act as coordinators for enzyme reactions and soluble factors cytochrome c and coenzyme Q (139). These enzymes are located in the inner mitochondrial membrane and the ETC is responsible for transferring electrons to oxygen (O_2), which is reduced to water, and the energy which is generated is then used to produce ATP. The tricarboxylic acid cycle is closely interlinked with the ETC, and provides NADH and $FADH_2$ which act as electron transport carriers, donating electrons to the ETC. Protein complexes I and II are responsible for the transfer of two electrons to coenzyme Q. These electrons can be donated by the electron carriers NADH and $FADH_2$, independently of the source, which can also be oxidation of fatty acids, metabolism of amino acids and choline metabolism. Complex III acts as a receptor for two electrons from coenzyme Q, transferring these to cytochrome c, to reduce oxygen to water. The ETC undergoes conformational changes provoked by these redox reactions, and as a result pumps H^+ into the intermembrane space, creating the mitochondrial membrane potential. The H^+ pumped into the intramembrane space is also used by complex V, also known as ATP synthase, to produce ATP by phosphorylating ADP.

However, OXPHOS leads to ROS production (140), primarily from the ETC and mitochondrial production of radical superoxide ($O^{\cdot}{}_2^-$), ultimately producing oxidative damage once antioxidant cell reserves have been depleted, eventually causing death (141,142). It is thought that stallions with high fertility levels may display higher levels of OXPHOS compared to their less fertile counterparts, leading to the paradoxical situation observed by several researchers that after a period of conservation spermatozoa from highly fertile stallions show higher levels of oxidative damage compared to those from less fertile

stallions (138). This paradoxical situation leads to the necessity for techniques which can prolong the functional life of these spermatozoa and allow sufficient time for transport to other geographical locations where mares may be located (143) or to allow coordination with ovulation. Therefore, a strict regulation of metabolism together with redox reactions are key factors, where electrons from reduced molecules are accepted by others such as nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and oxygen, causing reactive oxygen species by products such as hydrogen peroxide (H_2O_2) and superoxide radicals, amongst others. This inefficient transport of electrons in the ETC is the main origin of ROS. In order to combat the effects of ROS, spermatozoa are equipped with advanced antioxidant systems, both intracellularly (144–151) and in seminal plasma (52,90), their natural transport media. Failure to regulate the production of ROS plays a significant role in sperm malfunction (120,123,127).

Most reactions responsible for sperm malfunction occur within the mitochondria since $O^{\cdot}{}_2^-$ is anionic and this prevents its diffusion across membranes. These include reactions with iron sulphide (FeS) centres and nitric oxide ($^{\cdot}NO$) causing formation of peroxynitrite ($ONOO^-$), a powerful oxidant, as well as dismutation to H_2O_2, a weak oxidant. H_2O_2 also has regulatory functions (152), such as reversible oxidation of thiol in cysteine residues, and is able to diffuse across cell membranes. Although it is a weak oxidant and plays a regulatory role, its reaction with metal centres can provoke production of extremely toxic hydroxyl radicals ($^{\cdot}OH$). Peroxynitrite on the other hand is stable, but capable of reacting with CO_2 and electrophilic transition metal centres, and this leads to the formation of other strong oxidants such as nitrogen dioxide (NO_2), carbonate radicals ($CO_3^{\cdot-}$) and oxo-metal complexes, all of which cause oxidation, peroxidation, and nitration of multiple parts of the mitochondria.

Furthermore, there is also evidence from early proteomic studies that beta fatty acid oxidation can act as an energy source in equine spermatozoa (16) and also in humans (6), indicating that sperm metabolism is much more complex with a greater plasticity than previously anticipated (7,16,35,116). It is also probable that once sperm are in the female reproductive tract, female factors may provoke modifications and changes to the metabolic pathway favoured by spermatozoa for energy generation (153). In addition to their central role in cell metabolism mitochondria may also be responsible for controlling the lifespan of the spermatozoa (154).

Assisted reproductive technologies

Development and use of assisted reproductive technologies in equines, although now widely used, have, in general, lagged behind other production species. This has in a large part been due to restrictions imposed initially by stud books, limiting their use and inscriptions of offspring born using these methods. To this day some stud books such as that of the Thoroughbred still prohibit the use of artificial reproductive technologies and all foals registered must have been conceived via natural mating.

The use of techniques which allow storage of spermatozoa such as refrigeration or cryopreservation whether for use at a later date, or to allow transport or long-term storage have revolutionised equine breeding in recent decades, inevitably providing breeders with a greater degree of flexibility with respect to timing of ovulation in mares, in addition to other advantages such as a reduction in the risk of the spread of disease, with increased biosecurity and greater choice with regards to genetics.

Cryopreservation of spermatozoa in liquid nitrogen at -196°C is by far the most extensively used technique for long term preservation of male genetic material (155,156). However, despite its widespread use and considerable research into sperm biology and physiology, and the effects of different artificial reproductive technologies, there is still a lack of knowledge regarding the causes of individual variation and responses regarding post thaw survival of spermatozoa and accelerated senescence. Cryopreservation and subsequent thawing cause a significant proportion of the population of spermatozoa to undergo osmotic-induced necrosis, with surviving spermatozoa also suffering changes which affect functionality and lifespan (14). This means that artificial insemination with thawed spermatozoa requires extensive monitoring of the mare, with insemination performed once ovulation is detected (157).

The poor response of some stallion spermatozoa to cryopreservation means this technique is not a viable option for conservation in these individuals. In these cases, refrigeration is often used as an alternative, but provides less flexibility in terms of storage times, with a maximum of 48 hours from the time of extraction for insemination of mares using refrigerated doses for optimal results with currently available extenders. Even though refrigeration of spermatozoa is seen as an alternative short-term storage technique with advantages over cryopreservation in terms of how well it is tolerated by spermatozoa, there is still a population of stallions which produce sperm which either lack quality or are unable to tolerate the stress caused by chilling (158–160).

Both refrigeration and cryopreservation are effective for storage because they slow and limit metabolic activity (161,162), prolonging the viable lifespan of spermatozoa, but they come with a cost. Cryopreservation causes marked changes in the spermatozoa's membrane and mitochondria, and these are probably responsible in a large part for the loss in motility and fertility as well as necrosis that spermatozoa undergo after thawing with oxidative stress being a major component (163–165). During the process of cryopreservation spermatozoa are exposed to hyperosmotic shock during freezing and hypoosmotic shock at thawing (20) in addition to cold shock (166) and toxic cryoprotectants (167) used in cryopreservation media. The osmotic effects affect the mitochondria, causing changes in the ETC and increases in superoxide anion ($O^{\cdot}_{2}^{-}$) production. Eventually, this build-up causes oxidative stress by overwhelming antioxidant defence mechanisms in the spermatozoa causing lipid peroxidation and oxidative damage to DNA, lipids, and proteins (123). Eventually irreversible changes occur, affecting sperm functionality, fertilisation capacity and the ability to support early embryo development (100,137). Proportionately stallion spermatozoa see a greater drop in post thaw motility compared to other species with up to a 50% reduction of pre-cryopreservation motility (37) considered to be normal.

The short-term option for storage, refrigeration, requires the addition of extenders, either with or without additional centrifugation which help to ensure acceptable sperm quality and fertility for up to 48 hours. Addition of substrates and antioxidants to media to provide materials for energy production and scavenge unwanted by-products of OXPHOS (122) and minimise build-up of ROS are essential for ensuring sperm longevity. However, the perfect extender formulation is yet to be determined and there does not appear to be a one size fits all solution with regards to extenders.

The fact that spermatozoa are capable of surviving up to a week in the mares' reproductive tract (168) is indicative that the formulation of extenders is likely suboptimal. Many extenders intended for refrigeration are formulated using extremely high concentrations of glucose, which are much higher than physiological concentrations found in either the stallion or mare reproductive tract and is indicative that these concentrations are likely to be harmful to spermatozoa (3,169). These high glucose

concentrations lead to production of toxic metabolites such as glyoxal and methylglyoxal, as described previously and triggering of metabolic pathways which lead to the overproduction of ROS and mitochondrial damage through the activation of diacylglycerol protein kinase C and NADPH oxidase (170,171), ultimately causing a loss of sperm functionality.

The cost to sperm quality as a result of cold shock has led to interest in the possibility of storing spermatozoa at room temperature, potentially avoiding the negative effects of chilling (122). This concept however brings with it other complications as the metabolic rate is not slowed and the preference of equine spermatozoa for the OXPHOS metabolic pathway (138) can lead to significant ROS production (140), which can negatively affect sperm function (141,172).

Individual variation

The lack of selection in equines for fertility means that encountering differences in sperm quality is commonplace. There are also significant differences between individuals in terms of response of how ejaculates tolerate conservation (17), and this variation is often greater than that between breeds. This means that a one-size fits all protocol for different procedures used for sperm storage is not a practical solution in this species and has led to a general lack of standardisation in a clinical setting. Protocols therefore regularly need to be adjusted in order to achieve the best possible results for individual stallions, which may still be less than optimal in terms of meeting standard commercial quality requirements.

As differences in energy requirements in mitochondria become apparent between species and in individuals of the same species (122,125,127), handling and storage techniques tailored specifically to individuals to achieve best results are becoming inevitable. Providing appropriate energy substrates and using handling techniques to ensure minimal damage to spermatozoa during storage and to prolong sperm longevity will likely become the norm, particularly when dealing with valuable individuals, as the large variation between stallions is a cause of significant economic losses.

Identification through proteomic analysis of specific requirements in certain stallions, as a result of spermatozoa and seminal plasma protein composition is therefore key to understanding the molecular behaviour of the equine spermatozoa in general, and at an individual level. This knowledge can then be applied to optimise components such as extender composition or to adapt cryopreservation protocols or refrigeration, such as removal of seminal plasma, to achieve optimal results for sperm storage.

Biomarkers

Biomarkers are unique biological or biologically derived objective, quantifiable indicators of a process, event, or condition that can be measured, evaluated, and compared (198).

In the context of reproduction identification of both diagnostic and predictive biomarkers (199) is the first step towards understanding modifications and abnormalities at a molecular level which may lead to infertility and subsequently improvement of infertility management in the male (200). As many of the currently used tests to classify sperm quality are often normal, this unfortunately leads to the necessity for further, more invasive tests, such as testicular biopsy, which carries its own risks (201,202). Therefore, the identification of techniques which are non-invasive and can precisely diagnose and

identify, as well as provide a prognosis for specific causes of fertility problems or reactions to ART's are essential.

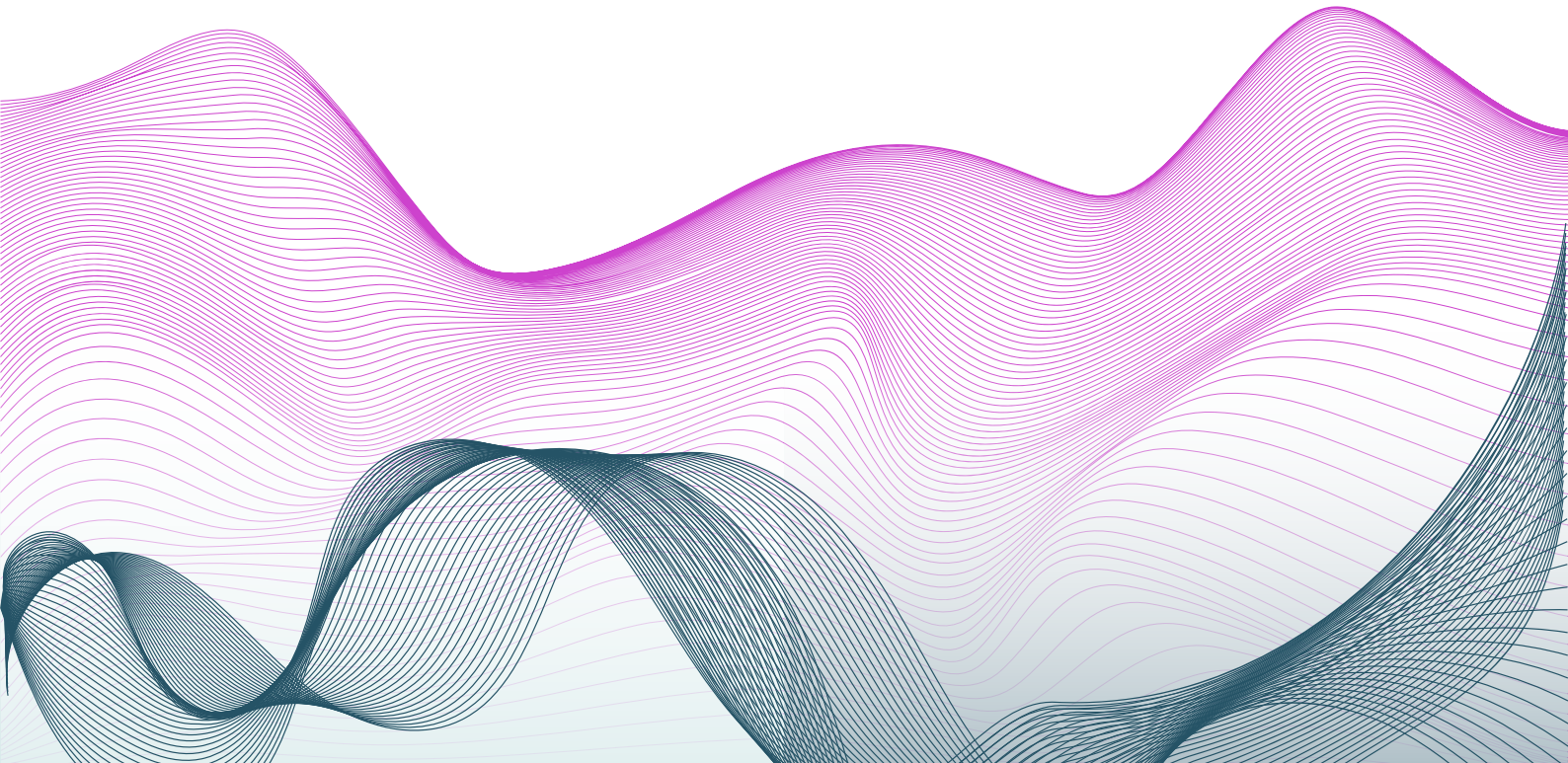
Proteomics

Spermatozoa are ideal cells for proteomic analysis due to their post transcriptional and translational silence, in addition to being individual cells which are easy to isolate. Proteomic analysis is typically performed using mass spectrometry, which allows large scale, high throughput characterisation and quantification of proteins, and includes identification, modification, quantification, and localisation. The use of mass spectrometry for large scale analysis of protein characterisation entails the transfer of polar, non-volatile, and thermally unstable proteins and peptides into the gas phase, whilst avoiding degradation of samples (173) and the particular approach used depends on the sample complexity and final goals of the analysis (174). Proteomics comprises one of the "omics" techniques and is highly complementary to the already well implemented genomics, transcriptomics and metabolomics technologies, providing extremely useful, interrelated information, essential for understanding how proteins, the cellular building blocks, directly assert potential functions of genes via enzymatic catalysis, molecular signalling, and physical interactions (175).

Although in humans the first research which looked at the use of proteomics for identification of variability in semen samples was published nearly two decades ago (176), the implementation of the use of proteomics for the identification of biomarkers indicative of fertility in clinical practice in equine spermatozoa is relatively recent. However, it is proving to be a powerful tool in this and other species, allowing in depth analysis and comparison of the protein composition of spermatozoa under different conditions (i.e. healthy versus disease, or for the study of the pathogenesis of sperm cryo-damage with the use of different ART's), uncovering pathological mechanisms at a phenotypic level (177) as sperm proteins are known to be crucial in the maintenance of normal sperm morphology, motility, acrosome formation and reaction, capacitation and fertilisation (178). The use of proteomics has led to incredible advances in the understanding of the biology of these cells in a very short space of time, especially regarding knowledge of sperm metabolism and its unexpected plasticity (6,10,16,35,95,96,179,180).

Existing research has shown that numerous diseases and disorders are linked to defects in post translational modifications of proteins, which are essential for the maintenance of normal cellular states (181), and there is also data indicating that some conditions affecting male reproductive capacity, including sperm motility are a result of deregulation of post translational modifications which can be both reversible and irreversible (182). It is widely recognised that phosphorylation of certain proteins in spermatozoa is one of the most important post translational modifications, affecting an estimated one-third of all cell proteins (183), many of which are phosphorylated at one or more sites (serine, threonine and tyrosine) (184). These changes play an essential role in regulating some of the processes which occur in spermatozoa and are vital for their correct function, including fertilisation, motility, capacitation, and acrosome reactions (185). Studies in different species looking at phosphoregulation in numerous other processes including sperm formation (186,187), maturation (188), capacitation (189–193) and motility (185,194–197) have found that it is highly significant for proper regulation of spermatozoa, and subsequently indicative of quality. In the future phosphoproteomics will allow a more detailed analysis of the biochemical basis of poor semen quality and is already being used to identify potential biomarkers which can be used to categorise pathologies or conditions leading to infertility.

O B J E C T I V E S



Understanding of the molecular function of equine spermatozoa is constantly evolving, and the concept of oxidative stress in the spermatozoa has evolved from the excessive production of reactive oxygen species to the disruption of redox regulation. Yet there are still large gaps in our understanding of this cell at a molecular level, which is largely influenced by its protein composition. Therefore, the purpose of this thesis is to further contribute to knowledge of protein composition and molecular function of the equine spermatozoa and how this affects responses to technologies and processes used for its preservation. For this, specific objectives were proposed:

1. Development of proteomics techniques in the stallion for analysis of equine seminal plasma composition:

   1.1. Analysis of the protein composition of equine seminal plasma using UHPLC/MS/MS improving the understanding of molecular function.

   1.2. Identification of discriminate biomarkers in equine seminal plasma predictive of sperm quality.

   1.3. Identification of discriminate biomarkers in equine seminal plasma predictive of responses to commonly used artificial reproductive technologies such as cooling, centrifugation and cryopreservation.

2. Proteomic study of the equine spermatozoa and changes induced in this cell by cryopreservation:

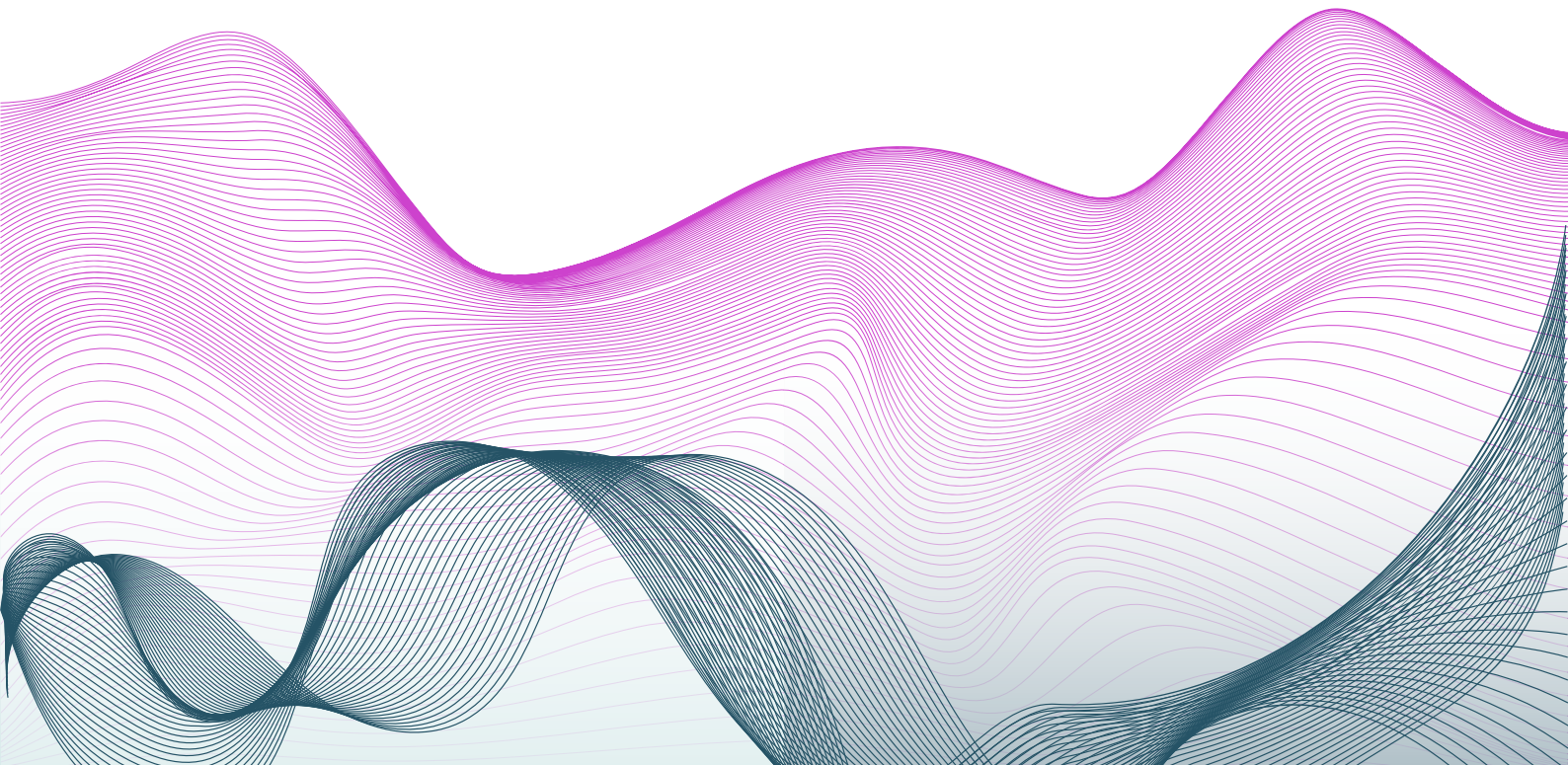
   2.1. Analysis of the protein composition of equine spermatozoa using UHPLC/MS/MS aiding understanding of molecular function and key metabolic pathways.

   2.2. Identification of proteins associated with dysregulation of the equine spermatozoa due to processing for cryopreservation.

   2.3. Identification of discriminate biomarkers in equine spermatozoa which are predictive of outcomes after cryopreservation procedures.

3. Study of the stallion sperm phosphoproteome to identify changes in phosphoproteins after cryopreservation of spermatozoa, providing an improved understanding of molecular changes induced by the procedure.

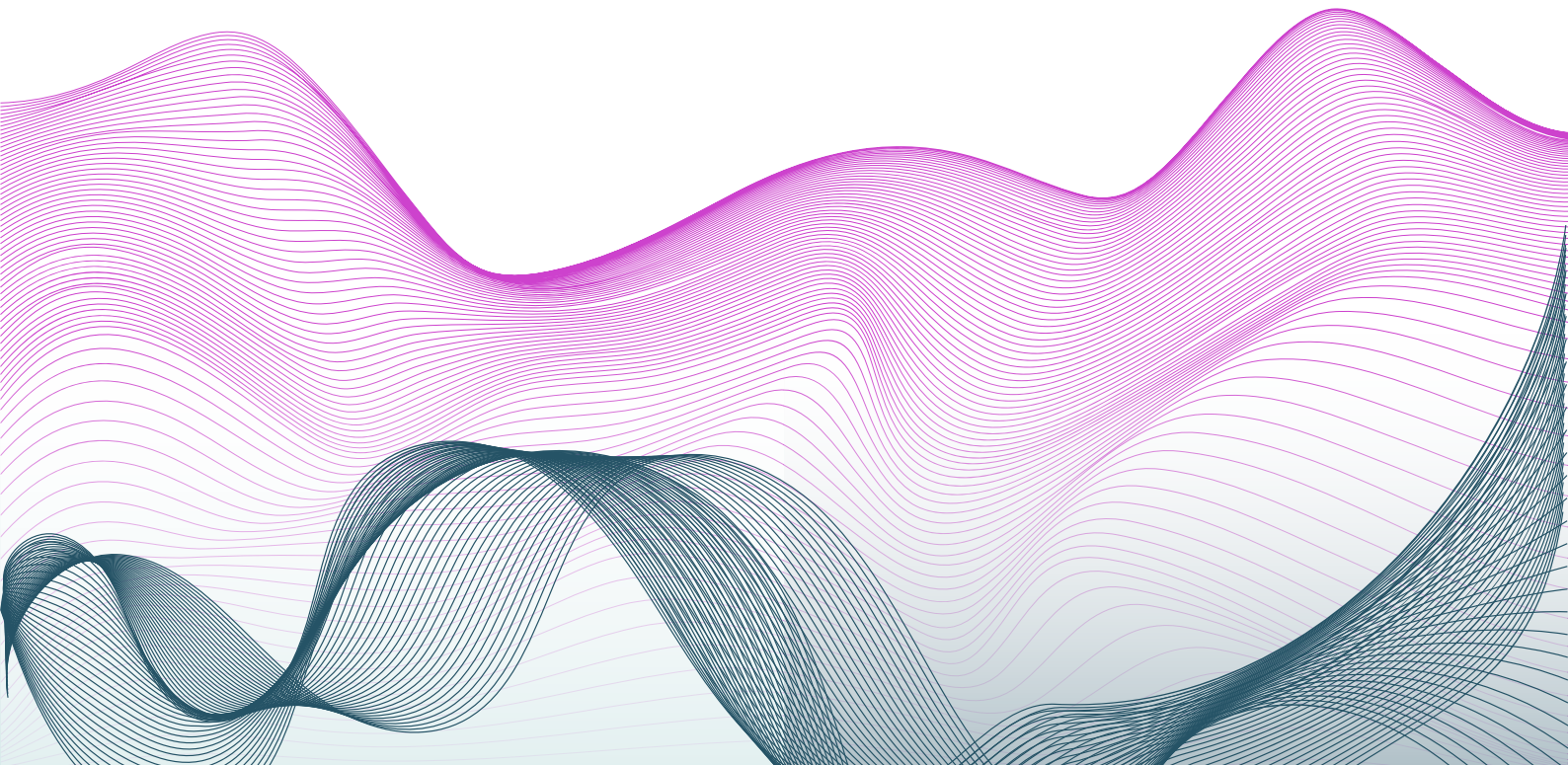
J U S T I F I C A T I O N



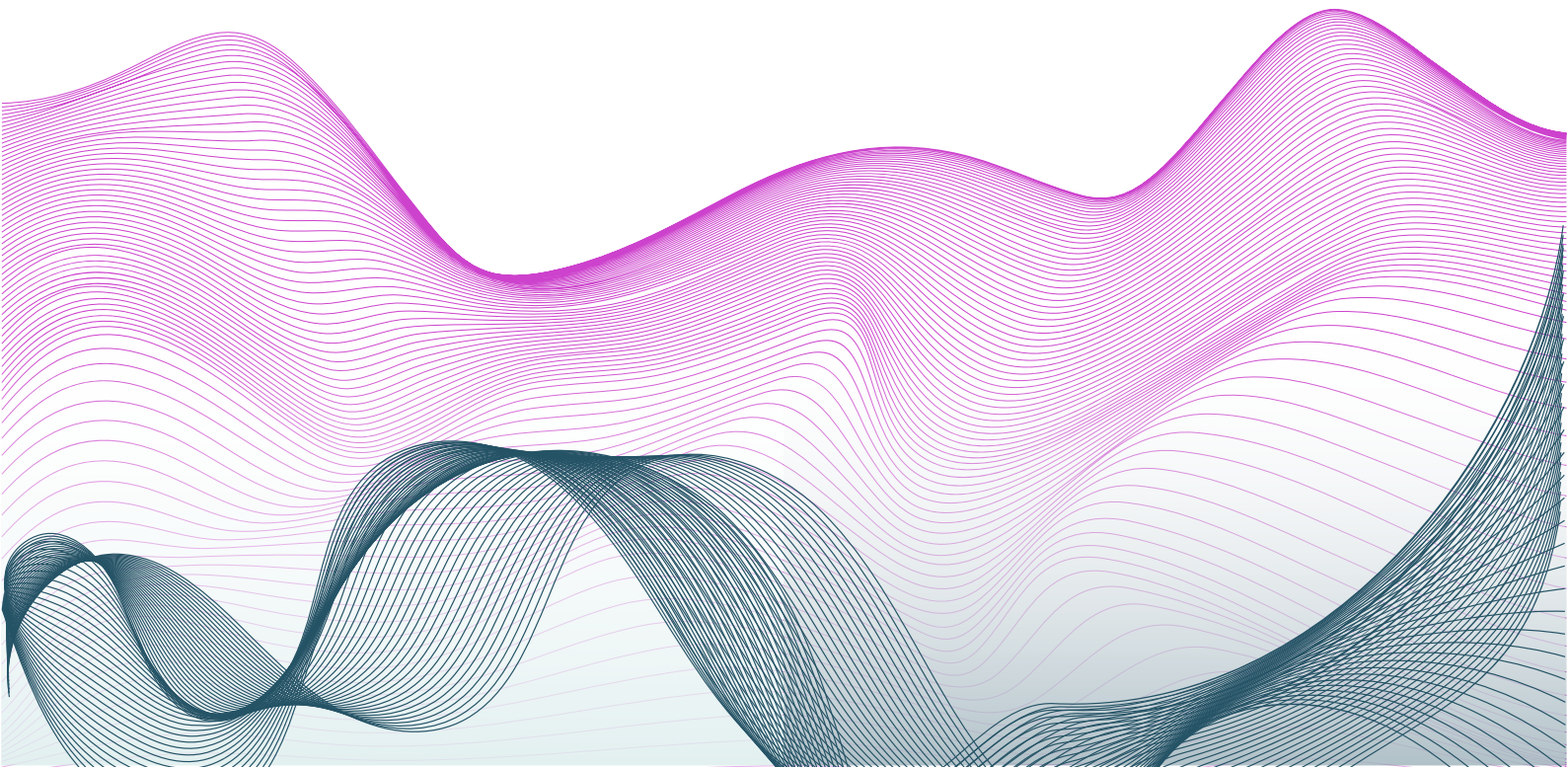
Despite the now widespread commercialisation of equine semen in both refrigerated and cryopreserved forms, there has been a lack of in depth understanding of the intricate workings of the metabolism and cellular biology of the equine spermatozoa and factors which affect the varying behaviours of spermatozoa from this species, in response to different artificial reproductive technologies. This lack of understanding and variation between individuals has meant there is a need for standardisation of protocols for seminal conservation, in addition to varying outcomes regarding spermatic quality which have traditionally been widely accepted.

More recent widespread access to the use of the omics technologies is shedding new light on factors which influence sperm quality and how spermatozoa from different individuals react to different reproductive technologies used for conservation. In this thesis, shotgun proteomics was used for the first time in this species to identify differences in the protein composition of seminal plasma and sperm of individual stallions and look at how particular protein biomarkers can be used to identify and predict the responses of spermatozoa to certain reproductive technologies.

RESULTS



ARTICLE 1



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Research Article

Research Article

Seminal plasma AnnexinA2 protein is a relevant biomarker for stallions which require removal of seminal plasma for sperm survival upon refrigeration†

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Abstract

Some stallions yield ejaculates that do not tolerate conservation by refrigeration prior to artificial insemination (AI), showing improvement after removal of most of the seminal plasma (SP) by centrifugation. In this study, the SP-proteome of 10 different stallions was defined through high-performance liquid chromatography with tandem mass spectrometry and bioinformatic analysis in relation to the ability of the ejaculates to maintain semen quality when cooled and stored at 5°C. Stallions were classified into three groups, depending on this ability: those maintaining good quality after direct extension in a commercial extender (*good*), stallions requiring removal of seminal plasma (RSP) to maintain seminal quality (*good-RSP*), and stallions, unable to maintain good semen quality even after RSP (*poor*). Pathway enrichment analysis of the proteins identified in whole equine SP using human orthologs was performed using g: profiler showing enriched Reactome and the Kyoto Encyclopedia of Genes and Genomes pathways related to hexose metabolism, vesicle mediated transport, post translational modification of proteins and immune response. Specific proteins overrepresented in stallions tolerating conservation by refrigeration included a peroxiredoxin-6 like protein, and transcobalamin-2, a primary vitamin B12-binding, and transport protein. Also, the protein involved in protein glycosylation, ST3 beta-galactoside alpha-2,3-sialyltransferase 1 was present in *good* stallions. These proteins were nearly absent in *poor* stallions. Particularly, annexinA2 appeared as to be the most powerful discriminant variable for identification of stallions needing RSP prior to refrigeration, with a $P = 0.002$ and a q value $= 0.005$.

Overall this is the first detailed study of the equine SP-proteome, showing the potential value of specific proteins as discriminant bio-markers for clinical classification of stallions for AI.

Summary sentence

The seminal plasma protein Annexin A2 identifies ejaculates needing removal of seminal plasma prior to conservation in refrigeration.

Key words: seminal plasma, UHPLC/MS/MS, proteins, artificial insemination, horse.

Introduction

Despite artificial insemination (AI) being successfully used in equine breeding, several obstacles exclude valuable stallions from wider use in AI with refrigerated, extended semen. Semen from these stallions does not tolerate storage at 5°C for a sufficient period of time to allow for shipping; this is manifested as a drop in motility below acceptable values for a commercial dose [1]. However, motility improves in the ejaculates from many of these stallions when most of the seminal plasma (SP) is removed by centrifugation [2, 3], suggesting a component of the SP plays a part in how tolerant the ejaculate is of being stored by refrigeration [4]. SP in stallions is composed of secretions from the epididymis and the accessory glands of the male genital tract, particularly those from the prostate. Stallions produce ejaculates with large volumes and the SP is involved in different functions; from acting as a vehicle, nourishing, and regulating spermatozoa viability [5–9] to interacting with the mare's endometrium [5, 10–12]. Although many proteins in equine SP have previously been characterized [6, 13–19], detailed proteomic studies of equine SP using shot gun proteomics are not yet available. The use of different omics is rapidly advancing our knowledge of sperm biology [20–24], and despite the importance of proteomics, it is a relatively recent field in spermatology and, in the particular case of equines, only two papers have been published to date using shot gun proteomics [25, 26]. However, there is a previous paper addressing the proteome of equine SP [18]. Nevertheless, proteomic studies are providing striking new findings in sperm biology. Owing to the enormous need to link novel findings in sperm biology, including the complexity and plasticity of metabolism and the presence of proteins involved in transcription and translation [20, 27, 28] suggesting a role in early embryo development; the present study aimed to: first provide an in depth description of the proteome of equine SP, and second to test the hypothesis that specific bio-markers which indicate the ability of ejaculates to withstand refrigeration for AI can be detected in equine SP.

Material and methods

Reagents and media

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

Semen collection and processing

Semen was collected from 10 stallions maintained as indicated under European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). All stallions were of proven fertility, with a median age of 11 years (range 7 to 20 years old), including 5 Andalusians, 1 Spanish Sport Horse, 1 Lusitano, 1 Arab, 1 Anglo-Arabian, and 1 Spanish-Arabian horse. The study was supervised and approved by the ethical committee of the University (IB16030). Semen collection was carried out using a warmed Missouri model artificial vagina following standard protocols at our center, using a filter to separate the gel fraction. Immediately after collection, ejaculates were processed in the adjacent laboratory, and every ejaculate was processed using colloidal centrifugation [29, 30] to isolate SP. In brief, ejaculates were processed by single layer colloidal centrifugation using Equipure (Nidacon Mölndal, Sweden) following the instructions provided by the manufacturer, and centrifuged for 20 min at 300 *g* at room temperature.

Experimental design

Three ejaculates from each of the 10 stallions with different abilities to withstand semen extension and refrigeration for AI were used in this study (30 biological replicates plus two technical replicates for each). Additional independent ejaculates from the same stallions were used for sperm functional analysis, where ejaculates were split into two aliquots and one was directly extended in INRA96 (IMV Technologies, L'Aigle, France), while the other had the SP removed by centrifugation before extension. Both aliquots were extended to 25×10^6 sperm/mL in INRA 96 and kept refrigerated in volumes of 20 mL at 5°C for 48 h. After 24 and 48 h of incubation, total motility and velocities were measured in both groups (with and without SP) using computer assisted sperm analysis (CASA). Based on this analysis stallions were classified into three groups: *good* if total motility after 48 h was > 50% and curvilinear velocity (VCL) was >100 µm/s, *good after removal of seminal plasma (good-RSP)*, if after centrifugation and removal of the bulk of SP motility after 48 h was >50% and VCL was >100 µm/s and *poor*, for those ejaculates where even after RSP total motility after 48 h was <50%, and VCL was <100 µm/s.

Sample preparation

Samples were processed immediately after collection. Aliquots of isolated SP were kept frozen at −80 °C until further analysis. Phase contrast microscopy was used to control the absence of spermatozoa, moreover SP was filtered (0.22 µM) before snap freezing and further processing.

Protein solubilization

Aliquots of SP were solubilized in lysis buffer (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7 M urea, 2 M thiourea, and 40 mM Tris (pH 10.4) and incubated under constant rotation at 4°C for 1 h.

Protein quantification

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

In-solution trypsin digestion

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

UHPLC–MS/MS analysis

The UHPLC/MS system used was an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High Speed Binary Pump coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. The MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01) controlled the UHPLC and Q-TOF. Every sample was injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 µm, 150 × 2.1 mm, Agilent Technologies), thermostatted at 55°C at a flow rate of 0.4 mL/min. The gradient program started with 2% of buffer B (water/acetonitrile/formic acid, 10:89.9:0.1) that remained in isocratic mode for 5 min and then increased linearly up to 45% B over 40 min, further increasing up to 95% B for 15 min and remaining constant for an additional 5 min. After this 70 min run, 5 min of post-time followed using the initial condition for conditioning of the column for the next run. The mass spectrometer was operated in positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 L/min at 250°C. The sheath gas flow was set to 300°C. The peak to peak voltages of the capillary spray, fragmentor and octopole RF were 3500, 340, and 750 V, respectively. Profile data were acquired for both mass spectrometry (MS) and tandem mass spectrometry (MS/MS) scans in extended dynamic range mode, where the MS and MS/MS mass ranges were 50–1700 m/z and scan rates were 8 spectra/s for MS and 3 spectra/s for MS/MS. Auto MS/MS mode was used with precursor selection in abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of −4.8. The same ion was rejected after two consecutive scans.

Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH] + 50–10000 m/z; maximum precursor charge +5; retention time and m/z tolerance ±60 s; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against the appropriate and updated protein database (https://www.uniprot.org/uniprot/?query=Equus+caballus&sort=score, accessed 4/07/20) was performed following these criteria: non fixed modifications were selected, and the following variable modifications were selected: carbamidomethylated cysteines, tryptic digestion with a maximum of five missed cleavages, ESI-Q-TOF instrument, minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation of reversed database scores. Validation of peptide and protein data was performed using auto thresholds with a false discovery rate (%FDR) of 1.2%. The results for proteins were obtained as protein summarized using all validations; score > 4 and scored peak intensity (%SPI: the percentage of the extracted spectrum that is explained by the database search result) 60.

SP proteome analysis

The proteins identified in SP were queried for gene ontology (GO) terms (http://geneontology.org) according to cellular component (CC), biological process (BP), and molecular function (MF) and classified using (PANTHER v.14.0) (http://www.pantherdb.org), and g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) following detailed published protocols [31–33]. The lists of proteins were queried against the equine proteome database (https://www.uniprot.org/taxonomy/9796) for significant enrichment using the Fisher's exact test corrected with a FDR set at $P < 0.05$.

Enrichment analysis of pathways in the SP proteome

PANTHER (http://www.pantherdb.org/pathway/pathwayList.jsp) and KEGG pathway (https://www.genome.jp/kegg/) [34–37] analysis were used to identify biological pathways likely to be active in SP. The significance of the presence of the protein list was queried against the equine proteome database using a FDR < 0.05 and Fisher's exact test. g:Profiler was also used to perform an enrichment analysis [33]. Because of the increased depth of the human proteome in terms of annotation, the equine annotations were transformed to their human orthologs using g:Profiler (https://biit.cs.ut.ee/gprofiler/orth) and a pathway enrichment analysis and visualization was performed again using g:Profiler and Cytoscape analysis using Reactome (https://reactome.org).

Network analysis

Cytoscape (https://cytoscape.org) plug in ClueGo was used to identify functionally grouped GO terms in equine SP as described previously [38, 39].

STRING (https://version-10-5.string-db.org) was used to identify potential functional partners of specific proteins.

Identification of discriminant proteins in SP

Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins in the SP based on spectral counts among stallions classified in function of the period of time that their ejaculates can be stored under refrigeration. Variance filtering was used to reduce the noise and improve the detection of discriminant variables [40]. Identification of discriminant variables between subgroups of stallions (*good*, *good-RSP*, and *poor*) was performed by fitting a linear model for each variable with the condition of semen lifespan under refrigeration as predictor. *P*-values were adjusted for multiple testing with the Benjamini–Hochberg method [41], variables with adjusted P values < 0.1 were considered significant. T-tests between two different conditions were also performed and differences were considered significant when $P < 0.05$. Data were Log_2 transformed and normalized, and comparisons were performed filtered by fold-change > 2.

Table 1. Panther overrepresentation test for proteins of interest identified by UHPLC/MS/MS GO analysis of equine (*Equus caballus*) seminal plasma.

GO biological process (BP)	Fold-enrichment	*P* value
Hemoglobin metabolic process (GO:0020027)	40.14	1.32E-04
ATP generation from ADP (GO:0006757)	21.15	1.28E-07
Glycolytic process (GO:0006096)	21.15	1.28E-07
ADP metabolic process (GO:0046031)	16.81	5.05E-07
Purine ribonucleoside diphosphate metabolic process (GO:0009179)	14.90	1.04E-06
Purine nucleoside diphosphate metabolic process (GO:0009135)	14.90	1.04E-06
Pyruvate metabolic process (GO:0006090)	14.57	1.20E-06
Ribonucleoside diphosphate metabolic process (GO:0009185)	14.25	1.37E-06
Nucleoside diphosphate phosphorylation (GO:0006165)	13.95	1.55E-06
Nucleotide phosphorylation (GO:0046939)	13.66	1.77E-06
Cellular response to hydrogen peroxide (GO:0070301)	12.32	8.74E-05
Response to hydrogen peroxide (GO:0042542)	11.02	3.01E-05
Carbohydrate catabolic process (GO:0016052)	10.86	1.54E-06
Nucleoside diphosphate metabolic process (GO:0009132)	10.57	8.28E-06
Cellular response to antibiotic (GO:0071236)	8.65	1.05E-04
ATP metabolic process (GO:0046034)	5.59	1.37E-04
Cellular response to organic cyclic compound (GO:0071407)	4.16	1.16E-05
Carbohydrate metabolic process (GO:0005975)	3.89	2.39E-05
Response to drug (GO:0042493)	3.10	8.73E-05
CC morphogenesis (GO:0032989)	2.82	1.03E-04
Regulation of response to stress (GO:0080134)	2.73	7.73E-06

PANTHER Overrepresentation Test (Released 2020-04-07) GO Ontology database Released 2020-02-21 Fisher's Test. ATP, adenosine triphosphate; ADP, adenosine diphosphate; FDR, false discovery rate; GO, Gene Ontology; CC, cellular component; UHPLC/MS/MS, ultra high-performance liquid chromatography with tandem mass spectrometry.
FDR, $P < 0.05$.

Computer-assisted Sperm Analysis (CASA)

Sperm motility and velocities were assessed with a CASA system (ISAS Proiser, Valencia, Spain) [30, 42]. Samples were loaded into a Leja chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 38°C. Sixty consecutive digitized images obtained using a ×10 negative phase-contrast objective (Olympus CX 41) and 500 spermatozoa per sample were then analyzed in random fields. Spermatozoa VAP > 35 µm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile. The following parameters were measured: percentages of linearly motile spermatozoa, circular (VCL) straight line (VSL) and average (VAP) velocities in µm/s.

Statistical analysis

The normality of the motility and sperm velocity data was assessed using the Kolmogorov–Smirnoff test. Paired t-tests and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 7.00 for Mac, (La Jolla, CA, USA, (www.graphpad.com).

Results

Three ejaculates from each of 10 stallions were used in this study, and 3544 different proteins were identified and matched to the equine proteome database. The complete list of SP-proteins is provided as supplementary material (Supplementary Table 1).

GO analysis of SP proteins

GO analysis (BPs) of SP-proteins returned enriched terms related to metabolism (Table 1), only terms with a fold-enrichment > 2 are provided. Interestingly, terms related to redox regulation were also present including the terms *cellular response to hydrogen peroxide* (GO:0070301) and *response to hydrogen peroxide* (GO:0042542). GO terms for CCs are provided in Table 2 and include terms related to *immunological synapse* (GO:0001772) responses and *extracellular space* (GO:0005615) and *extracellular region* (GO:0005576). The g:profiler analysis of orthologs revealed, in addition to terms related to metabolism, an enrichment in GO terms (CCs) related to *extracellular vesicles* (GO:1903561), *extracellular exosome* (G0:0070062) *and vesicle-mediated transport* (GO:0016192) (Figure 1.), suggesting a role for SP in promoting cell-to-cell communication. Notably, the term *animal organ development* (GO:0048513) was observed in the enrichment.

Pathway enrichment analysis

The results of the pathway enrichment analysis using g:profiler with human orthologs are given in the Manhattan plot in Figure 2 and accompanying table. Reactome and KEGG pathways related to *hexose metabolism*, *vesicle mediated transport, post translational modification of proteins*, and pathways related to the *immune response* were enriched.

Functional network analysis

The ClueGo analysis employed GO and KEGG data resources to classify the proteome of the equine SP in a functional network. In order to reduce the complexity of the network the fusion option was applied. The network obtained is presented in Figure 3. The functional network obtained suggested important roles of SP proteins in the regulation of ovulation, of transforming growth factor beta (TGF-β) and of mitochondrial membrane permeability.

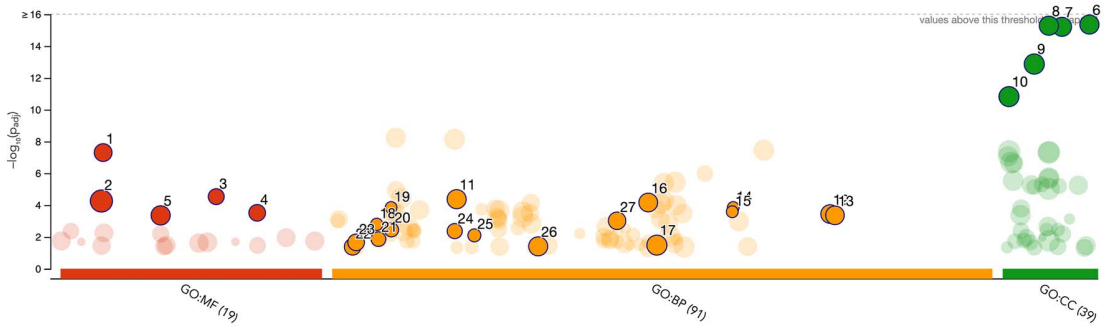
Impact of the equine SP-proteome on the preservation of chilled semen

Stallion ejaculates were collected and evaluated in terms of life span after extension and refrigeration at 5°C for 48 h. Three different

Table 2. Panther overrepresentation test (GO CC complete) of proteins of interest identified by UHPLC/MS/MS and GO analysis of equine (*Equus caballus*) SP.

GO Cellular component (CC)	Fold-enrichment	*P* value
Immunological synapse (GO:0001772)	13.38	3.46E-04
Mitotic spindle (GO:0072686)	6.76	1.19E-04
Microtubule cytoskeleton (GO:0015630)	2.38	1.78E-04
Cytoskeleton (GO:0005856)	2.31	2.01E-06
Extracellular space (GO:0005615)	2.23	2.06E-04
Extracellular region (GO:0005576)	1.98	1.36E-04
Intracellular non-membrane-bounded organelle (GO:0043232)	1.91	1.94E-07
Nonmembrane-bounded organelle (GO:0043228)	1.91	1.96E-07

PANTHER Overrepresentation Test (Released 20200407) GO Ontology database Released 2020-02-21 Fisher's Test. CC, cellular component; FDR, false discovery rate; GO, gene ontology; SP, seminal plasma; UHPLC/MS/MS, ultra high-performance liquid chromatography with tandem mass spectrometry.
FDR, $P < 0.05$.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0008092	cytoskeletal protein binding	4.973×10^{-8}
2	GO:MF	GO:0005515	protein binding	5.547×10^{-5}
3	GO:MF	GO:0045296	cadherin binding	2.938×10^{-5}
4	GO:MF	GO:0050839	cell adhesion molecule binding	3.071×10^{-4}
5	GO:MF	GO:0019899	enzyme binding	4.437×10^{-4}
6	GO:CC	GO:1903561	extracellular vesicle	4.411×10^{-16}
7	GO:CC	GO:0070062	extracellular exosome	6.122×10^{-16}
8	GO:CC	GO:0043230	extracellular organelle	5.231×10^{-16}
9	GO:CC	GO:0031982	vesicle	1.354×10^{-13}
10	GO:CC	GO:0005615	extracellular space	1.505×10^{-11}
11	GO:BP	GO:0016192	vesicle-mediated transport	4.254×10^{-5}
12	GO:BP	GO:0120036	plasma membrane bounded cell projection organization	3.658×10^{-4}
13	GO:BP	GO:0140352	export from cell	4.389×10^{-4}
14	GO:BP	GO:0061718	glucose catabolic process to pyruvate	1.411×10^{-4}
15	GO:BP	GO:0061615	glycolytic process through fructose-6-phosphate	2.629×10^{-4}
16	GO:BP	GO:0046903	secretion	6.879×10^{-5}
17	GO:BP	GO:0048513	animal organ development	3.292×10^{-2}
18	GO:BP	GO:0006007	glucose catabolic process	1.626×10^{-3}
19	GO:BP	GO:0006735	NADH regeneration	1.411×10^{-4}
20	GO:BP	GO:0006757	ATP generation from ADP	3.563×10^{-3}
21	GO:BP	GO:0006090	pyruvate metabolic process	1.378×10^{-2}
22	GO:BP	GO:0002283	neutrophil activation involved in immune response	4.206×10^{-2}
23	GO:BP	GO:0002446	neutrophil mediated immunity	2.201×10^{-2}
24	GO:BP	GO:0016052	carbohydrate catabolic process	4.308×10^{-3}
25	GO:BP	GO:0019320	hexose catabolic process	8.009×10^{-3}
26	GO:BP	GO:0033554	cellular response to stress	3.915×10^{-2}
27	GO:BP	GO:0045055	regulated exocytosis	9.652×10^{-4}

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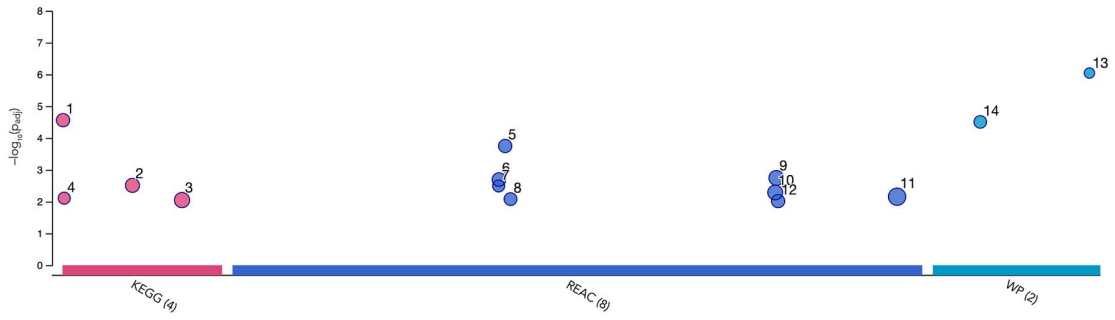
organism	hsapiens

g:Profiler

Figure 1. g:GOST multiquery Manhattan plot showing enrichment analysis of whole equine seminal plasma proteins. The whole equine seminal plasma proteome was queried against the equine proteome database. Gene Ontology (GO) terms for molecular function (MF) are in red, for biological process (BP) in orange, and for cellular component (CC) in green. The *P* values are depicted on the y axis and in more detail in the results table below present the image.

outcomes were evident (Figure 4); *good* were those stallions in which total motility and VCL were >50% and 100 µm/s respectively. Some stallions required SP-elimination to remain above these values, *good-RSP* and a third group classified as *poor* was unable to maintain these values despite RSP. The proteome was studied in these three groups, and differences observed between groups with significant differences in the number of proteins over or underrepresented in each group. In particular, numerous proteins were detected as



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	KEGG	KEGG:00010	Glycolysis / Gluconeogenesis	2.747×10^{-5}
2	KEGG	KEGG:04066	HIF-1 signaling pathway	3.081×10^{-3}
3	KEGG	KEGG:05132	Salmonella infection	8.960×10^{-3}
4	KEGG	KEGG:00051	Fructose and mannose metabolism	7.784×10^{-3}
5	REAC	REAC:R-HSA-70171	Glycolysis	1.785×10^{-4}
6	REAC	REAC:R-HSA-70326	Glucose metabolism	2.028×10^{-3}
7	REAC	REAC:R-HSA-70263	Gluconeogenesis	3.217×10^{-3}
8	REAC	REAC:R-HSA-3214842	HDMs demethylate histones	8.338×10^{-3}
9	REAC	REAC:R-HSA-2990846	SUMOylation	1.804×10^{-3}
10	REAC	REAC:R-HSA-3108232	SUMO E3 ligases SUMOylate target proteins	5.166×10^{-3}
11	REAC	REAC:R-HSA-5653656	Vesicle-mediated transport	7.010×10^{-3}
12	REAC	REAC:R-HSA-4551638	SUMOylation of chromatin organization proteins	9.655×10^{-3}
13	WP	WP:WP4629	Computational Model of Aerobic Glycolysis	9.040×10^{-7}
14	WP	WP:WP534	Glycolysis and Gluconeogenesis	3.119×10^{-5}

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organism	hsapiens

g:Profiler

Figure 2. g:GOST multiquery Manhattan plot showing comparative enrichment analysis of whole equine seminal plasma proteins. The whole equine seminal plasma proteome was queried against the equine proteome database. Kyoto Encyclopedia of Genes and Genomes (KEGG) are depicted in red, Reactome pathways in dark blue and Wikipathways in light blue (all using human orthologs). The *P* values are depicted on the y axis and in more detail in the results table present below the image.

being underrepresented in the *poor* group of stallions. In order to identify specific proteins in each of the groups that may explain the differences observed, an ANOVA was performed and a heat map constructed (Figure 5), specific proteins overrepresented in both groups of *good* stallions included a *peroxiredoxin-6 like protein*, and *transcobalamin-2*, a primary vitamin B12-binding and transport protein. Also, the protein involved in protein glycosylation, *ST3 beta-galactoside alpha-2,3-sialyltransferase 1* was present in the *good* stallions (Figure 6). These proteins were nearly absent in *poor* stallions and the glucagon receptor was absent in samples from stallions in the *poor* group. Following this, volcano plots were used to explore potential candidates that may explain differences between stallions, and revealed that *poor* stallions were characterized by low levels of antioxidant proteins, particularly *catalase, superoxide dismutase* and *peroxiredoxin 6 like protein* (Figure 7C), while *good* stallions presented high amounts of peroxiredoxin 6 like protein (Figure 7A) and *annexin A2* was enriched in stallions requiring SP-removal (Figure 7B).

More specifically, and due to its practical importance, differences between stallions that need RSP to withstand refrigeration, from those that withstand refrigeration after extension in the presence of SP were explored, focusing on which proteins were differentially expressed by two groups of stallions classed as: *good* or *good-RSP*. In the volcano plots, two proteins (*annexinA2* and *spectrin beta chain)* were underrepresented in *good* stallions while overrepresented in *good-RSP* stallions (Figure 8A and B). In particular, annexinA2 was revealed as a powerful discriminating variable for identification of *good-RSP* stallions (Figure 9), with a $P = 0.002$ and a q value = 0.005. STRING was used to identify potential functional partners of this protein, and included *Protein S100-A10, Kalirin, Obscurin, Plasminogen, Plasminogen activator, Trio Rho guanine nucleotide exchange factor, Annexin A11, Caspase4*, and *Caspase 9* (Figure 8C).

Discussion

This study investigated the proteome of SP in horses using a shot gun proteomics approach and a bioinformatic enrichment analysis. Although previous studies have addressed equine SP [12, 16, 43–48], this is, to the authors' knowledge, the first study focused not only on the detailed description of the SP-proteome but also linking specific SP proteins with the ability of the ejaculate to withstand storage by refrigeration at 5°C for 48 h. Enrichment analysis suggested that equine SP proteins play important roles in the metabolism of hexoses, extracellular vesicles, and in response to hydrogen peroxide, as these terms were significantly enriched. Hexose metabolism and extracellular vesicles are linked, acting as a support mechanism for spermatozoa. Prostasomes, a specific class of extracellular vesicles, express glycolytic enzymes with capacity for ATP production [49–51]. Interestingly, the abundance of SP proteins involved in carbohydrate metabolism relates to stallion fertility [18]. The proteins involved in these metabolic pathways are present in equine prostasomes [50] which can be linked to the enrichment findings in our study. Another relevant function of SP, enriched in our analysis, was

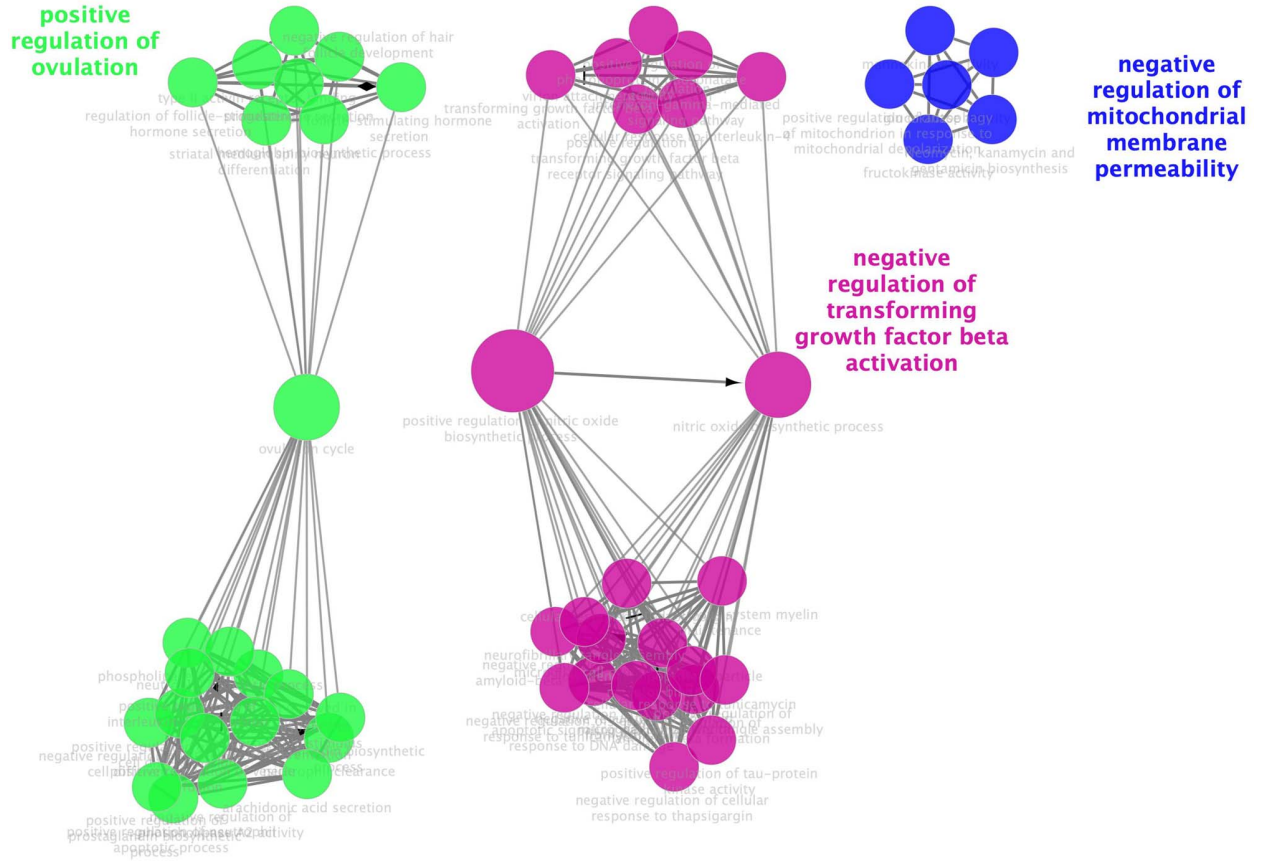

Figure 3. CLueGO network analysis of proteins in equine seminal plasma. To reduce redundancy of GO terms, the fusion option was selected. GO/KEGG pathway functionally grouped networks with terms are indicated as nodes (Benjamini–Hochberg *P* value < 0.05), linked by their kappa score level (≥ 0.35) where only the label of the most significant term per group is shown.

the response to stress and to hydrogen peroxide. Catalase has been reported as a key antioxidant present in equine SP that is derived primarily from prostatic secretions [47]. In addition, in relation to redox regulation, the GO term nicotinamide adenine dinucleotide regeneration (GO:0006735) was significantly enriched in equine SP. Finally, another group of enriched terms were those related to post-transcriptional modification of proteins, particularly SUMOylation, with the reactome pathways *SUMOylation* and *SUMO E3 ligases SUMOylate target proteins*. As spermatozoa are translationally and transcriptionally silent cells, it relies heavily on post-translational modifications for its regulation [52, 53]. This finding suggests a major role of SP in regulating sperm functionality; further supported by the study of functional networks. ClueGO analysis revealed a highly interconnected network of SP proteins, with particularly relevant functions concerning the regulation of ovulation, TGF-β and mitochondrial membrane permeability. The existence of ovulation inductor factors in equine SP has been suggested previously [54] and further research is warranted.

In addition to enrichment analysis, the proteome in 10 stallions routinely used in a commercial facility producing seminal doses was studied. They were classified into three groups according to the ability of their ejaculates to withstand extension and posterior conservation by refrigeration with or without removal of SP. Differences in the proteome among these groups were investigated, in an attempt to identify specific proteins or groups of proteins, that may explain the different ability of ejaculates to withstand conservation by refrigeration at 5°C. Numerous proteins were less abundant in samples with a reduced life span under refrigeration, many of these proteins were related to redox regulation, including catalase, peroxiredoxin 6, and superoxide dismutase. Differences among stallions in superoxide dismutase and glutathione peroxidases content have been previously reported [55]. In addition, another protein with antioxidant activity, HSP-90 [56] was found to be less abundant in *poor* samples. Interestingly, alteration of redox regulation inactivates HPS-90 due to the action of reactive electrophilic aldehydes generated during the loss of redox homeostasis [57]. All together, these findings support the hypothesis that ineffective redox regulation is behind the limited period of time that these ejaculates can be conserved by refrigeration. Other proteins related to metabolism, like oxoglutarate dehydrogenase, glyceraldehyde 3 phosphate dehydrogenase or proteins involved in cell viability like PI3K were also reduced in this group of stallions. The total absence of the glucagon receptor in *poor* samples found in our study is also noteworthy. This receptor has recently been described in human spermatozoa [58]. In this study glucagon fueled phosphatidylinositol-3-kinase (PI3K)/AKT signaling and was reversed by the protein kinase inhibitor H89, indicating a dependence of glucagon signaling on protein kinase A. A stimulatory action of glucagon on lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities was observed in the above mentioned study. Peroxiredoxin 6 was

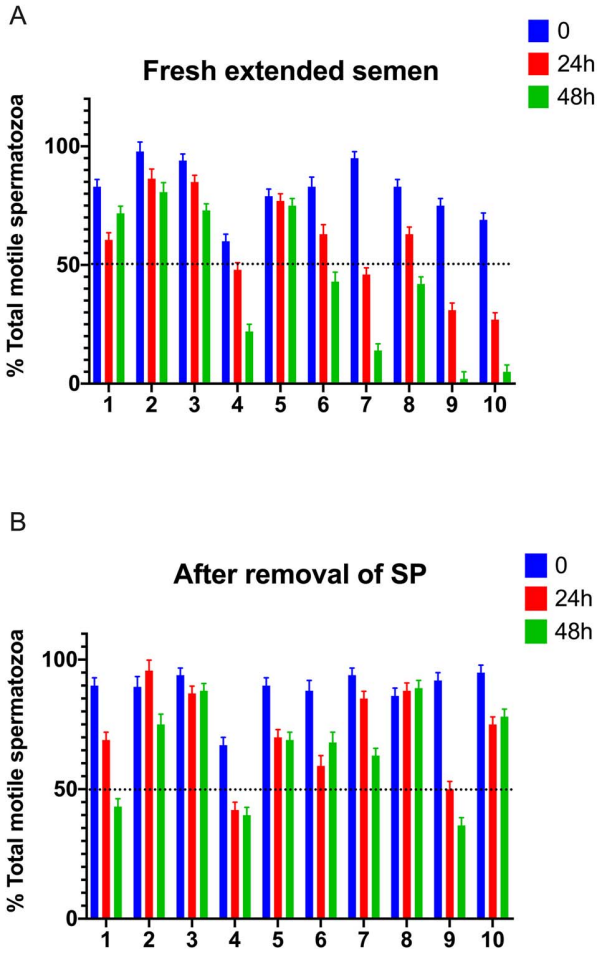

Figure 4. Storage under refrigeration resistance test of the 10 stallions used in a commercial AI program used in the present study. Ejaculates were collected and split into two subsamples, one was extended in INRA 96 to 25 × 10^6 spm/mL, and the other half was extended after removal of seminal plasma by centrifugation and chilled at 5°C for up to 48 h. Sperm motility using computer assisted sperm analysis (CASA) classified stallions into three groups: *good* if motility after 48 h was >50% and VCL was >100 µm/s, *poor* if motility after 48 h was <50% and VCL was <100 µm/s and *good-RSP*, if 48 h after centrifugation and removal of the bulk of seminal plasma, motility was >50% and VCL was >100 µm/s. In A) stallions 1, 2, 3, and 5 were *good* stallions, in B) stallions 6, 7, 8, and 10 were *good-RSP*, stallions 4 and 9 were *poor* and did not improve after removal of seminal plasma. (a–c) The percentage of total motile spermatozoa after 24 and 48 h of conservation by refrigeration at 5°C are given in the three groups of stallions. (d–f) Circular velocity is given. Data are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$; n.s., not significant.

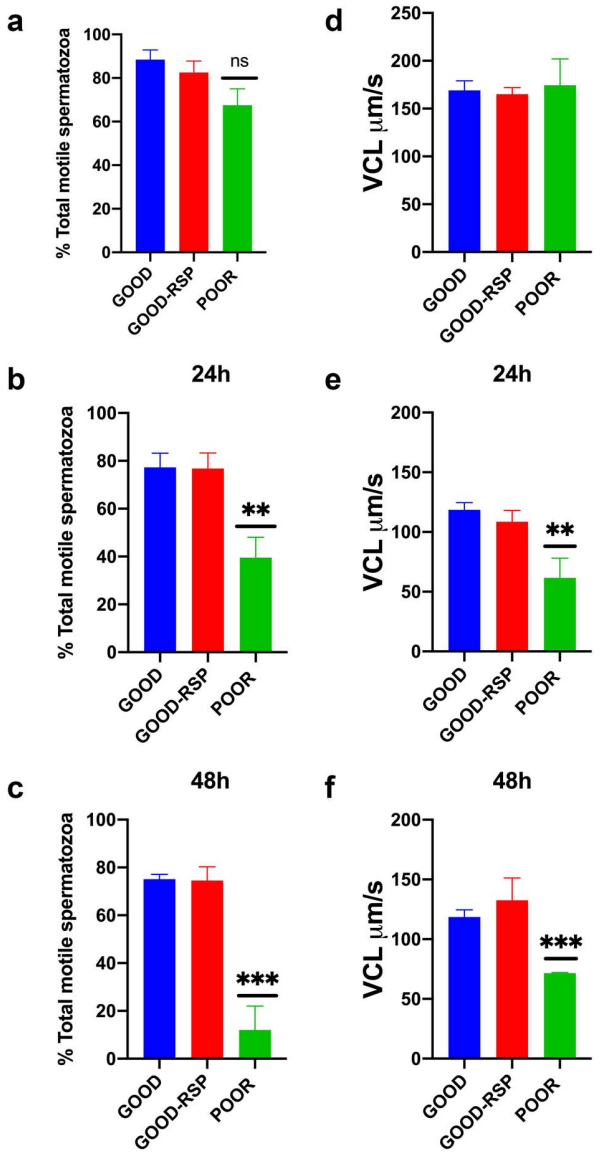

Figure 4. Continued.

found to be most abundant in *good* stallions, and catalase was also abundant but with no difference in levels from stallions that needed RSP to achieve a good period of conservation.

The protein *ST3 beta-galactoside alpha-2,3-sialyltransferase 1* (ST3GAL1) was highly enriched in *good* stallions. This protein plays a role in post-transcriptional modification of proteins, and interestingly the gene for ST3GAL1 has previously been associated with sperm storage duration in the oviduct in chickens [59]. This finding falls in line with the special dependence of post transcriptional modifications for the proper functioning of the spermatozoa. The comparison between stallions that needed removal of most SP (*good-RSP*) and those which did not require this procedure (*good*) identified a number of proteins as better candidates for biomarkers, with annexin highly enriched in *good-RSP* stallions, while barely present in *good* stallions. Annexins have a wide variety of cellular functions, including cell proliferation, differentiation, apoptosis, migration, membrane repair, and inflammatory response [60]. Although annexin can be considered a good potential marker to distinguish samples that will require SP-removal, the reasons behind this remain to be fully understood, but potential mechanisms involved may include an increase in inflammatory responses [61] or accelerated progression of apoptosis in these stallions [62, 63]. Equine annexins may stimulate the production of reactive oxygen species, a mechanism involved in these responses [61]. The network analysis also supported this hypothesis, with annexin interacting with proteins involved in cell survival and immune regulation.

In conclusion, this study provides the first detailed description of the proteome of equine SP. Bioinformatic analysis revealed major roles related to hexose metabolism, which may be mediated by

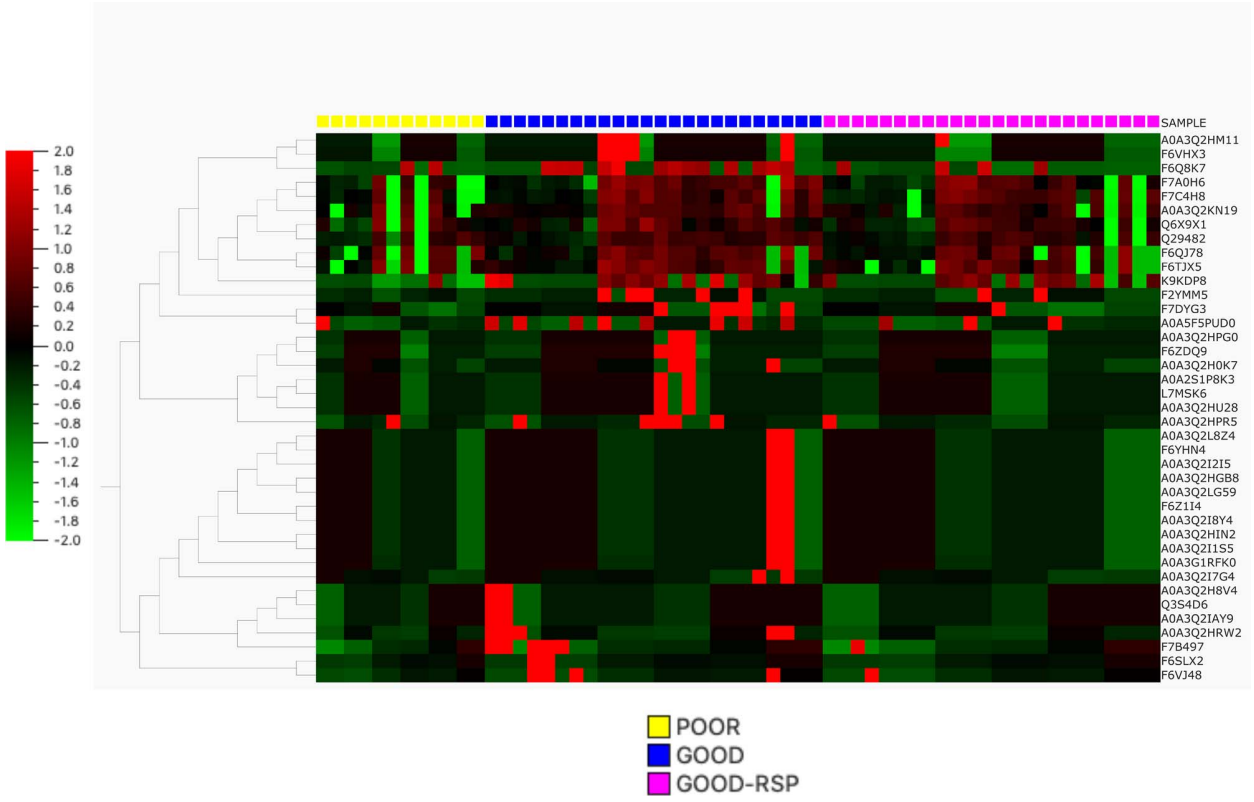

Figure 5. Heat map showing the identification of discriminant variables (seminal plasma [SP] proteins) for the group of *good* stallions in comparison with *good-RSP* and *poor* with a fold change > 2, $P < 0.05$ and $q = 0.1$.

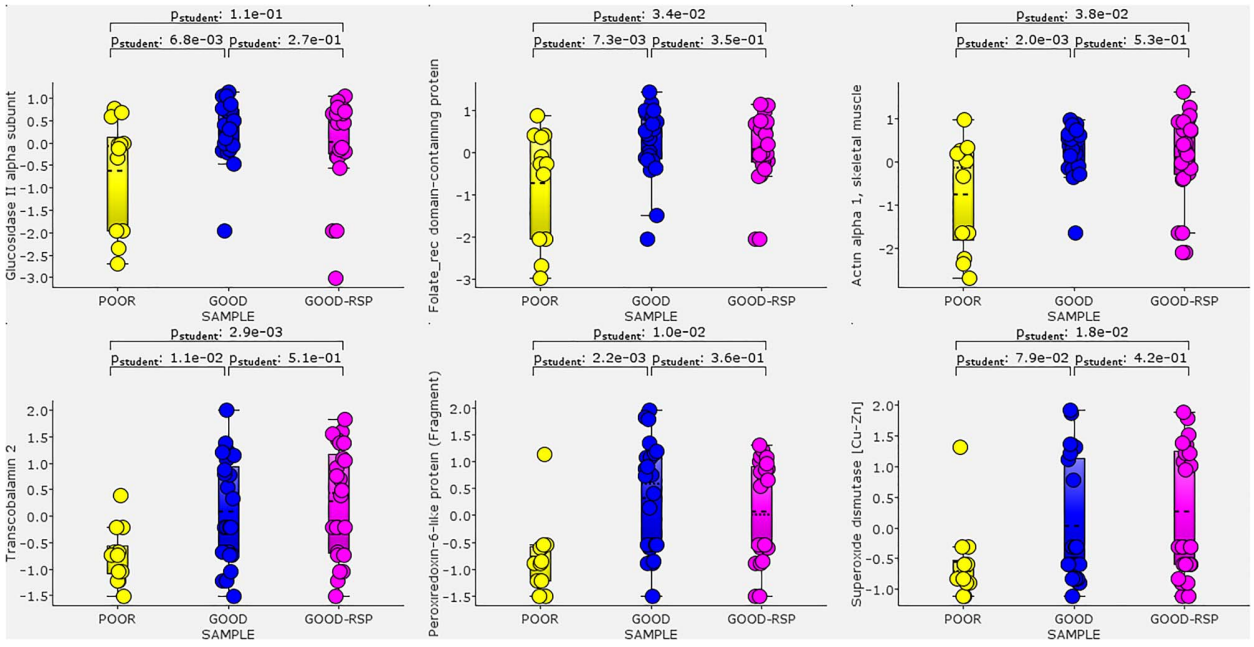

Figure 6. Differences in the amount of specific representative proteins in the three groups of stallions, *good*, *good-RSP*, and *poor*. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins in the seminal plasma (SP) based on spectral counts among stallions classified in function of the period of time that their ejaculates can be stored under refrigeration. *Poor* samples are represented in yellow boxes, *good* samples in blue boxes, and *good-RSP* in pink boxes.

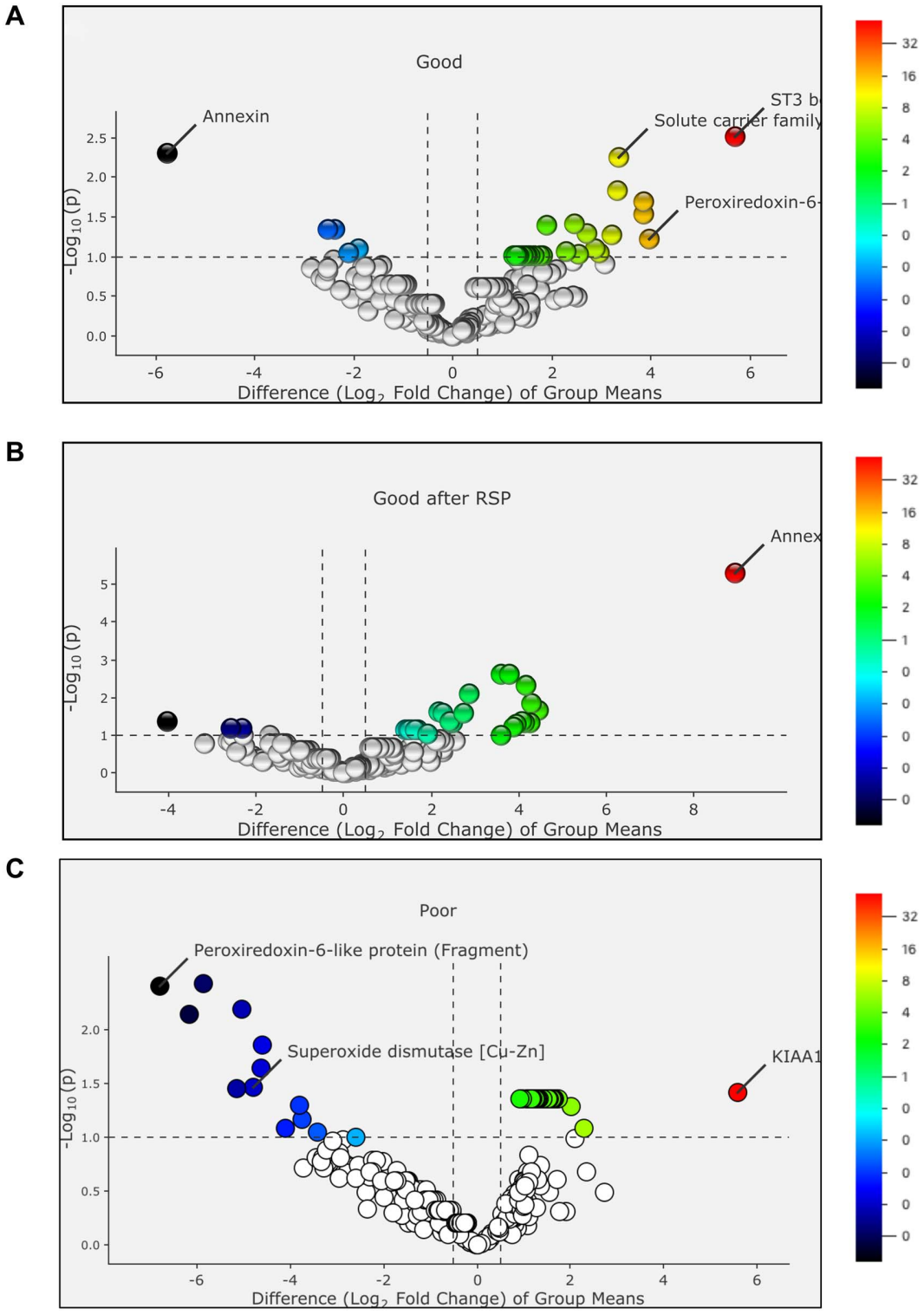

Figure 7. Volcano plots showing proteins enriched or underrepresented in the three groups of stallions (A: *good*, B: *good-RSP*, and C: *poor*). Representative proteins identified in each group are depicted.

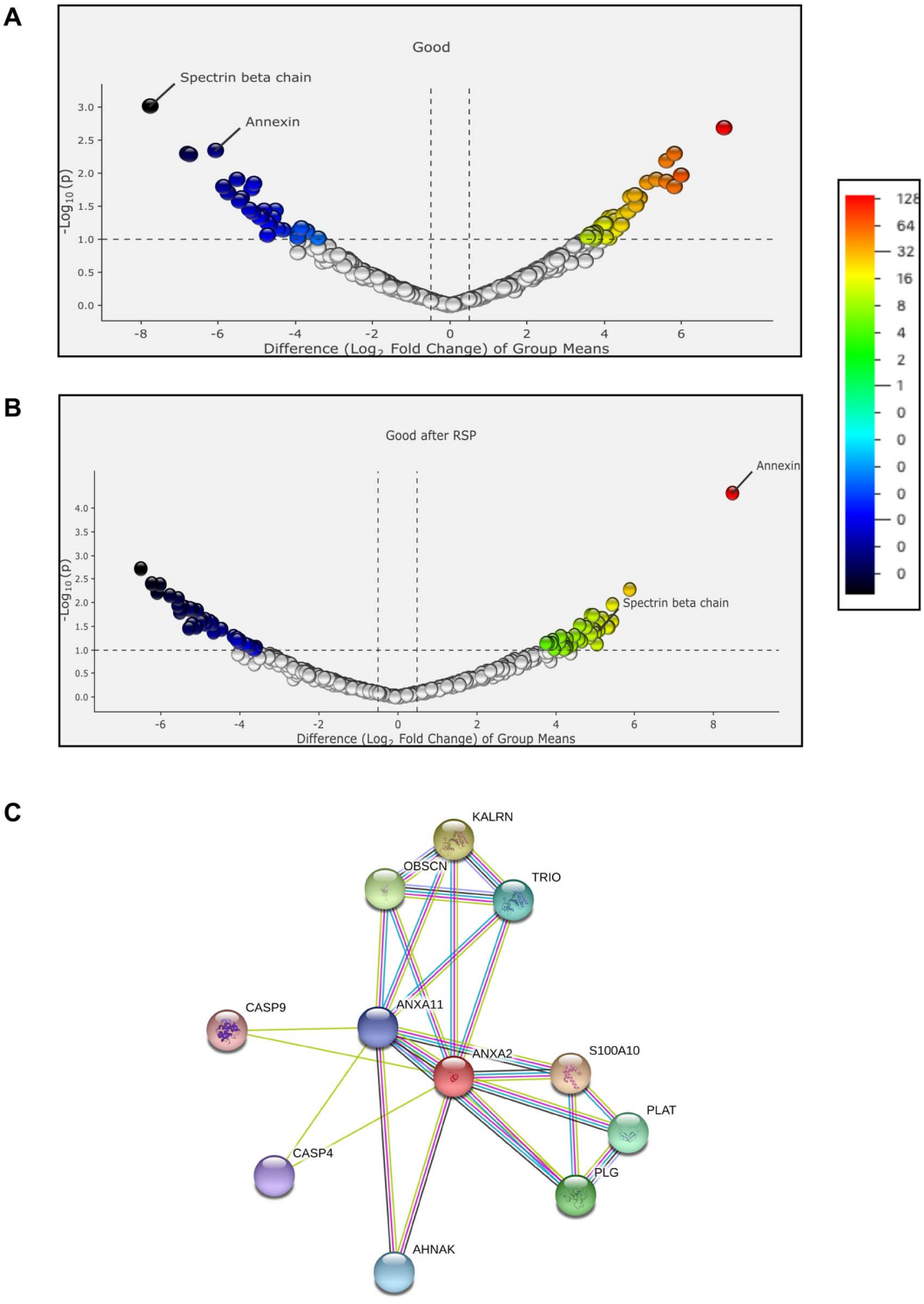

Figure 8. Volcano plots showing proteins with the highest power as discriminant variables between *good* and *good-RSP* stallions. AnnexinA2 appeared as a powerful discriminant variable to identify stallions needing removal of seminal plasma (SP) prior to chilled preservation, with a $P = 0.002$ and a q value = 0.005. C STRING analysis (https://string-db.org) was used to identify potential functional partners of this protein.

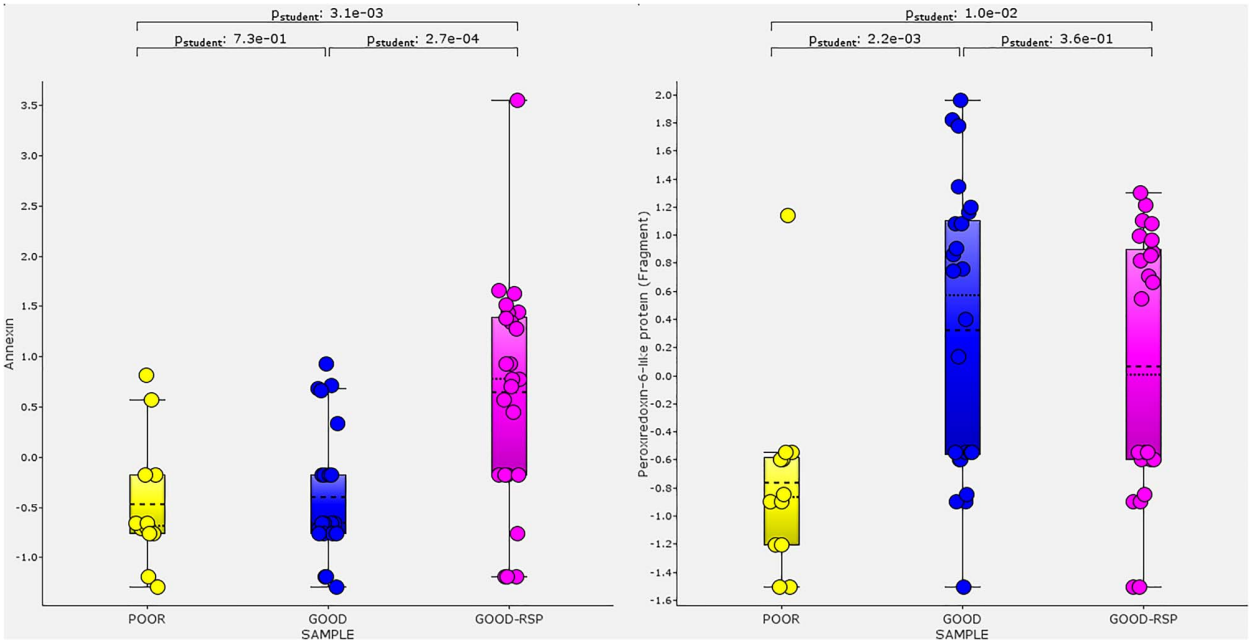

Figure 9. Amount of specific seminal plasma (SP) proteins in specific groups of stallions. Annexin A2 (A0A3Q2LPE6) and Peroxiredoxin-6 like protein (K9KDP8). Annexin was present in significantly larger amounts in the group of stallions requiring removal of SP (left panel), while the presence of Peroxiredoxin-6 like protein was significantly reduced in the SP of stallions classified as *poor* (right panel).

extracellular vesicles, probably involving prostasomes. In addition, findings are suggestive of major roles of SP in redox regulation and immune response regulation. The finding that the varied presence of specific proteins from groups of SP proteins has potential value as markers for the ability of ejaculates to withstand refrigeration, and whether or not they may need SP-removal to achieve this goal is of particular practical interest. Moreover, findings offer new potential lines of research, with applications both in sperm biotechnologies and reproductive medicine.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Conflict of interest

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

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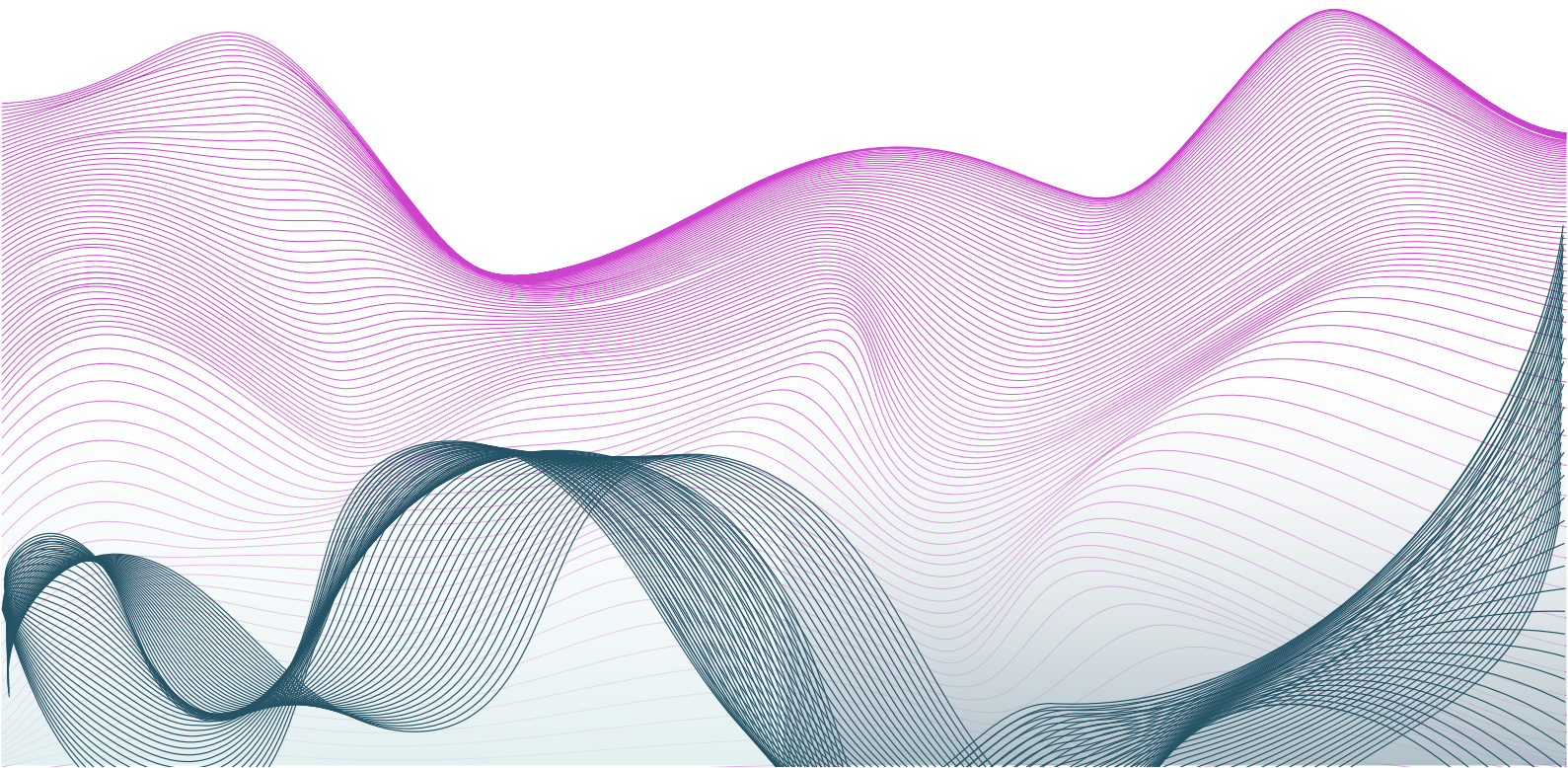

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Seminal plasma proteins as potential biomarkers for sperm motility and velocities

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ABSTRACT

Seminal plasma proteins have important roles in sperm functionality, and different mechanisms including micro-vesicle transport of proteins are involved in the regulation of sperm biology. Due to the role of seminal plasma, we hypothesized that specific proteins present in seminal plasma may be used as discriminant variables with potential to identify stallions producing different quality ejaculates; 10 fertile stallions, with different motility and velocity values (although within normal ranges) were used in this study. Motilities and velocities were studied using computer assisted sperm analysis (CASA), while protein composition of the seminal plasma was studied using UHPLC-MS/MS. Specific proteins were more abundant in samples with poorer percentages of total motility, average path velocity and circular velocity, and were: *Secreted phosphoprotein 1, Fructose-bisphosphate aldolase* (p = 1,95E-09; q = 0.0005) and *Malate dehydrogenase 1* (p = 1,41E-11; q = 0.002), to the contrary samples with better straight-line velocity values were enriched in *Glutathione peroxidase (p=0.00013; q=0.04) and Triosephosphate isomerase (p=0.00015; q=0.04).*

1. Introduction

The stallion ejaculate is composed of a mixture of spermatozoa and the secretions from the accessory sex glands [1]. Numerous proteins are vehiculated in the seminal plasma, mainly in micro vesicles such as prostasomes; microRNAs are also vehiculated in this way [2,3]. Seminal plasma influences sperm functionality in different ways, for example the presence of high amounts of Annexin A2 may impair the ability of the ejaculates to sustain prolonged conservation periods under refrigeration [4]. However seminal plasma also contains proteins that support sperm metabolism, probably through vesicle mediated transport [4]; this group of proteins support sperm metabolism and also help in the redox regulation of these cells [2,3,5,6]. Seminal plasma proteins may interact with the spermatozoa vehiculated in micro-vesicles; thus, these proteins may influence and regulate sperm functionality [6]. Nowadays, the use of mass spectrometry allows for the identification of numerous proteins in the spermatozoa and the seminal plasma, in addition to the increased availability of bioinformatic software and on line platforms which allow curation of the information gathered from large data sets [7–10]. We hypothesized that bioinformatic analysis of seminal plasma proteins may provide candidates for biomarkers of sperm quality in stallions, with potential to be used as discriminant variables to forecast the quality of a particular ejaculate.

2. Material and methods

2.1. Reagents and media

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

2.2. Semen collection and processing

Semen was collected from 10 stallions (three ejaculates per stallion) of various breeds maintained as indicated in institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). Data on the reproductive

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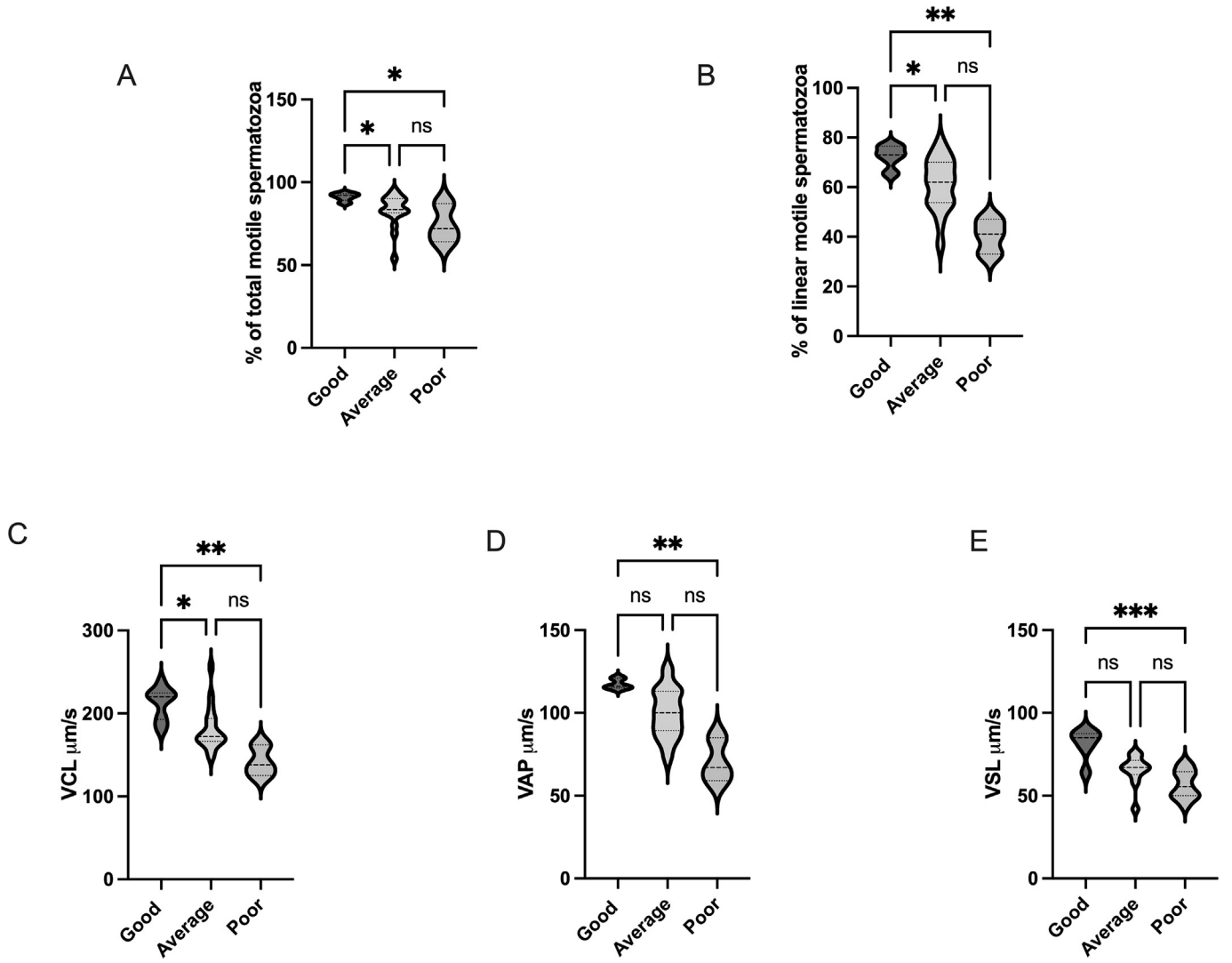

Fig. 1. Computer assisted sperm analysis in 10 stallions (3 ejaculates each, n = 30). Ejaculates were obtained as described in the material and methods and diluted to 50 × 10^6 spermatozoa in Tyrode's media. Aliquots were then loaded into a Leja chamber and at least 500 spermatozoa were analyzed. Stallions were classified into three categories according to their performance in total and linear motility, with stallions showing ejaculates with median percentages of total motility over 90% classified as "good", those with total motility over 80% classified as average and those with percentages below 80% classified as poor. The values for good, average, and poor percentages of linear motile spermatozoa were 70% 60% and 40% respectively. Violin plots were used showing the data distribution in each group. A) Percentage of total motile spermatozoa B) Percentage of linear motile spermatozoa C) Circular velocity (VCL; µm/s) D) Average path velocity (VAP; µm/s) E) Straight line velocity (VSL; µm/s) *$P < 0.05$**$P < 0.01$, ns = non-significant.

record of the stallions used are presented in Supplementary Table 1. All stallions were of proven fertility, with a median age of 10.8 years old. Semen was collected from all stallions on a regular basis following the standard protocol in our center in which collections are performed in the morning between 10 and 12 h. Ejaculates used in this study were collected after depletion of the extragonadal sperm reserves, during the 2019 breeding season. The University ethics committee approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina, and the gel was removed with an inline filter. Semen was transported immediately to the laboratory after collection for evaluation and processing. Upon arrival at the laboratory, the seminal plasma (SP) was removed by serial centrifugation (2 × 1500g 10′) and stored at −80 °C until proteomic analysis. An aliquot of the ejaculate was processed through colloidal centrifugation [11,12] to remove dead spermatozoa, and contaminating cells, and then was re-suspended in Tyrode's medium (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM NaHCO3, 1 mM Na-Pyruvate, 21.6 mM Na-Lactate, 2 mM CaCl2*2H2O, 3.1 mM KCl, 0.4 mM MgSO4*7H2O, 0.3 mM NaH2-PO4*H2O, 0.3% BSA) 315 mOsm/kg and pH 7.4 [13], for assessment of motility and sperm velocities.

2.3. Sample preparation

Samples were processed immediately after collection. Aliquots of isolated SP were kept frozen at −80 °C until further analysis. Phase contrast microscopy was used to control the absence of spermatozoa, moreover SP was filtered (0.22 µM) before snap freezing and further processing.

2.4. Protein solubilization and quantification

Aliquots of SP were solubilized in lysis buffer and incubated under constant rotation at 4 °C for 1 h as described in previous studies [2,14]. The amount of protein was then normalized to obtain a final concentration of 100 µg of protein per sample.

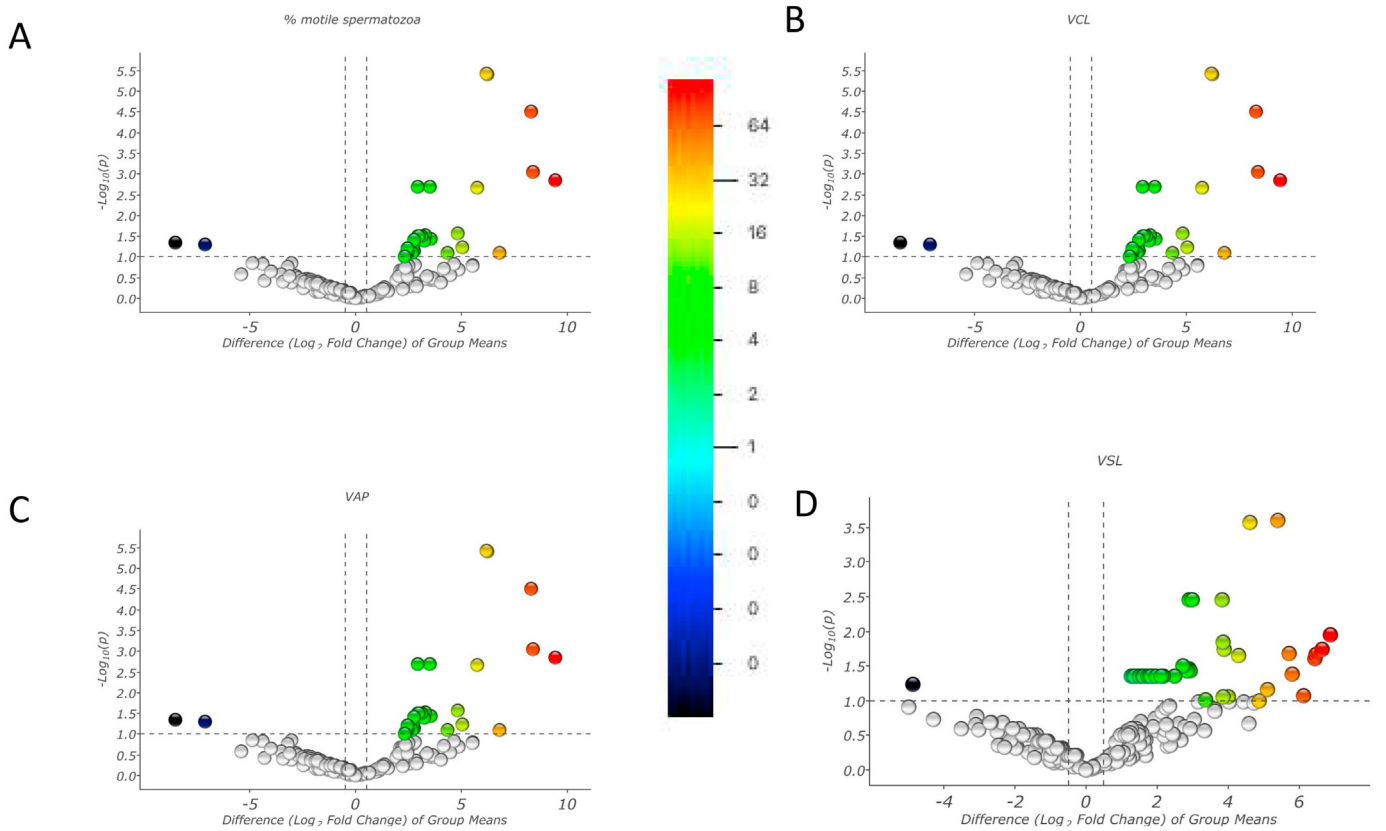

Fig. 2. Volcano plots showing differentially expressed proteins in the ejaculates of 10 stallions, different colors show different values of each protein represented by the z-scores represented in the heat map. The difference of protein content (log_2 fold change) is plotted against the significance of the difference $-log_{10}(p)$ between the two conditions (poor motility vs good and average for % total motility, VAP and VCL, or good versus poor and average in the case of VSL. Three independent ejaculates from 10 different stallions, in addition to two technical replicates (n = 60 samples) were used to derive results from.- A) Differential amounts of proteins in stallions showing poor values of % motility; these stallions showed increased amounts of proteins as seen in the upper right quadrant of the plot (in red). B) Stallions showing poorer values of VCL showing increased amounts of proteins depicted in the upper right quadrant of the plot (in red) C) Stallions showing poorer values of VAP showing increased amounts of proteins in the upper right quadrant (in red). D) Stallions showing better values of VSL had higher amounts of proteins depicted in the upper right quadrant (in green).

2.5. In-solution trypsin digestion

Trypsin digestion was performed as described in preceding studies [2,14,15]. In brief the proteins were mixed with a bicarbonate buffer, reduced with DTT and lastly alkylated. They were then digested by trypsin overnight.

2.6. UHPLC-MS/MS analysis

The separation and analysis of the samples were performed as previously described [2,14,16] using an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high-speed binary pump coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the UHPLC and Q-TOF was using Mass Hunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01).

2.7. Data processing

Data processing and analysis was performed using the Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) as previously described [14].

Table 1
The Biomarker Workbench in the bioinformatic software Qlucore Omics Explorer 3.7 (https://qlucore.com). was used to identify seminal plasma proteins able to identify poor and good samples in terms of motility and velocity. The number of variables identified specifically for each trait with a q value < 0.05 are given. Variables (seminal plasma proteins SPP) able to discriminate between stallions were found, and after correction for FDR 3 were able to differentiate between stallions with poorer motility, VCL and VAP and 2 proteins were able to identify stallions with better VSL.

Explanatory Variable	Explanatory Variable	Eliminated Factors	SPP p < 0.05	SPP q < 0.05
Type	Details			
Multi Group Comparison	STALLION		19	0
Two Group Comparison	motility [POOR > All]		17	3
Two Group Comparison	linear motility [POOR > All]		11	0
Two Group Comparison	VAP [POOR > All]		17	3
Two Group Comparison	VCL [POOR > All]		17	3
Two Group Comparison	VSL [GOOD > All]		84	2

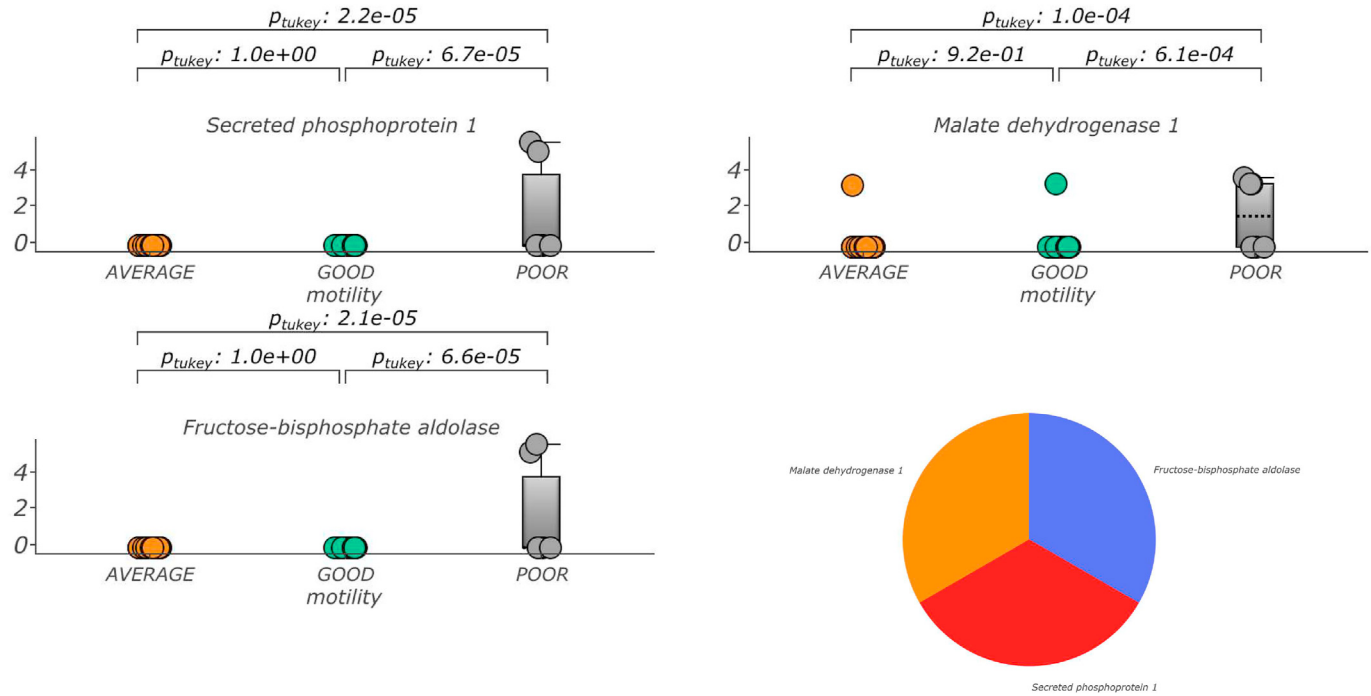

Fig. 3. Discriminant variables retrieved after bioinformatic analysis able to identify stallions with poor total motility, defined as stallions with motility within normal ranges, but below 80% total motility. Qlucore omics explorer bioinformatic software (Lund Sweden) was used to identify these variables, through the comparison of relative amounts of proteins based on spectral counts among stallions classified as good average or poor in terms of total motility. Proteins were Log_2 transformed and normalized, then variables with a corrected p value, $q < 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates ($n = 60$ samples) were used to derive results from.

2.8. Bioinformatic analysis

2.8.1. Variance filtering and PCA

Data were normalized and log_2 transformed using Qlucore Omics Explorer (https://qlucore.com) as described in previous publications [14]. Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of

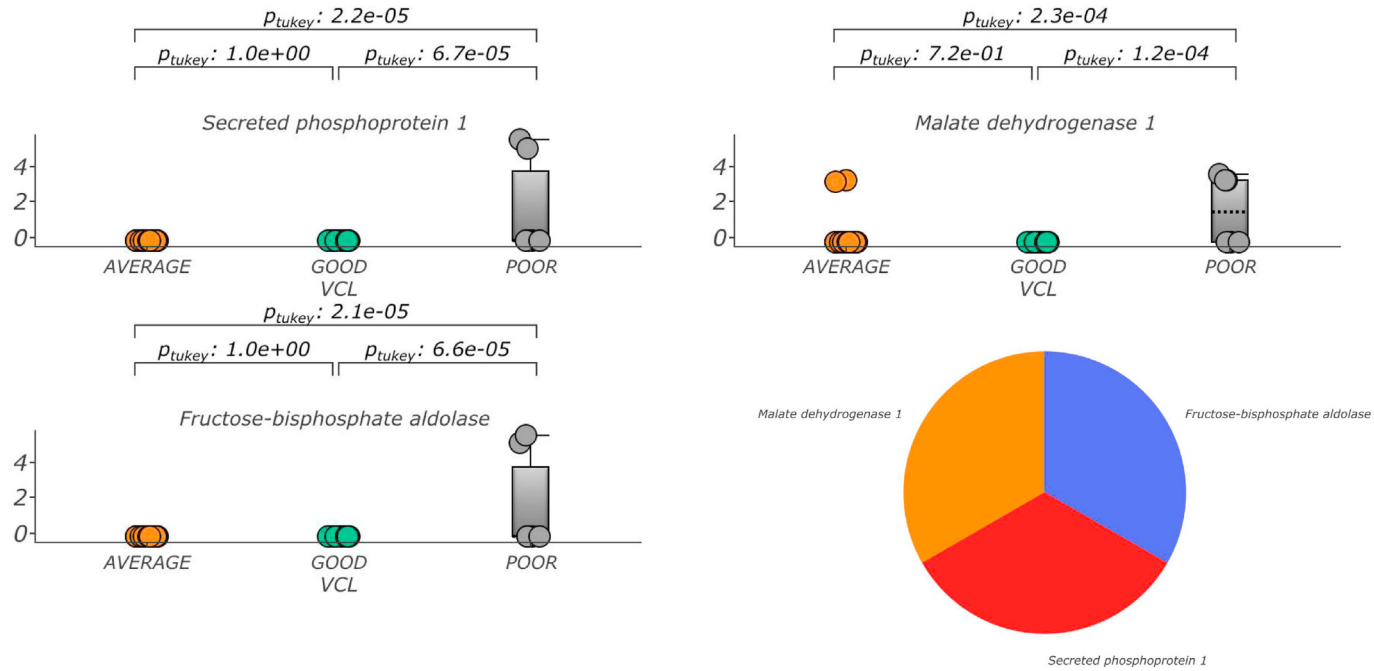

Fig. 4. Discriminant variables for stallions with poor VCL, these were normal fertile stallions but showed a VCL in the lower rank in our group of stallions with an average VCL of 141.7 ± 10.8 μm/s. Qlucore Omics Explorer bioinformatic software (Lund Sweden) was used to identify these variables through the comparison of the relative amounts of proteins based on spectral counts among stallions classified as good, poor and average in terms of VCL. Proteins were Log_2 transformed and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates ($n = 60$ samples) were used to derive results from.

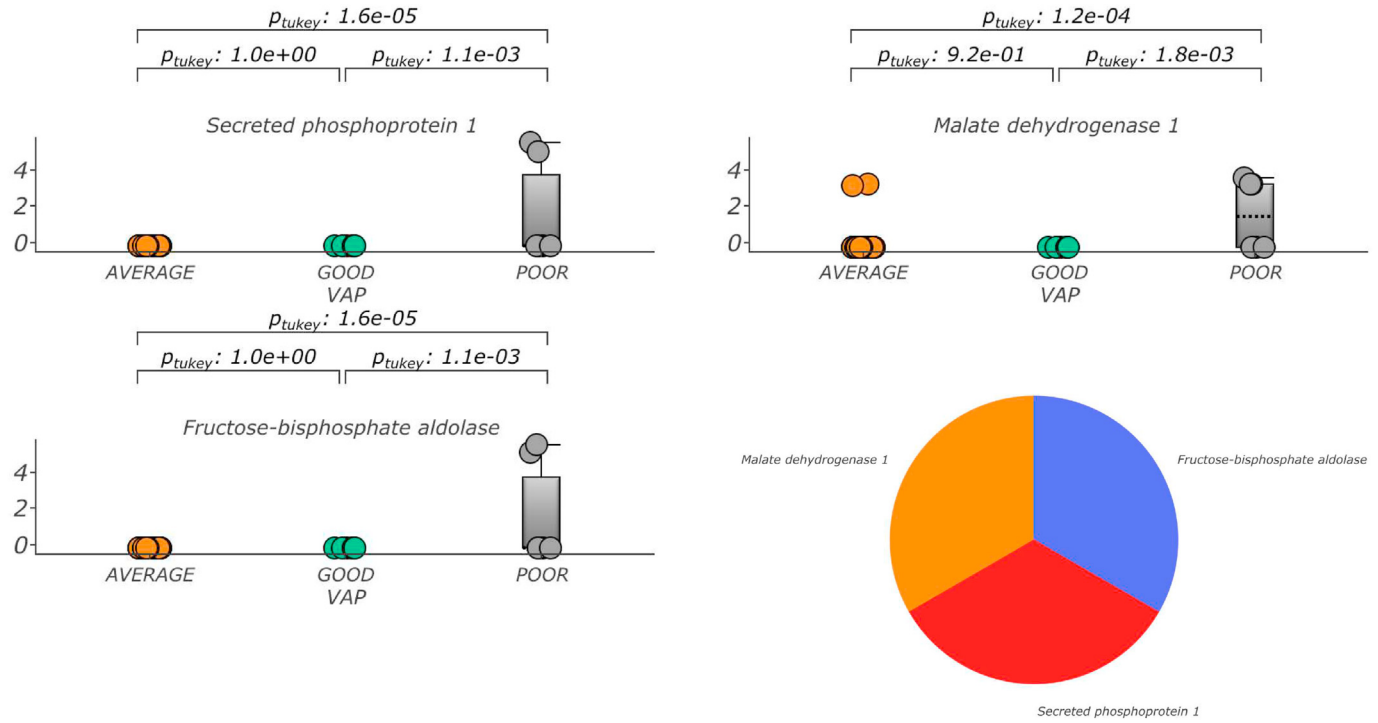

Fig. 5. Discriminant variables for stallions with poor VAP, consisting of those stallions with VAP of 70.3 ± 7.6 8 μm/s. Qlucore Omics Explorer bioinformatic software (Lund Sweden) was used to identify discriminant variables measuring the relative amounts of proteins based on spectral counts among stallions classified as good, poor and average. Proteins were Log_2 transformed and normalized and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates n = 60 samples were used to derive results from.

noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [17] was used to determine the optimal filtering threshold.

2.8.2. - Identifying discriminating variables

Qlucore Omics Explorer Ver. 3.7 (https://qlucore.com) was used to identify the discriminating variables able to find differences in motility, and velocities (VCL, VAP and VSL) among the stallions. This software works by fitting a linear model for each variable with condition proteins of the seminal plasma from stallions showing different values of the above parameters as predictors, including the stallion, breed, and age as nuisance covariates. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [18,19] and variables with adjusted p-values (q values, equivalent to false discovery rate FDR) below 0.1 were considered significant. Then, to further validate the potential biomarkers previously identified (seminal plasma proteins) we also used the Biomarker Workbench in Qlucore version 3.7.21; this functionality allows the simultaneous analysis of multiple variables. A model including, age, breed, individual stallion, and the computer assisted sperm analysis (CASA) derived parameters: percentage of total motile spermatozoa, percentage of linear spermatozoa, circular velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) was used. In this analysis variables were considered potential biomarkers only if q values were below 0.05.

2.9. Computer-assisted sperm analysis (CASA)

Sperm motility and velocities were assessed with a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) [12,20]. Samples were loaded into Leja® chambers with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 38 °C. Sixty consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus C×41), and 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with an average path velocity (VAP) > 35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

2.10. Statistical analysis

The following parameters were measured: percentages of total and linear motile spermatozoa, circular (VCL), straight line (VSL) and average (VAP) velocities in μm/s.

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

3. Results

3.1. Identification of stallions with different motility and velocities

The percentage of motile sperm ranged from 74.3 ± 6.7 to 92.0 ± 1.1% ($P < 0.05$; Fig. 1). Stallions showing ejaculates with median total motility over 90% were classified as "good", those with total motility over 80% were classified as "average" and those with percentages below 80% were classified as "poor" (Fig. 1A). The values for good, average, and poor percentages of linearly motile spermatozoa were 70% 60% and 40% respectively (Fig. 1B). Sperm velocities also showed significant variation, in terms of circular velocity, good stallions had VCL values of 212.3 ± 6.7 μm/s, for stallions classified as average VCL was 181.1 ± 6.17 μm/s and for stallions classified as poor VCL was 141.7 ± 10.8 μm/s (Fig. 1C). Average path velocity in the good, average, and poor groups was

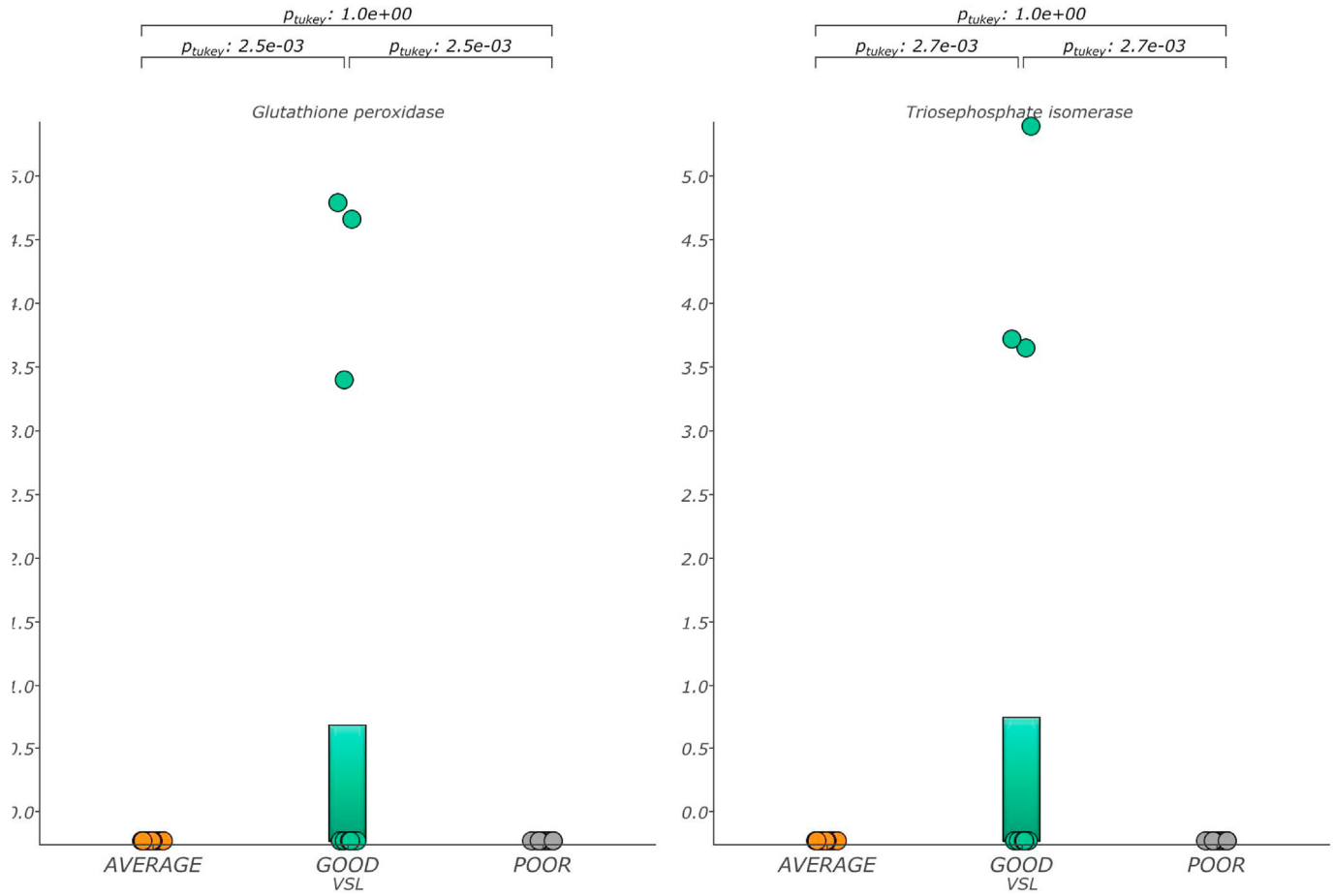

Fig. 6. Discriminant variables identifying stallions with good VSL, (mean value of 81.5 ± 3.7 μm/s). Qlucore Omics Explorer (Lund Sweden) bioinformatic software was used to compare relative amounts of proteins based on spectral counts among stallions classified as good, poor and average for VSL. Proteins were Log_2 transformed and normalized and then variables with a corrected p value, $q \leq 0.05$ were considered as biomarkers; 3 independent ejaculates from 10 different stallions in addition to two technical replicates ($n = 60$ samples) were used to derive results from.

117.3 ± 1.8, 99.9 ± 3.0 and 70.3 ± 7.6 respectively (Fig. 1D). Finally, VSL was 81.5 ± 3.7 μm/s in good stallions, 65.5 ± 2.6 μm/s in average and 51.3 ± 2.4 μm/s in the poor group (Fig. 1 E)

3.2. Seminal plasma proteins differ in stallions with good and poor motility and velocities

Raw data were uploaded to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026375 [21]. Volcano plots were constructed to obtain an overview of seminal plasma proteins differentially expressed in stallions showing poorer motility (Fig. 2A), lower VCL and VAP (Fig. 2 B and C) and better (faster) VSL (Fig. 2D). Next, discriminant variables ($p = 9.6e\text{-}04$; $q = 0.069$) were identified for % total motility for VCL and VAP ($p = 8.7x\ e\text{-}4$, $q = 0.0069$) and for VSL ($p = 0.001$; $q = 0.08$). We identified 6 proteins in seminal plasma with discriminant power for stallions with lower percentages of total motility in their ejaculates, then these proteins were further curated ($q < 0.05$) using the biomarker workbench (Table 1) to find the proteins with the highest potential as biomarkers, and *fructose-bisphosphate aldolase* ($p = 2.56e\text{-}5$; $q = 0.0070$), *secreted phosphoprotein 1* ($p = 2.58e\text{-}5$; $q = 0.0070$) and *malate dehydrogenase 1* ($p = 0.00015$; $q = 0.028$) were identified as strong biomarkers of ejaculates with poor motility (Fig. 3). Discriminant variables were also identified for VCL and VAP (Figs. 4 and 5) and in both cases were found to be the same proteins as in the case of the percentages of total motile spermatozoa. However, in the case of straight-line velocity two proteins were identified as being discriminant variables for stallions with higher VSL (Table 1). Higher VSL values were better explained by two variables, *glutathione peroxidase* ($p=0.00013$; $q=0.04$) and *triosephosphate isomerase* ($p=0.00015$; $q=0.04$) (see Fig. 6).

4. Discussion

Stallion ejaculates presenting percentages of total motility and velocities which were different, but within normal ranges, differed in the amounts of specific seminal plasma proteins, present in ejaculates. These findings should be considered in the context of in vitro situation. Many proteins present in seminal plasma are vehiculated in exosomes (epididysomes, prostasomes and vesiculosomes), in fact it has already been discovered that many seminal plasma proteins attach to stallions sperm membranes [22]. Although further research is needed, presence of these proteins in the seminal plasma may reflect the secretory activity of the male genital tract and thus its functionality [3,6]. Specific proteins were associated with samples showing lower percentages of total motility, VCL and VAP. To the contrary, two specific seminal plasma proteins were present in higher amounts in samples with higher VSL. Interestingly, the same discriminant proteins were able to predict samples which had poor motility, VCL and VAP. Fructose bisphosphate aldolase catalyzes the reversible reaction that splits

fructose 1, 6 bisphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3 -phosphate (GP3). These are intermediate metabolites in glycolysis, and in a subsequent step phosphate groups are eliminated. Irreversible elimination of the phosphate groups from DAHP and 3 GP forms methylglyoxal (MG) as a by product; this is a 2-oxoaldehyde that is a strong electrophile due to adjacent carbonyl groups that rapidly and spontaneously react with nucleophiles from proteins, lipids and DNA forming advanced glycation end products (AGEs) [23]. These compounds are potentially cytotoxic and mutagenic, although they may also be involved in regulatory functions. Besides, MG can form adducts with superoxide dismutase 1 (SOD1) impairing the antioxidant action of this enzyme and promoting oxidative stress [24]. In relation with this fact recent research from our laboratory identifies SOD1 as one of the most important antioxidant systems in the spermatozoa [14,25]. Furthermore we have recently described the toxic nature of MG for the stallion spermatozoa [26]. Extenders containing high amounts of glucose, produce high amounts of MG causing sperm malfunction including drops in motility and sperm velocities [26]. This provides an explanation to our findings linking high amounts of this enzyme in seminal plasma and poor motility, VCL and VAP. While relevant potential biomarkers were found for poor motility, average and circular velocity, to the contrary biomarkers for good straight-line velocity were evidenced. *Glutathione peroxidase* and *triosephosphatase isomerase* were biomarkers of good VSL. A BLAST analysis showed that the glutathione peroxidase found in our study had an 89% homology to the *epididymal secretory glutathione peroxidase* from *Sus scrofa*, 85.5% to the same protein in *Canis lupus familiaris* and 79.3% to the same protein in *Homo sapiens*. This enzyme catalyzes the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide by glutathione. This protein constitutes a glutathione peroxidase-like protective system against peroxide damage in sperm membrane lipids [27]. It is not surprising that a protein with roles in lipid peroxide detoxification relates to better characteristics of sperm kinematics. Spermatozoa are cells that need a tight redox regulation, and the loss of redox equilibrium rapidly leads to sperm malfunction [5,14]. Interestingly, our findings may also suggest a different regulation of different aspects of sperm kinematics, suggesting that motility, circular velocity, and average path velocities, may have similar regulation, while straight line velocity may have distinctive particularities.

In sum, seminal plasma proteins may have a major impact of sperm functionality, and specific seminal plasma proteins may be used as discriminant variables for poor motility, VCL and VAP, while discriminant variables for good VSL were also identified. Validation of these data and further research may help to develop potent biomarkers of sperm functionality which are rapidly applicable in clinical settings. These findings also underpin the role of seminal plasma in sperm functionality.

CRediT authorship contribution statement

Gemma Gaitskell-Phillips: Investigation, writing, reviewing and editing (English). **Francisco E. Martín-Cano:** Methodology, Investigation. **José M. Ortiz-Rodríguez:** Investigation. **Eva da Silva-Álvarez:** Investigation. **Javier Masot:** Supervision. **Eloy Redondo:** Supervision. **Maria C. Gil:** Investigation, Data curation. **Cristina Ortega-Ferrusola:** Investigation, Supervision, Data curation. **Fernando J. Peña:** Conceptualization, Validation, Formal analysis, Data curation, writing, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2021.10.007.

The authors confirm the following statements

1. That there has been no duplicate publication or submission elsewhere of this work.
2. That all authors have read and approved the manuscript, are aware of the submission for publication and agree to be listed as co-authors.

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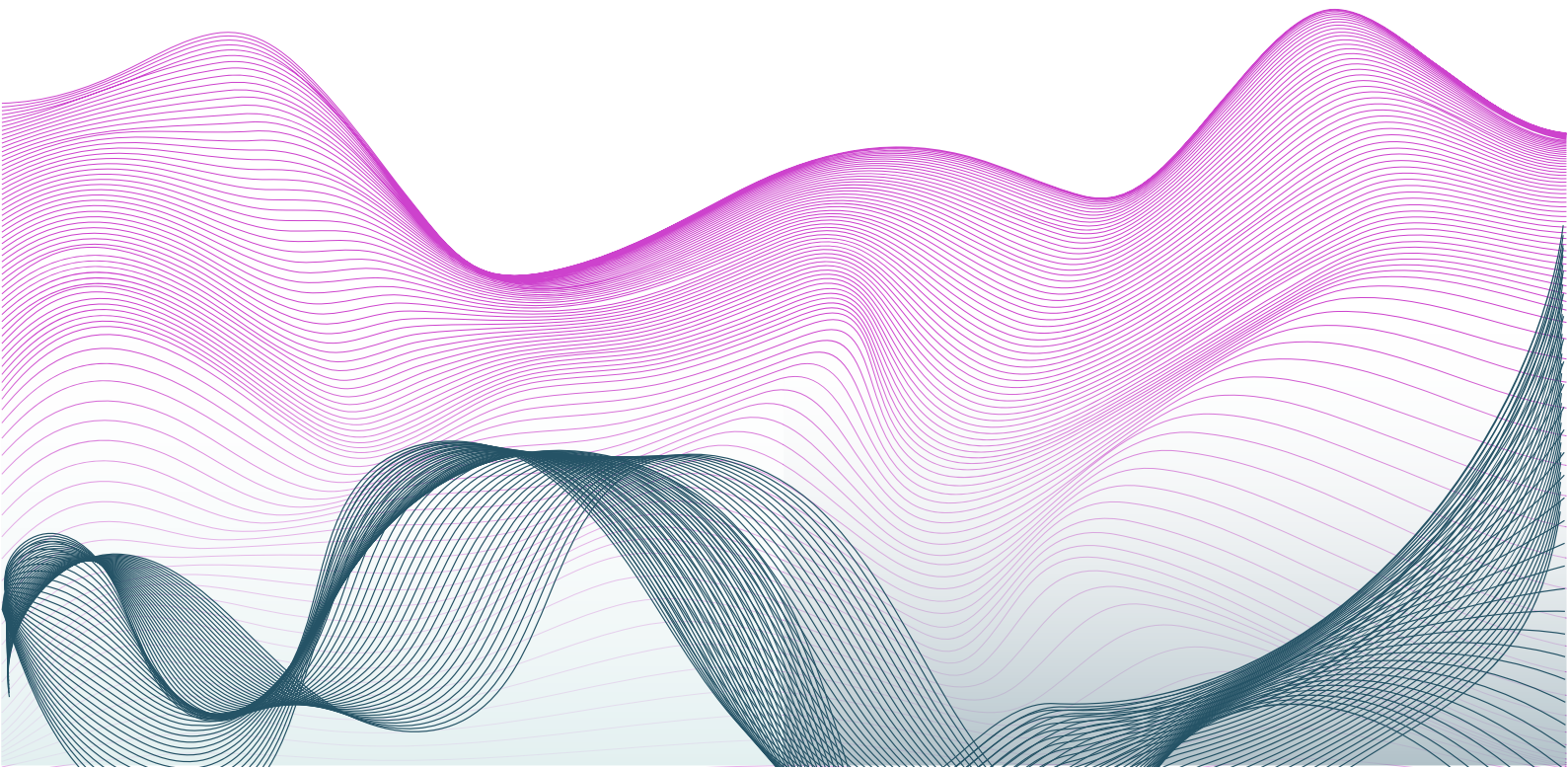

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The seminal plasma proteins *Peptidyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase and KIAA0825* are linked to better motility post thaw in stallions

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ABSTRACT

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

Seminal plasma proteins may play a major role in sperm functionality; being vehiculated through extracellular vesicles and participating in sperm physiology. Bioinformatic analysis identifies discriminant proteins able to predict the outcome of cryopreservation, identifying potential new biomarkers to assess ejaculate quality.

1. Introduction

Seminal plasma, is composed of secretions from the accessory sex glands and has immunosuppressive/immunoregulatory functions [1,2]. This fluid harbors numerous microvesicles, mainly prostasomes [3], with different functions nourishing spermatozoa and probably carrying RNAs and proteins with a role in early embryo development [4]. Despite its importance, equine seminal plasma has received little attention in comparison to the spermatozoa. However, the composition of equine seminal plasma has been the subject of several studies, some of them in relation to the freezeability of the ejaculate [5–8]. A detailed description of the proteome of equine seminal plasma has recently been published [9]. In this study, reactome and KEGG pathway analysis revealed an important role of seminal plasma in metabolism and vesicle mediated transport. Moreover, a specific protein in the seminal plasma, Annexin A2 served as a discriminant variable for stallions that needed removal of seminal plasma to maintain the quality of their ejaculates when conserved by refrigeration. However, little information is available regarding the potential relationship between the proteins present in seminal plasma and the freeze-ability of the ejaculates. While cryopreservation of stallion spermatozoa is a reproductive technology which use is increasing year after year,

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unresolved questions remain. One of the major drawbacks is the high stallion-to-stallion variability, that precludes many valuable stallions entering the international market for horse semen [10]. Undoubtedly, increasing knowledge around the factors that are involved in this variability will open new opportunities enabling its reduction. While sperm factors have received attention [11–14], the role of seminal plasma has not been so extensively investigated in relation with freeze-ability [6,15,16]. Previous reports indicate that equine seminal plasma plays important roles in hexose metabolism and is rich in antioxidants [6,9,17]. Cryopreservation causes osmotic induced necrosis in a high proportion of spermatozoa, while the surviving population experience a compromise in their energetic metabolism and redox regulation [18,19]. Since seminal plasma has roles in redox regulation and energetic metabolism through prostasome and other micro-vesicles that vehiculate, we hypothesized that differences in protein composition of the seminal plasma between stallions may help to explain differences in freeze-ability seen among them. The objective of this study was to identify proteins in seminal plasma that could be potential markers of freeze-ability using potent bioinformatic tools.

2. Material and methods

2.1. Reagents and media

All chemicals were purchased from Sigma-Aldrich (https://www.sigmaaldrich.com/spain), unless otherwise stated. JC-1, monochlorobimane (MCB), Annexin V 647 conjugated, CellEvent® Caspase 3/7 Green Detection Reagent, Hoechst 33342 and Ethidium homodimer (Eth-1) were purchased from Thermofisher (https://www.thermofisher.com/es/es/home.html) DRAQ7 was purchased from Beckman Coulter (https://www.beckmancoulter.com/es).

2.2. Experimental design

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

2.3. -Semen collection and processing

Semen was collected from stallions of different breeds maintained as indicated by specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). The ethical committee of the University approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina and the gel fraction of the ejaculate was removed with an inline filter. Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [21,22] and seminal plasma removed. The ejaculate was extended in freezing media and frozen using standard procedures that have been previously described by our laboratory [19]. In brief semen was diluted in the Cáceres freezing medium (University of Extremadura, Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 × 10^6 spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were ultrasonically sealed with an UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) machine and immediately placed in an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The following freezing curve was used. Straws were kept at 20 °C for 15 min, and they were then slowly cooled from 20 °C to 5 °C at a cooling rate of 0.1 °C/min. Thereafter the freezing rate was increased to −40 °C/min from 5 °C to −140 °C. The straws were then plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a water bath at 37 °C for at least 30 s.

2.4. Protein solubilization

Seminal plasma aliquots were solubilized in lysis buffer as previously described [9] and 20 μL of lysis buffer was added, the solution was vortexed and incubated under constant rotation at 4 °C for 1 h.

2.5. Protein quantification

Protein quantification was performed using the 2-D Quant Kit [9]. All samples were normalized to obtain a final concentration of 100 μg of protein per sample.

2.6. In-solution trypsin digestion

200 μL of the seminal plasma solution obtained from the previous stage were mixed with 100 μl of 25 mM ammonium bicarbonate buffer, pH 8.5 and the proteins were reduced and alkylated. Digestion was performed by adding 1 μL of Trypsin Proteomics Grade as previously described [9].

2.7. UHPLC-MS/MS analysis

Separation and analysis of the samples was performed following the protocol described in a previous study [9] with a UHPLC/MS system consisting of an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High Speed Binary Pump, coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the HPLC and Q-TOF was via MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01).

2.8. Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) following previously described protocols [9].

2.9. Computer-assisted sperm analysis (CASA)

Sperm motility and velocity were assessed using a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) in fresh and frozen and thawed spermatozoa according to standard protocols used at our center [23]. Semen samples were loaded into a Leja® chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at

37 °C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus C×41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with VAP >35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

2.10. Flow cytometry

Flow cytometry (FC) analyses were conducted using a Cytoflex®S flow cytometer (Beckman Coulter) equipped with violet, blue, yellow and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowjoV 10.7 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory [24,25].

2.10.1. Measurement of GSH, viability and mitochondrial membrane potential in stallion spermatozoa

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

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

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

2.11. Bioinformatic analysis

2.11.1. Variance filtering and PCA

Data were normalized and $\log_2$ transformed using Qlucore Omics Explorer (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [29] was used to determine the optimal filtering threshold.

2.11.2. Identifying discriminating variables

Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables that are most highly significantly different between good and poor freezers. The identification was performed by fitting a linear model for each variable with condition proteins in seminal plasma as a predictor of the outcome of cryopreservation and including the stallion nuisance covariate. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [30,31] and variables with adjusted *P*-values (q value) below 0.1 were considered significant.

2.12. Statistical analysis

End points measured were, the percentage of total motile spermatozoa and circular velocity after thawing, and the percentages of live spermatozoa, caspase 3 positive spermatozoa and the percentage of spermatozoa showing high mitochondrial membrane potential.

The normality of the motility, sperm velocity and flow cytometry data were assessed using the Kolmogorov-Smirnoff test. Paired t-tests and one-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 7.00 for Mac, La Jolla California USA, (www.graphpad.com).

3. Results

3.1. - Sperm quality post thaw differed between stallions

3.1.1. Motility and velocities

Significant differences were observed in total motility post thaw between the two groups of stallions, good and poor freezers, with good freezers showing a mean percentage of total motile spermatozoa of 37.7 ± 1.3% while the group of poor stallions showed a percentage of total motility post thaw of 21 ± 0.71% ($P < 0.001$) (Fig. 1A). A similar trend was observed for the percentage of linear motile spermatozoa (Fig. 1B). Sperm velocity (circular velocity) was also significantly different between both groups, with velocities of 125.6 ± 2.5 μm/s in good stallions, while VCL post thaw in poor freezers was 107.2 ± 2.5 μm/s ($P < 0.0001$) (Fig. 1C).

3.1.2. Viability and mitochondrial membrane potential

The percentage of live spermatozoa after thawing in the group of good stallions was 51.1 ± 1.6% while in the group of poor freezers it was 34.2 ± 1.6% ($P < 0.0001$) (Fig. 2A). Poor freezers showed higher percentages of caspase 3 positive spermatozoa (13.6 ± 0.6 vs 8.1 ± 04% in good freezers $P < 0.0001$ Fig. 2C). Good freezers showed a higher percentage of spermatozoa with high mitochondrial membrane potential (49.1 ± 1.5% vs 34.5 ± 1.1 in poor freezers, $P < 0.0001$, Fig. 2B).

3.2. Protein composition of seminal plasma differs in stallions with better motility post thaw. To determine possible differences in the composition of seminal plasma in stallions showing better values for motility, viability and mitochondrial activity post thaw, independent volcano plots were constructed in a first step comparing the proteome of the stallions showing significantly higher values in motility, viability and mitochondrial membrane potential with the proteome of the rest of the stallions (Fig. 3A–C). Volcano plots showed a different predominance of specific seminal plasma proteins among stallions showing better results after cryopreservation in the three categories considered (Fig. 3A–C). However, significant differences in the composition of the seminal plasma proteome were only observed between the group of stallions showing better motility post thaw. We identified 3116 proteins, and of these 34, were differentially expressed in stallions with better motility post

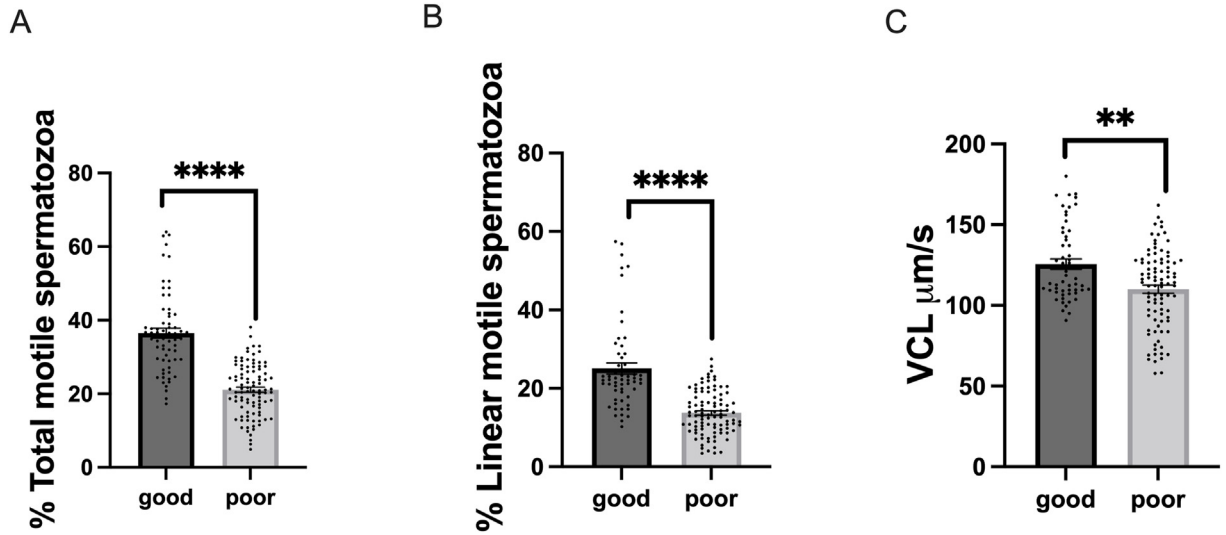

Fig. 1. Average values of motility (total and linear) and velocity (circular velocity) after freezing and thawing in ejaculates from 10 different stallions (3 replicates per stallion n = 30), classified as good (>35% total motility post thaw) or poor (<35% total motility post thaw). Semen was collected and processed as indicated in material and methods and the percentage of total motile spermatozoa (A), the percentage of linear motile spermatozoa (B) and the circular velocity (VCL) µm/s (C) were measured using computer assisted sperm analysis (CASA). Data are presented as means ± s.e.m. and derived from 3 identical replicates from each of the stallions (n = 30 ejaculates) $^{**}P < 0.01$, $^{****}P < 0.00001$.

thaw (Fig 3D), 4 of them were also differentially expressed in stallions with different percentages of linearly motile sperm post thaw and 1 protein, Midasin, was expressed in stallions showing high circular velocity post thaw (Fig. 3D–E). Next, bioinformatic analysis was conducted to identify discriminant variables; proteins in seminal plasma with potential to identify stallions that show good motility post thaw. Qlucore Omics Explorer (https://qlucore.com) was used to identify seminal plasma proteins that are most significantly different based on spectral counts in stallions with better motility post thaw. Proteins of which amounts differed in seminal plasma of stallions showing better motility after thawing were identified (Fig. 4C) with a fold change >2, P = 0.009 and

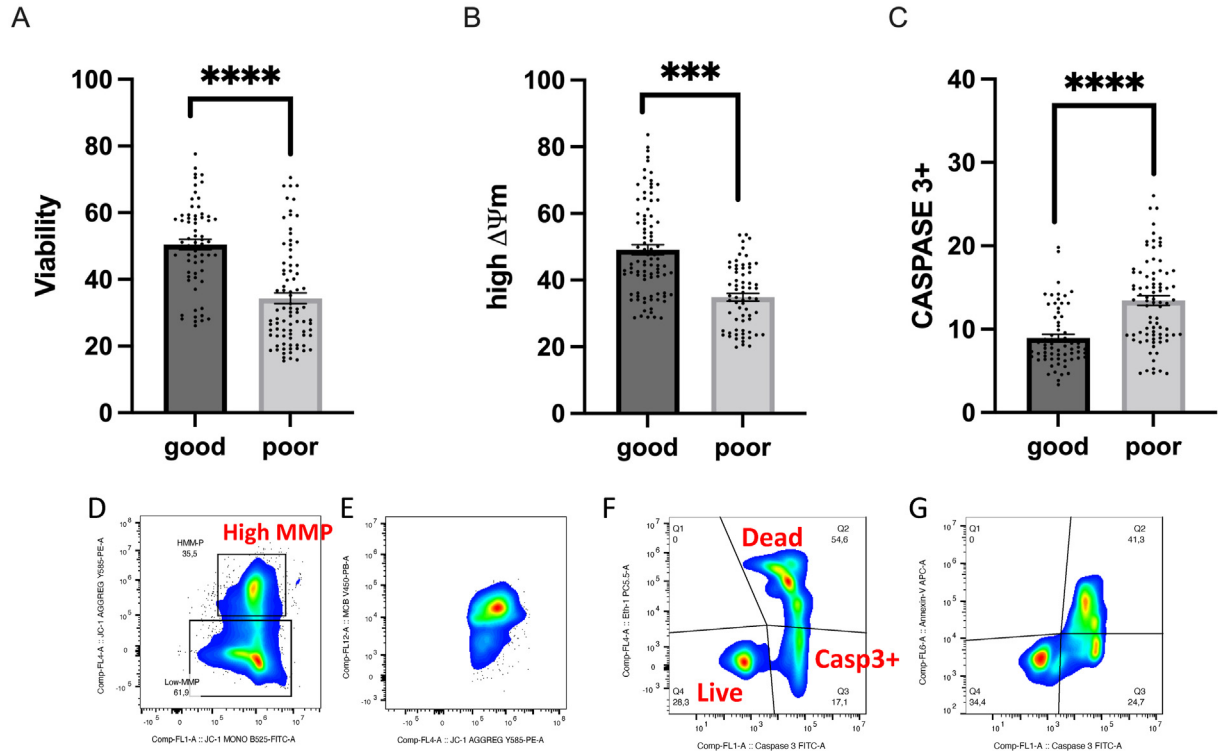

Fig. 2. Viability and mitochondrial membrane potential after freezing and thawing in ejaculates from 10 different stallions (3 replicates per stallion n = 30), classified as good (>40 viability) or poor (<40% viability post thaw). Semen was collected and processed as indicated in material and methods and the percentage of live spermatozoa (A), percentage of spermatozoa showing high mitochondrial membrane potential (B) and the percentage of caspase 3 positive spermatozoa (C) were measured using flow cytometry. D-F are representative cytograms of the assays. Data are presented as means ± s.e.m. and derived from 3 identical replicates from each of the stallions (n = 30 ejaculates) $^{***}P < 0.001$, $^{****}P < 0.00001$.

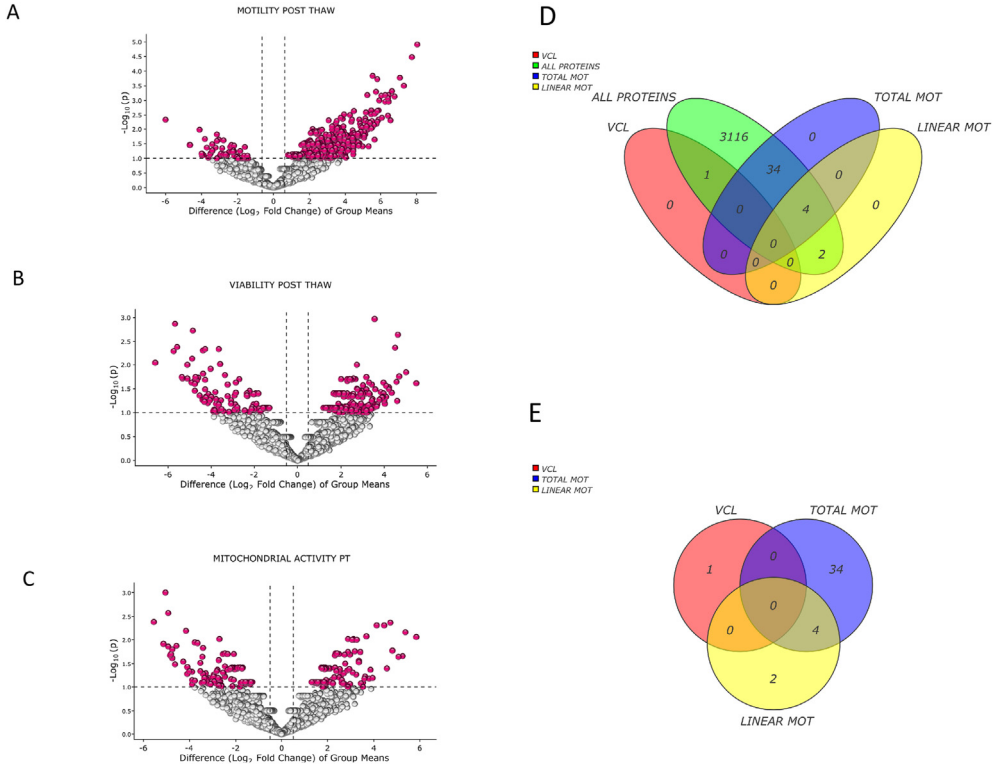

Fig. 3. Volcano plots showing seminal plasma proteins differentially expressed in ejaculates with better quality post thaw in terms of motility (A), viability (B) and mitochondrial membrane potential (C). Proteins which were more abundant are presented on the right-hand side of the volcano plot, proteins less abundant are presented on the left-hand side of the volcano plot. The difference in protein content (Log_2 fold change) is plotted against the significance of the difference -Log $_{10}$ (P) between the two conditions. D and E: Venn diagram showing different amounts of proteins in ejaculates showing better motility, linear motility and velocity post thaw (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis).

q = 0.098. A further filter was applied to the analysis to specifically identify proteins which were more abundant in stallions showing better motility post thaw, and then a much stricter criteria was applied to select discriminant proteins to obtain the most powerful discriminant variables. Proteins were filtered by a fold change of at least 5 between both conditions, with a P = 9.6e-04 and q = 0.05. Six proteins were identified as more abundant in the seminal plasma of stallions with better motility post thaw (Fig. 4D). These proteins were peptidyl arginine deiminase 2, rRNA adenine N (6)-methyltransferase, KIAA0825, Rho guanine nucleotide exchange factor 28, endoplasmatic reticulum protein 44, and two uncharacterized proteins F6SCY and A0A3Q2HPE3, one corresponding to the RAPGEF6 gene, with a guanyl nucleotide exchange factor activity as molecular function, involved in small GTPase mediated signal transduction. The second uncharacterized protein found corresponding to the ARHGEF18 gene, has guanyl-nucleotide exchange factor activity and participates in small GTPase mediated signal transduction processes.

In relation to the percentages of linear motile spermatozoa, significant differences were observed in the proteome of stallions showing higher percentages of linear motile spermatozoa post thaw (Fig. 5). With a fold change >4.75, *P* = 8.7e-4 and q = 0.071 four proteins in the seminal plasma were identified as discriminant variables for stallions showing different percentages of linearly motile spermatozoa post thaw (Fig. 5 D). These were *Peptitdyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase, KIAA0825 and an* Uncharacterized protein (A0A3Q2IAZ9), corresponding to the SCAF1 gene with RNA polymerase II C-terminal domain binding molecular function.

3.3. Stallions showing an overall better outcome after cryopreservation show differences in the composition of seminal plasma

Bioinformatic analysis was performed to identify discriminant variables (proteins) in seminal plasma potentially able to identify stallions with a better overall outcome after cryopreservation and six proteins were identified as discriminant variables, being more abundant in ejaculates scored in category 4 (p = 8.7e-4, q = 0.052 fold change >4). These proteins were the *ATR serine/threonine kinase, peptidyldeiminase 2, rRNA adedine N(6) methyltransferase*, an uncharacterized protein (A0A3Q2IAZ9), *KIAA0825* and the *Solute carrier family 25 member 37* (Fig. 6).

4. Discussion

In the present study the relationship between the proteome of seminal plasma in stallions and sperm quality post thaw were investigated. Post thaw sperm quality was defined in terms of the percentages of total and linear motility, circular velocity (VCL) in μm/s and percentages of viable sperm and spermatozoa showing high mitochondrial membrane potential. Three proteins were significantly enriched in the seminal plasma of stallions showing better motility (total and linear) post thaw, *peptidyl arginine deiminase 2, rRNA adenine N (6)-methyltransferase and KIAA0825.* Other proteins were observed in stallions with better motility post thaw including the *Rho guanine nucleotide exchange factor 28, endoplasmatic reticulum protein 44*, and two uncharacterized proteins *F6SCY and A0A3Q2HPE3, one corresponding to the RAPGEF6 gene,*

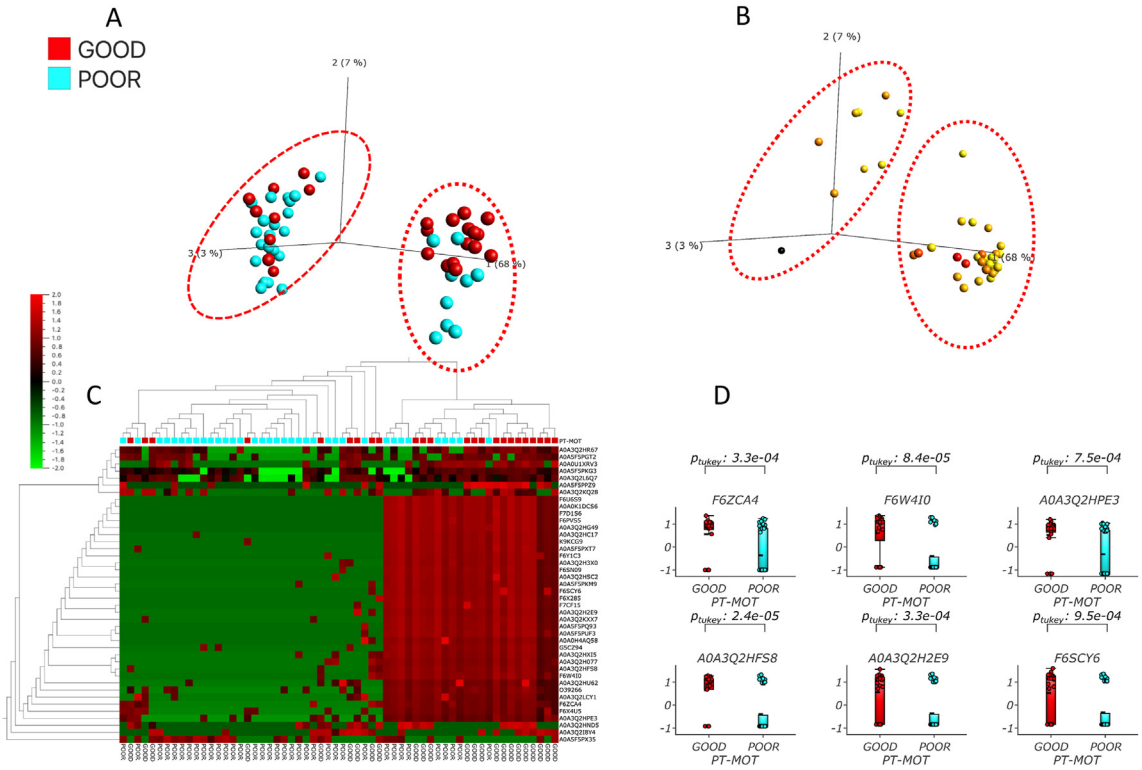

Fig. 4. Bioinformatic analysis of the proteins in stallion seminal plasma (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis), showing different amounts present in stallions with good and poor motility post thaw. A) Principal component analysis of the samples (ejaculates) B) Principal component analysis (variables) C) Heat map showing the different amounts of proteins in seminal plasma of stallions with good and poor motility post thaw D) Seminal plasma proteins identified as potential discriminant variables for stallions showing good (>35%) and poor motility (<35%) post thaw. Proteins were filtered by a fold change of at least 5 between both conditions, with a P = 9.6e-04 and q = 0.05.

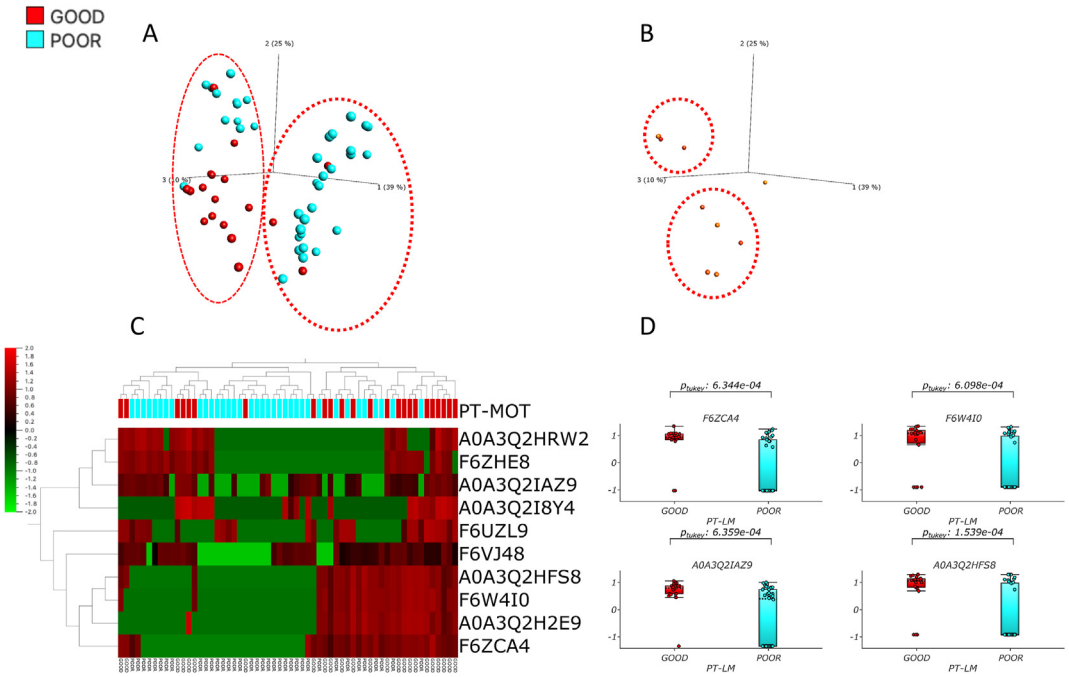

Fig. 5. Bioinformatic analysis of the proteins in stallion seminal plasma (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis), showing different amounts in stallions with good and poor linear motility post thaw. A) Principal component analysis of the samples (ejaculates) B) Principal component analysis (variables) C) Heat map showing the different amounts of proteins in seminal plasma of stallions with good and poor linear motility post thaw D) Seminal plasma Proteins identified as discriminant variables for stallions showing good and poor linear motility post thaw. With a fold change >4.75, *P* = 8.7e-4 and q = 0.071 four proteins in the seminal plasma were identified as discriminant variables.

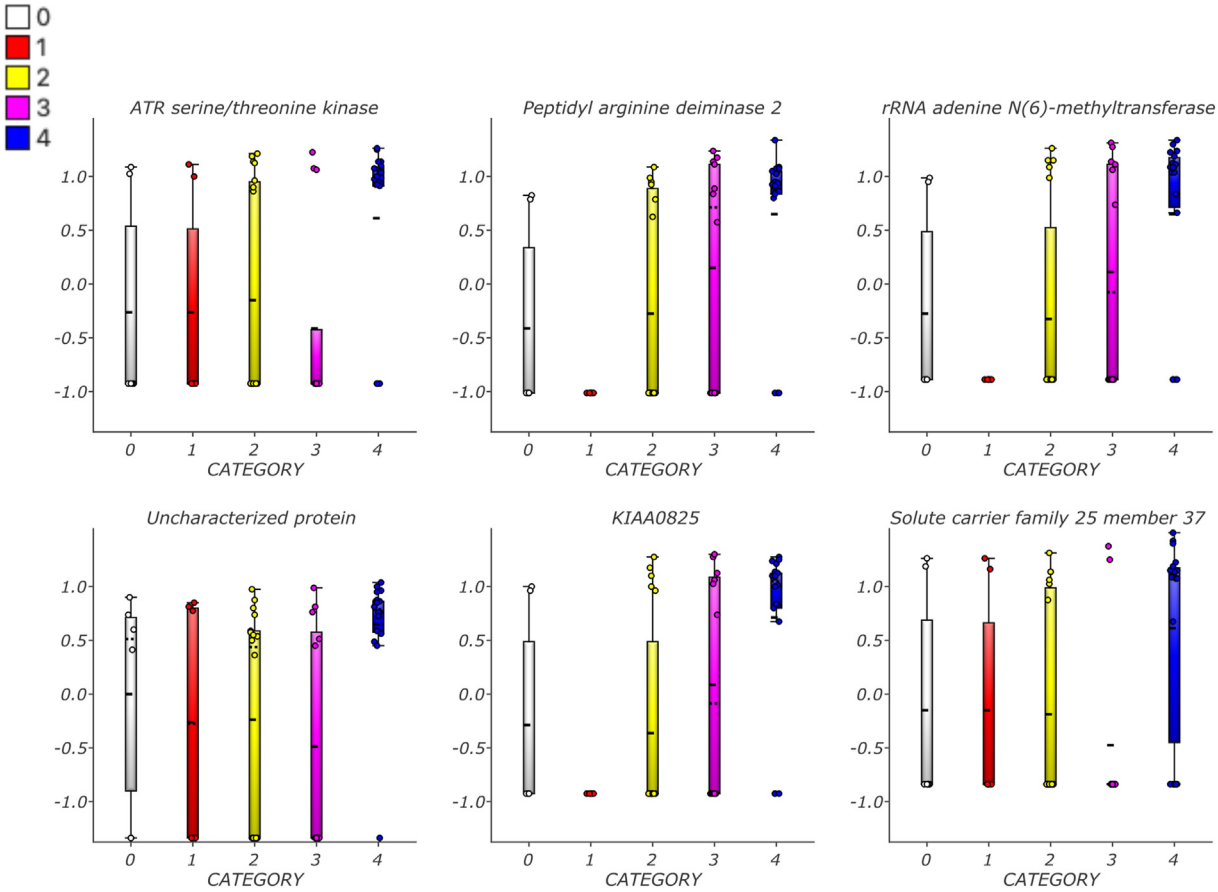

Fig. 6. Bioinformatic analysis of the proteins in stallion seminal plasma showing different amounts in stallions classified in 5 categories according to post thaw sperm quality, with 0 being the worst and 4 the best. Seminal plasma proteins identified as discriminant variables for stallions classified in category number 4. Six proteins were identified as discriminant variables, being more abundant in ejaculates scored in category 4 ($p = 8.7e\text{-}4$, $q = 0.052$, fold change >4).

with a guanyl nucleotide exchange factor activity as molecular function, involved in small GTPase mediated signal transduction. The second uncharacterized protein found corresponded to the *ARHGEF18* gene, which has guanyl-nucleotide exchange factor activity and participates in small GTPase mediated signal transduction processes. *Peptidyl arginine deiminase 2* catalyzes the deamination of arginine residues of proteins leading to citrulline. Citrullination is a poorly understood post translational modification that has been related to modulation of epigenetic events, immunity, and transcriptional regulation [32–34]. This protein has potential roles in fertility, since it has been reported that the female human knockout phenotype for the gene coding for a similar protein (PADI6) is sterile due to a cleavage failure of their fertilized eggs [35,36]. The possibility that the spermatozoa vehiculates this protein to the oocyte must be considered and warrants further investigation, taking into account that a set of embryo proteins are exclusively of paternal origin [4]. The *Rho guanine nucleotide exchange factor 28* belong to the family of guanine nucleotide exchange factors, this family of proteins have key regulatory roles in embryo development [37], through regulation of differentiation, proliferation and morphogenesis. *KIAA0825* (A0A3Q2HFS8) was also more abundant in the seminal plasma of stallions showing better total and linear motility post thaw. This is a protein of unknown function, however the murine ortholog is known to be expressed during limb development. It has also been reported that variants of this gene are linked to post axial polydactyly in humans [38]. Other proteins enriched in the seminal plasma of stallions showing better motility post thaw were *rRNA adenine N(6)-methyltransferase*, this protein is involved in rRNA methylation as codified by the transcription factor B1 mitochondrial (TFB1M) gene. The knockouts for this gene show altered mitochondrial function, reduced ATP production and increased levels of reactive oxygen species (ROS) in response to cellular stress [39], providing a potential link between the presence of higher levels of this protein and resistance to cryopreservation. The protein A0A3Q2IAZ9 was also more abundant in the seminal plasma of stallions showing higher percentages of linearly motile sperm post thaw, this protein corresponds to the SCAF1 gene. This gene codifies for a protein with RNA polymerase II C-terminal domain binding. Potential functions in early embryo development could be attributed to this protein, considering recent developments indicating the importance of paternal proteins vehiculated in semen and seminal plasma for early embryo development [4]. We also searched for variables able to discriminate between stallions showing better overall performance post thaw. Four proteins were also discriminant for good motility post thaw, *peptidyl arginine deiminase 2, rRNA, adenine N(6) methyltransferase, KIAAA0825* and an uncharacterized protein corresponding to the SCAR-1 gene, as well as two specific proteins from this group consisting of an ATR serine/threonine kinase and the solute carrier family 25 member 37 (SLC25A37). The *ATR serine/threonine kinase* is activated in the presence of single stranded DNA [40], participating in DNA repair and playing an important role in meiosis in the male germinal epithelium [41], probably linking the higher presence of this protein with better sperm quality, and thus more resistance to the stresses of cryopreservation. The mitochondrial metal transporter *mitoferrin1* (SLC25A37), plays a major

role in mitochondrial iron homeostasis, as well as in the functionality of oxidative phosphorylation proteins [42], this protein is probably related with improved mitochondrial functionality and thus cryo-resistance.

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

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CRediT authorship contribution statement

Gemma Gaitskell-Phillips: Investigation, Writing – review & editing. **Francisco E. Martín-Cano:** Methodology, Investigation. **José M. Ortiz-Rodríguez:** Investigation. **Eva da Silva-Álvarez:** Investigation. **Maria C. Gil:** Investigation, Data curation. **Cristina Ortega-Ferrusola:** Investigation, Supervision, Data curation. **Fernando J. Peña:** Conceptualization, Validation, Formal analysis, Data curation, Writing – original draft, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no conflicts of interest to disclose.

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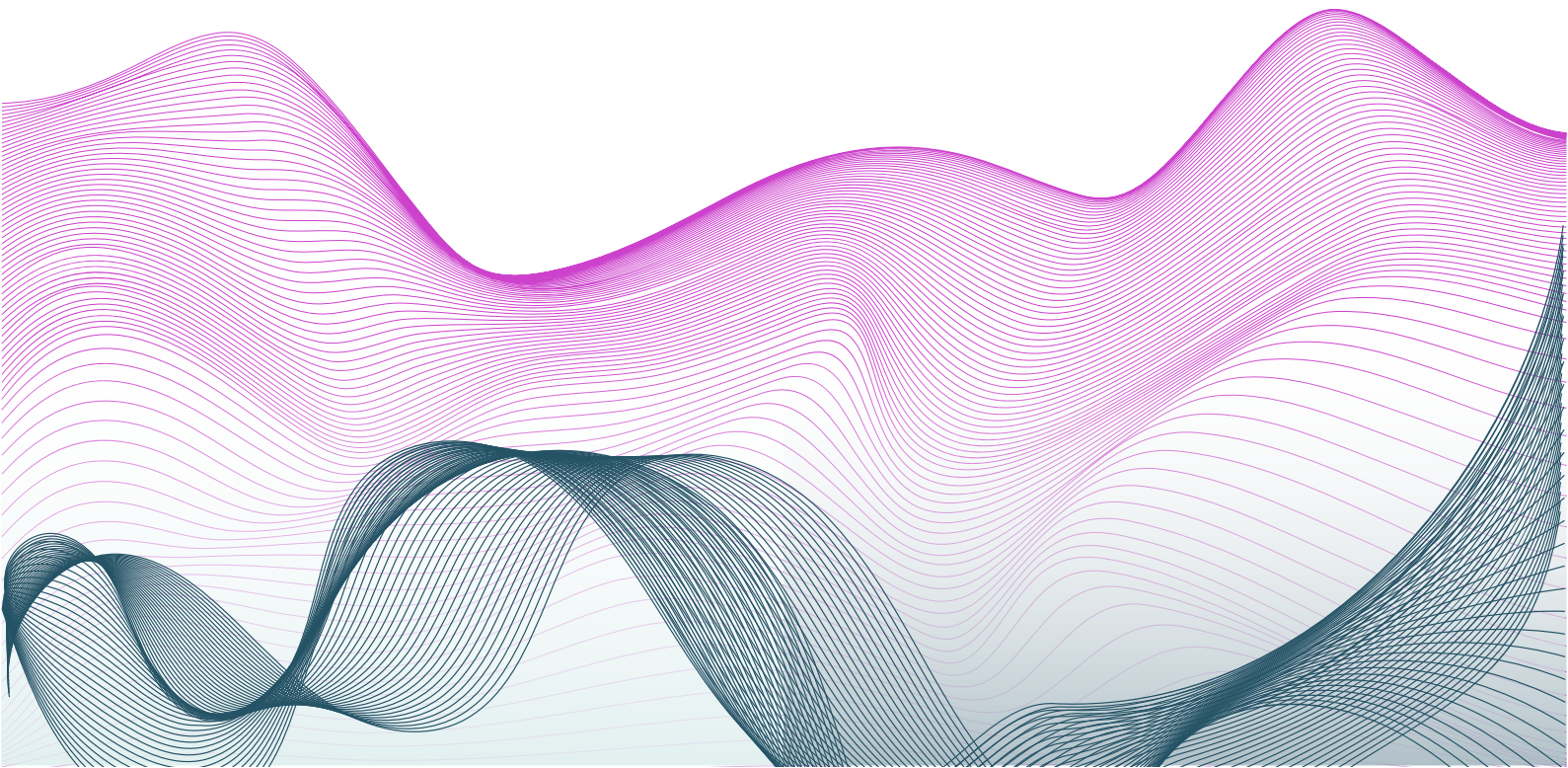
ARTICLE 4



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

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ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

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

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

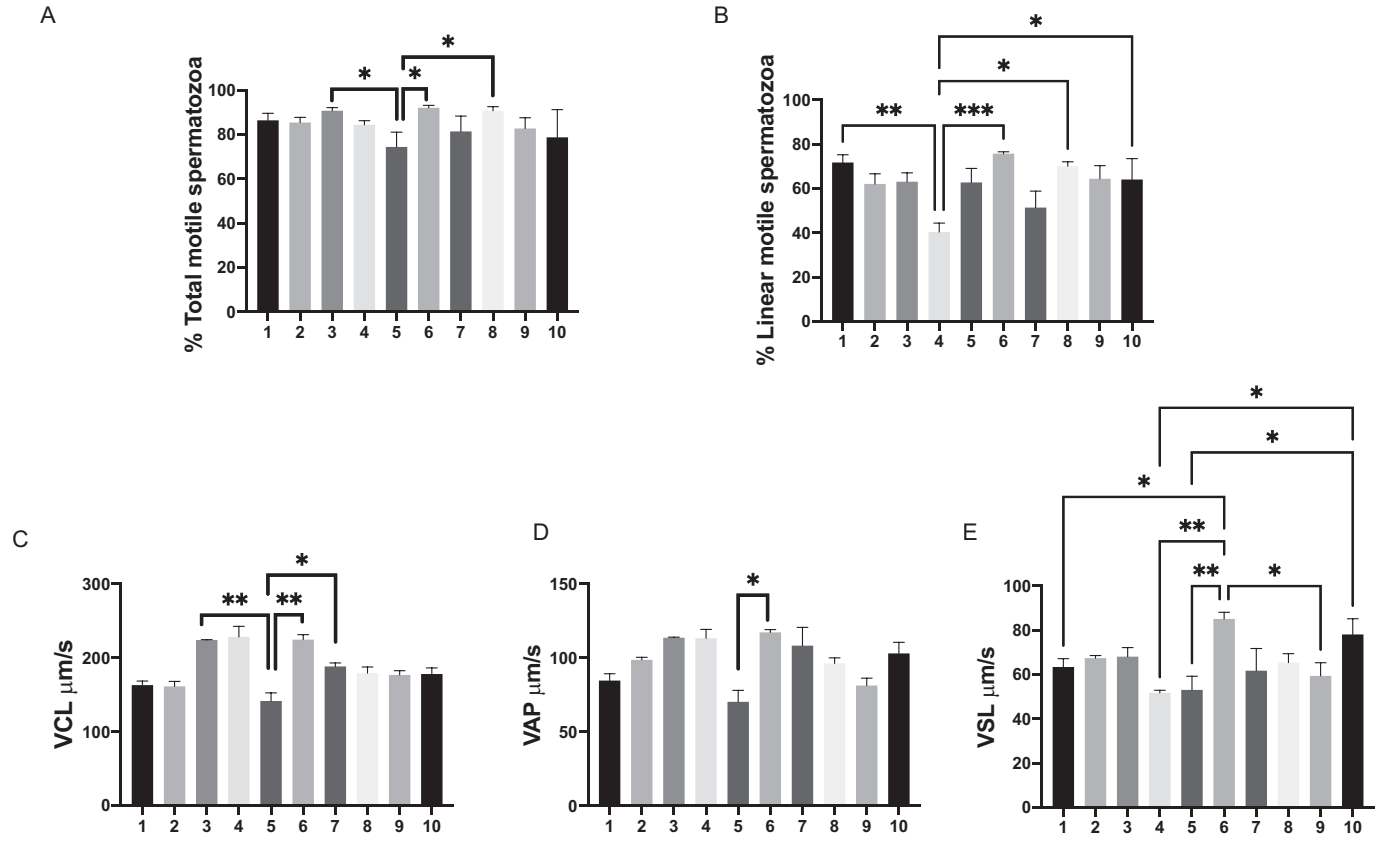


Fig. 1. Computer assisted sperm analysis (CASA), ejaculates were obtained and processed as described in material and methods and the percentages of total motile, linear motile, circular velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) in µm/s determined. A) percentages of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

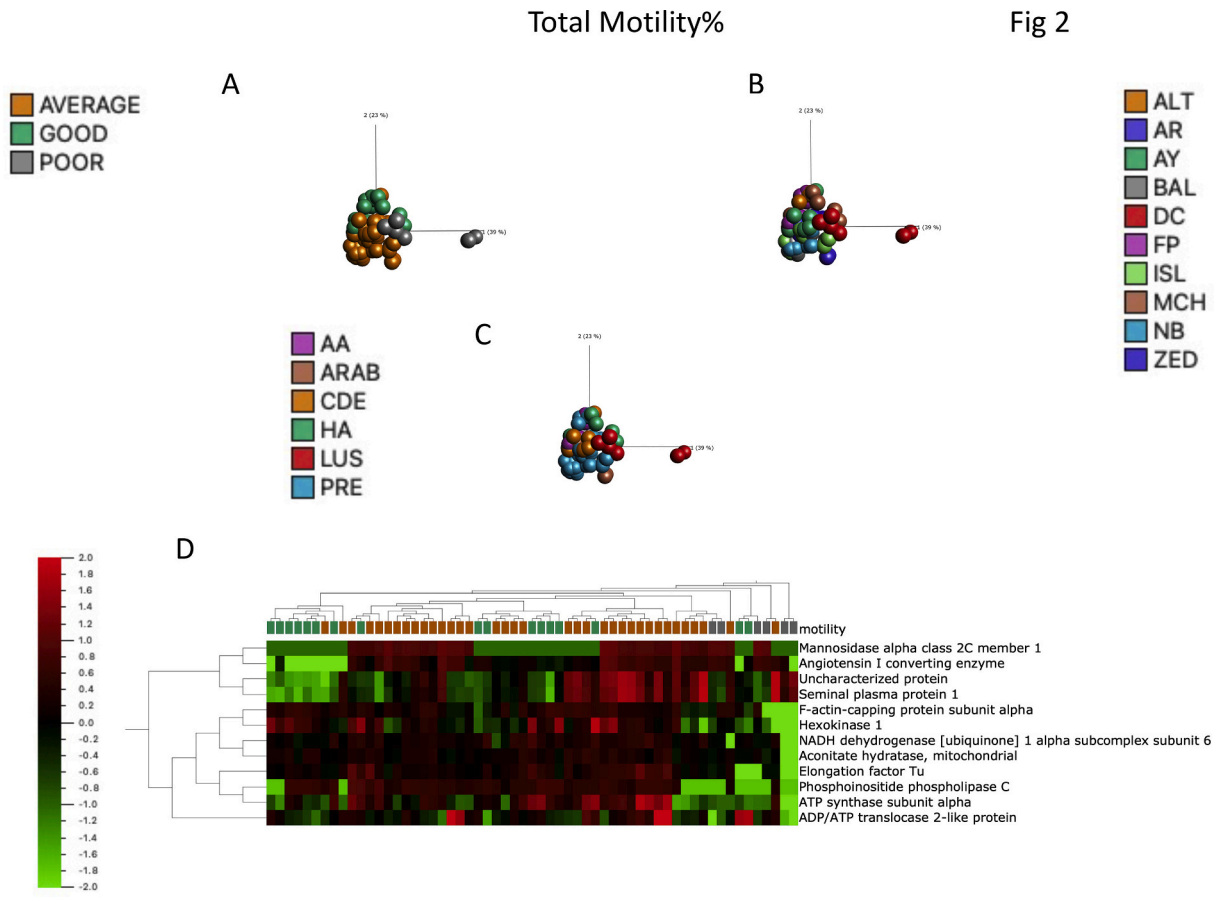


Fig. 2. Bioinformatic analysis of proteins differentially expressed in stallions with different percentages of total motility A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions, average, good, and poor B) 3D PCA overlayed with color codes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

2. Material and methods

2.1. Reagents and media

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

2.2. Semen collection and processing

Semen was collected from 10 stallions of various breeds (5 Andalusians, 1 Spanish Sport Horse, 1 Lusitano, 1 Arab, 1 Anglo-Arabian and 1 Spanish-Arabian horse) maintained as indicated under specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). All stallions were of proven fertility with registered foals and embryos produced as indicated in Table 1 with a median age of 10.8 years old. Semen was collected from all stallions on a regular basis (2–3 times per week), and ejaculates used in this study were collected after depletion of extragonadal sperm reserves. The ethical committee of the University approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina. The gel was removed using an inline filter. Semen was immediately transported to the laboratory after collection for evaluation and processing. Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [35,36] to remove dead spermatozoa, seminal plasma and contaminating cells, and then was re-suspended in Tyrode's medium (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM $NaHCO_3$, 1 mM Na-Pyruvate, 21.6 mM Na-Lactate, 2 mM $CaCl_2$*2H2O, 3.1 mM KCl, 0.4 mM $MgSO_4$*7H2O, 0.3 mM NaH_2PO4*H2O, 0.3% BSA) 315 mOsm/kg and pH 7.4 [37]. An aliquot was immediately removed to measure motility and velocity assessment and the rest was processed for proteomic analysis.

2.3. Sperm preparation

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

2.4. Protein solubilization

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

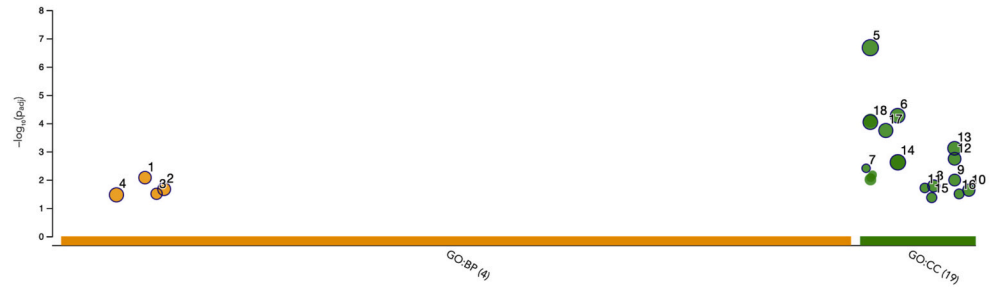
2.5. Protein quantification

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

2.6. In-solution Trypsin digestion

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

A

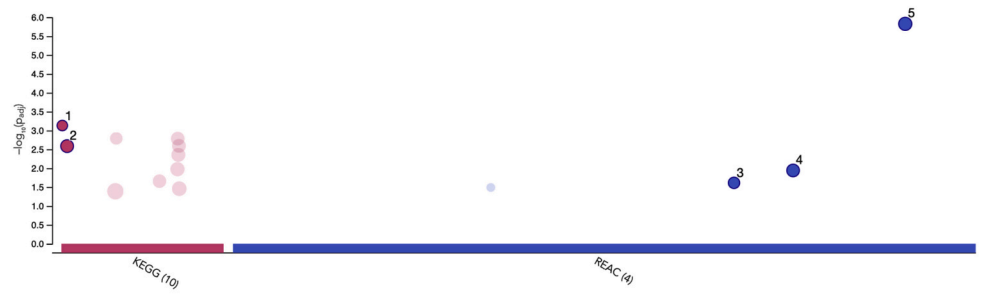


ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:BP	GO:0007338	single fertilization	8.373×10^{-3}
2	GO:BP	GO:0009566	fertilization	2.133×10^{-2}
3	GO:BP	GO:0009060	aerobic respiration	3.118×10^{-2}
4	GO:BP	GO:0006091	generation of precursor metabolites and energy	3.405×10^{-2}
5	GO:CC	GO:0005739	mitochondrion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

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

g:Profiler

B



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

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

g:Profiler

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

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

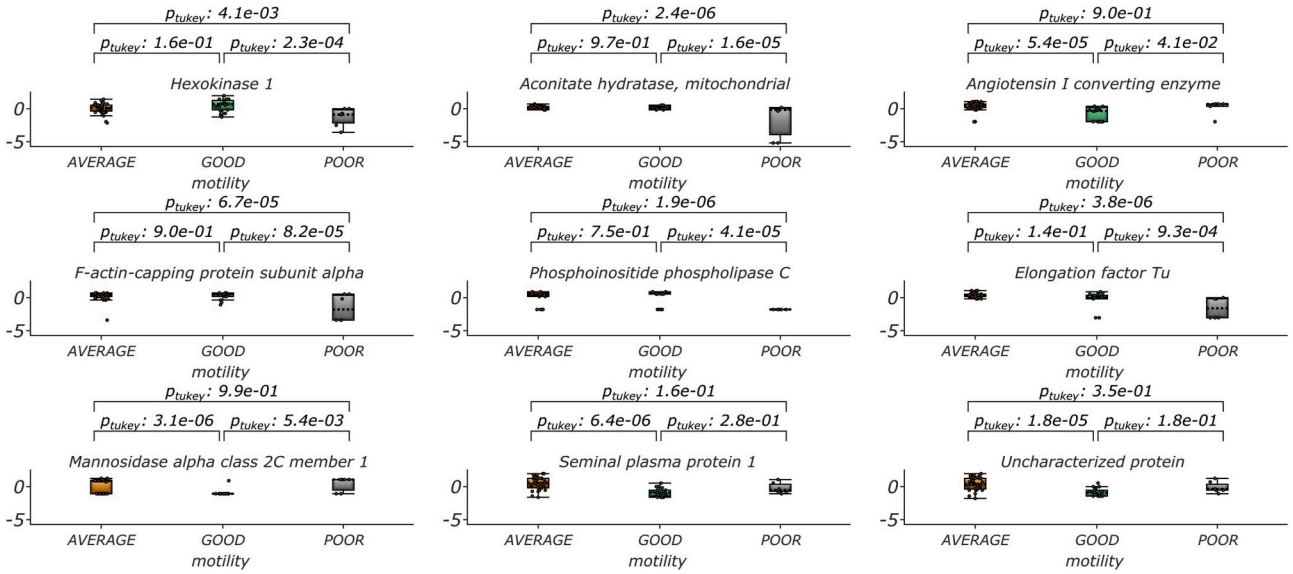


Fig. 4. Differences in the amount of specific representative proteins in stallions with different percentages of total motility. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category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

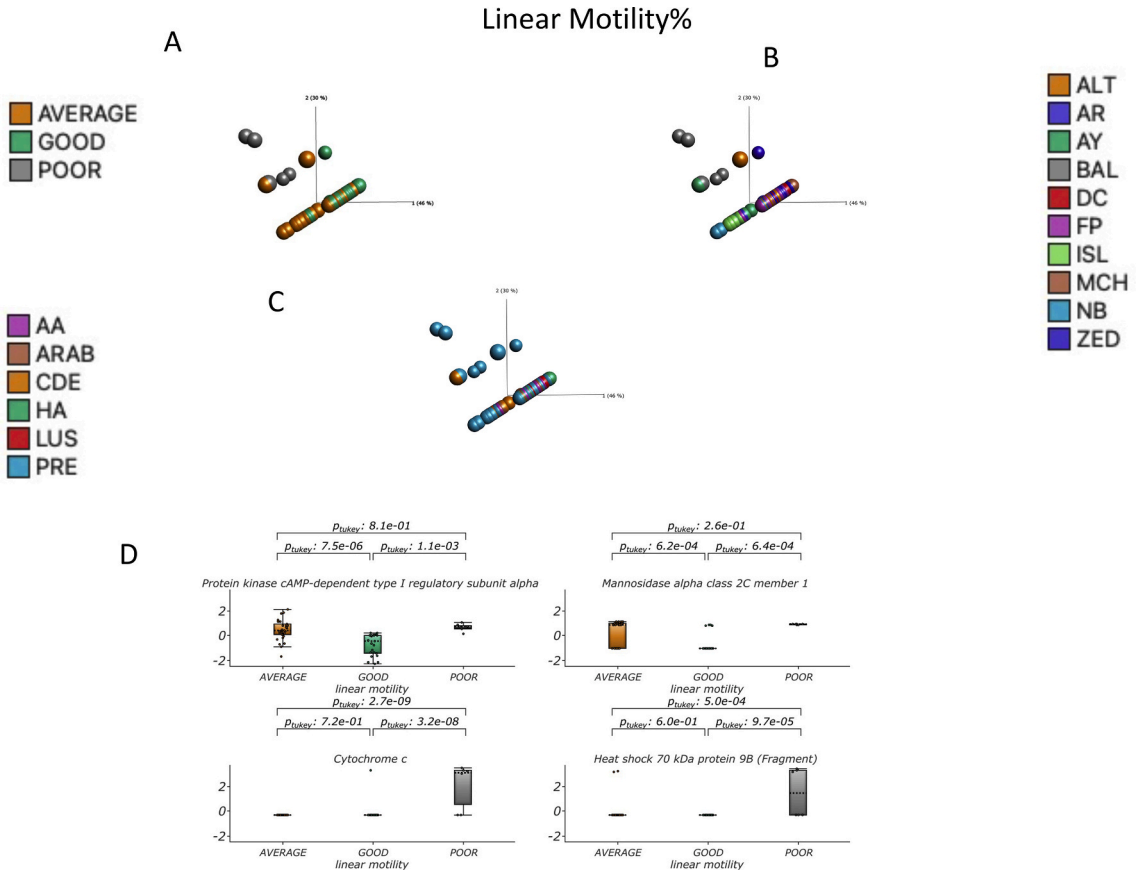


Fig. 5. Differences in the amount of specific representative proteins in stallions with different percentages of linear motility. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category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

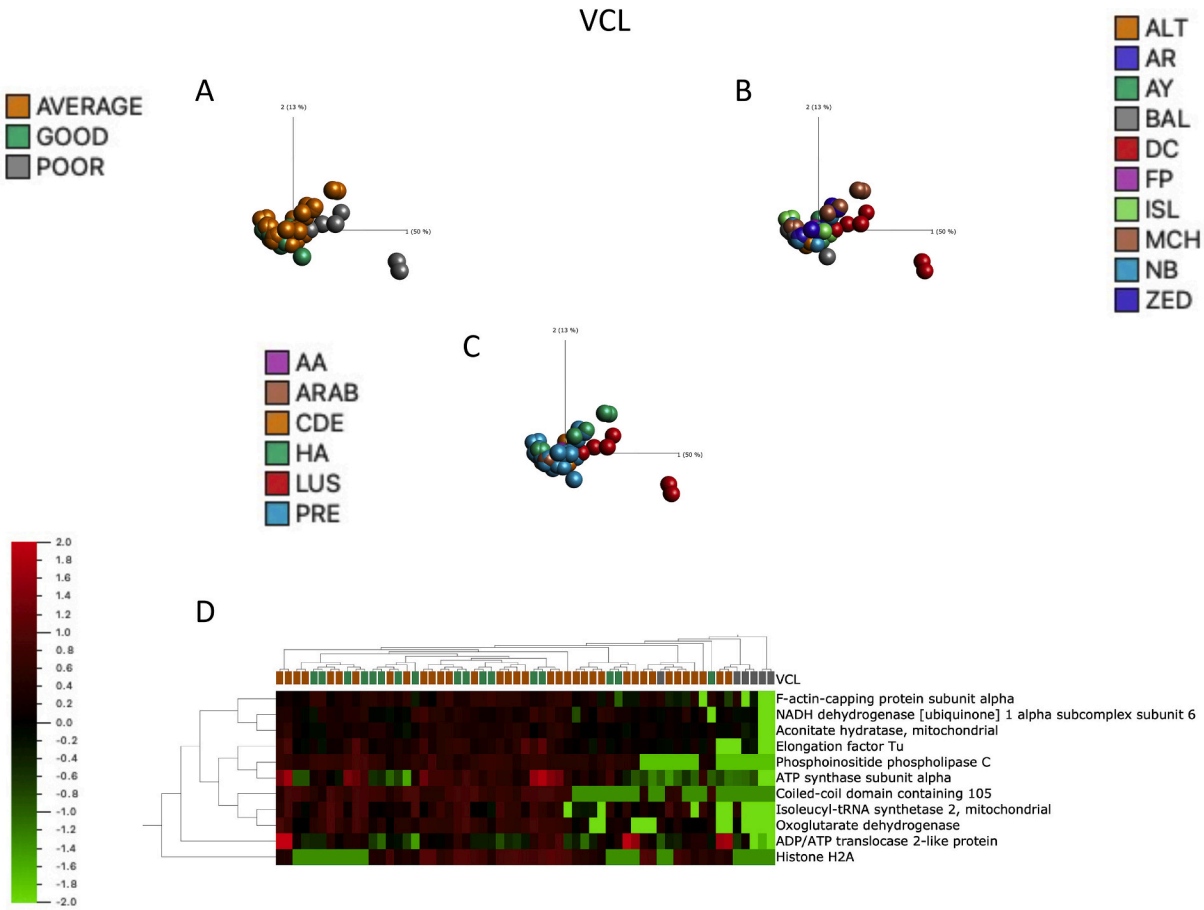


Fig. 6. Bioinformatic analysis of proteins differentially expressed proteins in stallions with different circular velocity (VCL) A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions B) 3D PCA for sperm proteins C) 3D PCA of the samples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

2.7. UHPLC-MS/MS analysis

Separation and analysis of the samples was performed with an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high speed binary pump, and coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the UHPLC and Q-TOF was using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). The sample was processed as previously described [38], in brief was injected onto an Agilent AdvanceBio Peptide Mapping UHPLC column (2.7 μm, 150 × 2.1 mm, Agilent technologies), thermostatted at 55 °C, at a flow rate of 0.4 mL/min. The gradient program started with 2% of B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) that remained in isocratic mode for 5 min, and then increased linearly up to 45% B over 40 min, increasing up to 95% B over 15 min and then remaining constant for 5 min. After this 65 min of run, 5 min of post-time followed, using the initial condition for conditioning of the column for the next run. The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 L/min at a temperature of 250 °C, and the sheath gas flow was set to 12 L/min at 300 °C. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500 V, 340 V and 750 V respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range were 50–1700 m/z and scan rates were 8 spectra/s for MS and 3 spectra/s for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of −4.8. The same ion was rejected after two consecutive scans.

2.8. Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) as previously described [17]. Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH] + 50–10,000 m/z; maximum precursor charge +5; retention time and m/z tolerance ±60 s; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against Uniprot/Horse (https://www.uniprot.org/taxonomy/9796) was performed as follows: non fixed modifications were selected and as a variable modification: carbamidomethylated cysteines and tryptic digestion with 5 maximum missed cleavages were selected. ESI-Q-TOF instrument with minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation of reversed database scores. The autovalidation strategy used was auto-threshold, in which the peptide score is automatically optimized for a target % FDR (1.2%). Protein polishing validation was then performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0%).

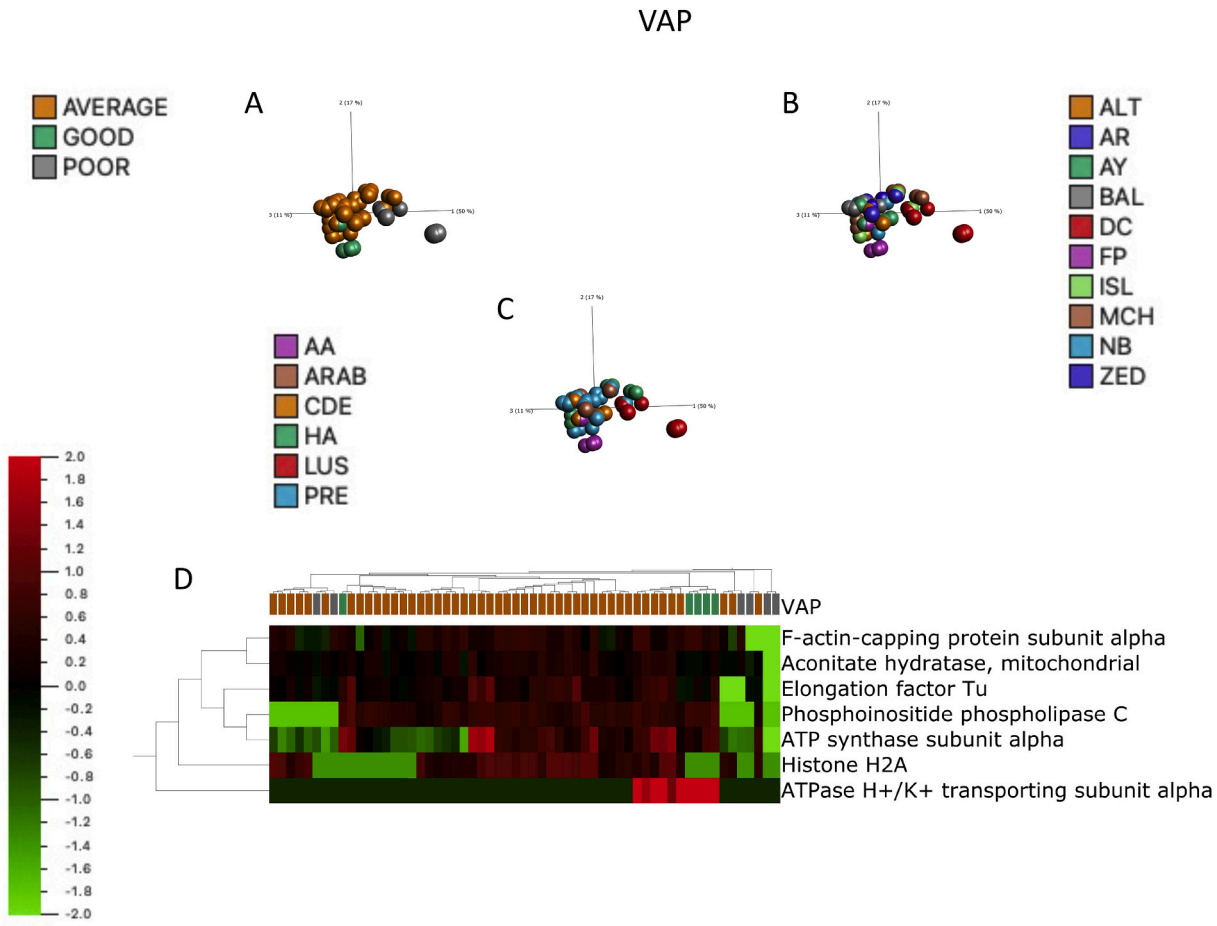


Fig. 7. Bioinformatic analysis of proteins differentially expressed in stallions with different average path velocity (VAP) A) 3D principal component analysis (PCA) of the samples, different color codes are applied to each group of stallions B) 3D PCA for sperm proteins C) Heat Map showing different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

2.9. Computer-assisted sperm analysis (CASA)

Sperm motility and velocity were assessed in fresh and frozen thawed samples using a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to standard protocols used at our center [39]. Semen samples were loaded into a Leja® chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37 °C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10 x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with a VAP > 35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

2.10. Bioinformatic analysis

2.10.1. Variance filtering and PCA

Data were normalized and $\log_2$ transformed using Qlucore Omics Explorer (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [40] was used to determine the optimal filtering threshold.

2.10.2. Identifying discriminating variables

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

2.10.3. Enrichment analysis of pathways in the sperm proteome

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

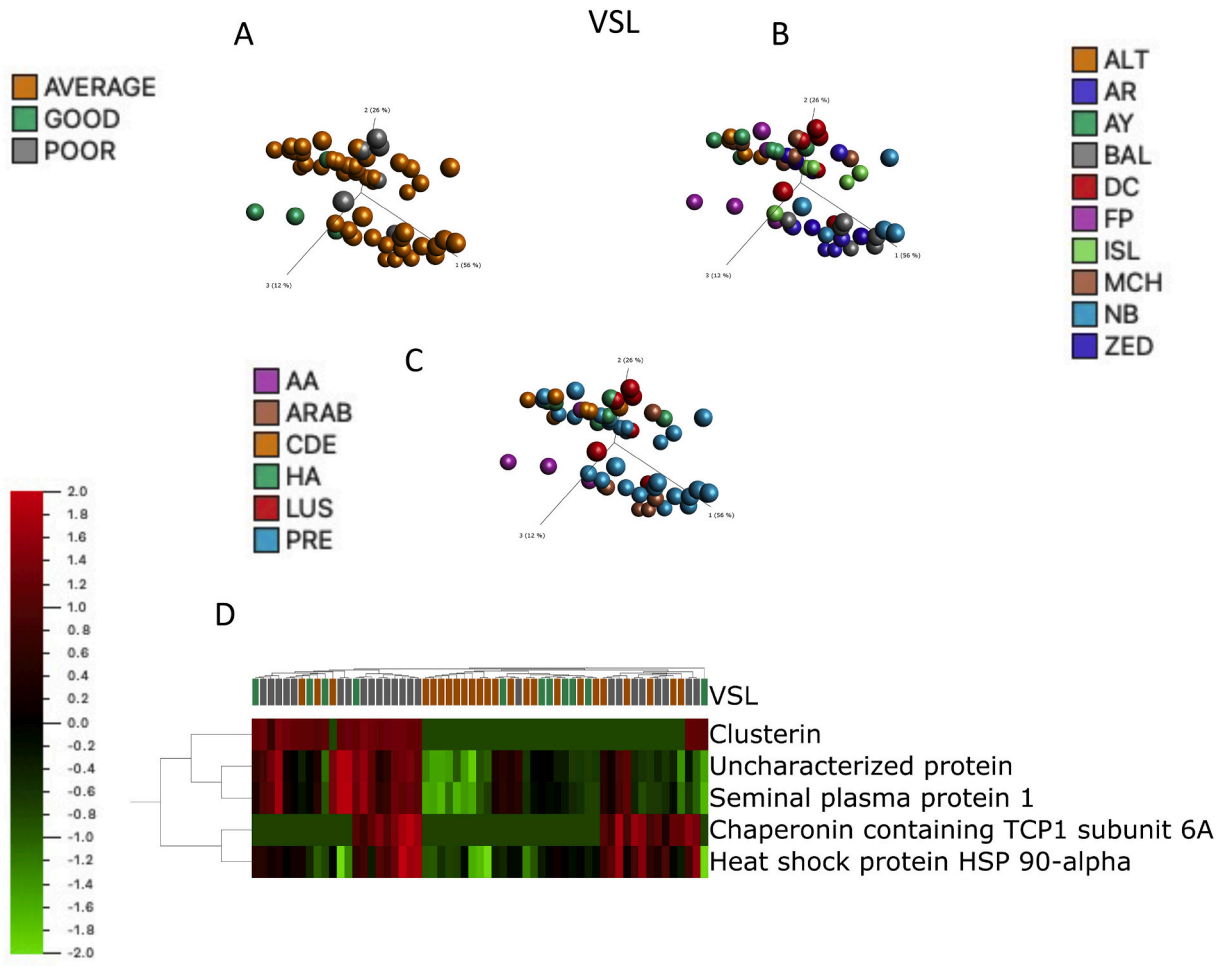


Fig. 8. Differences in the amount of specific representative proteins in stallions with different percentages of VSL. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts. Proteins in each category are represented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

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

2.11. Western blotting

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

2.12. Statistical analysis

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

3. Results

3.1. Sperm motility and velocity

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

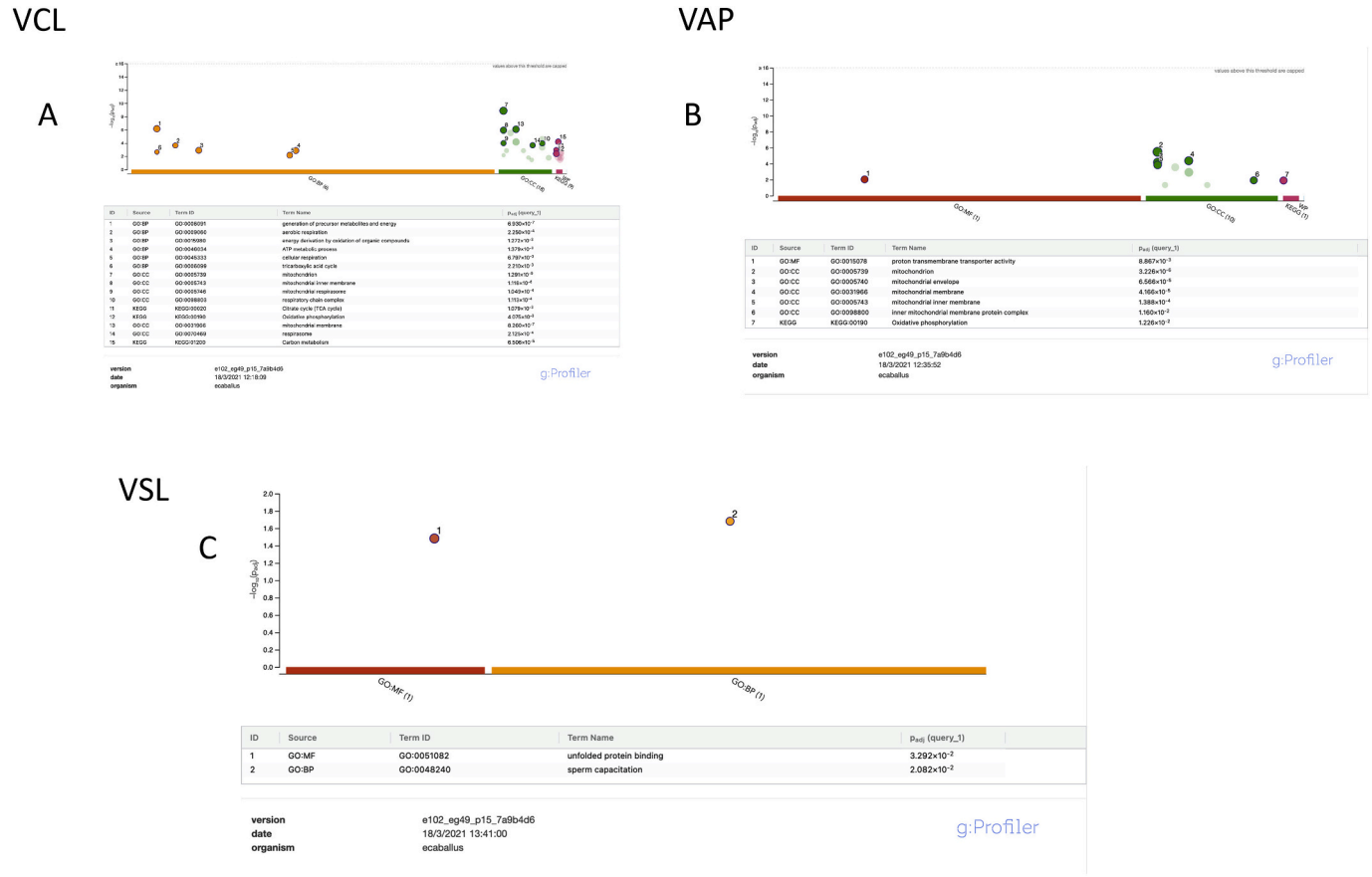


Fig. 9. g:GOST multiquery Manhattan plot showing enrichment analysis of proteins present in different amounts in stallions with different VCL, VAP and VSL. The sperm proteome was queried against the equine proteome database. A) Gene Ontology (GO) terms for biological process (BP) are in orange, and those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

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

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

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

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

Differences in protein composition were related to differences in

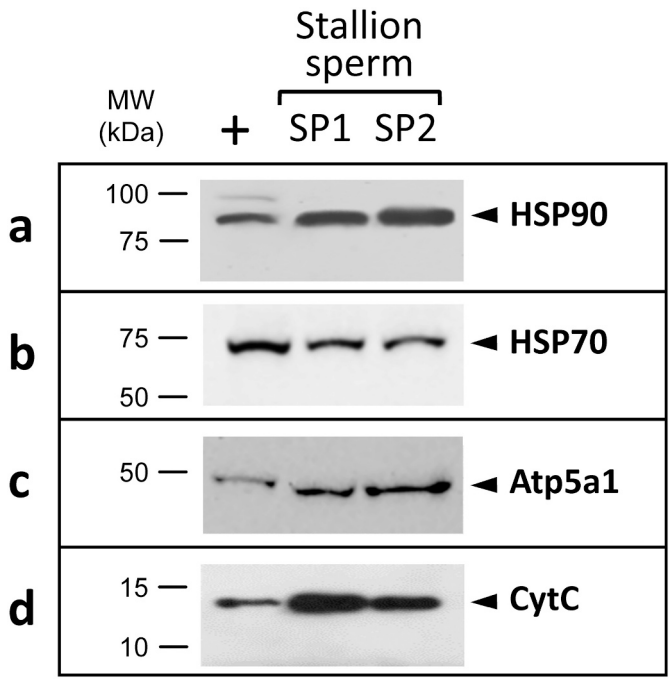


Fig. 10. Representative subset of proteins identified were validated through western blotting analysis.Sperm lysates from two different stallions were used. a) HSP90 b) HSP70 c) ATP5A1 and d) Cytochrome C. Positive controls used were brain lysates for ATP5A1, and Hela cells for HSP70, HSP90 and cytochrome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

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

4. Discussion

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

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

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

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

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

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

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

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

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

Declaration of competing interest

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

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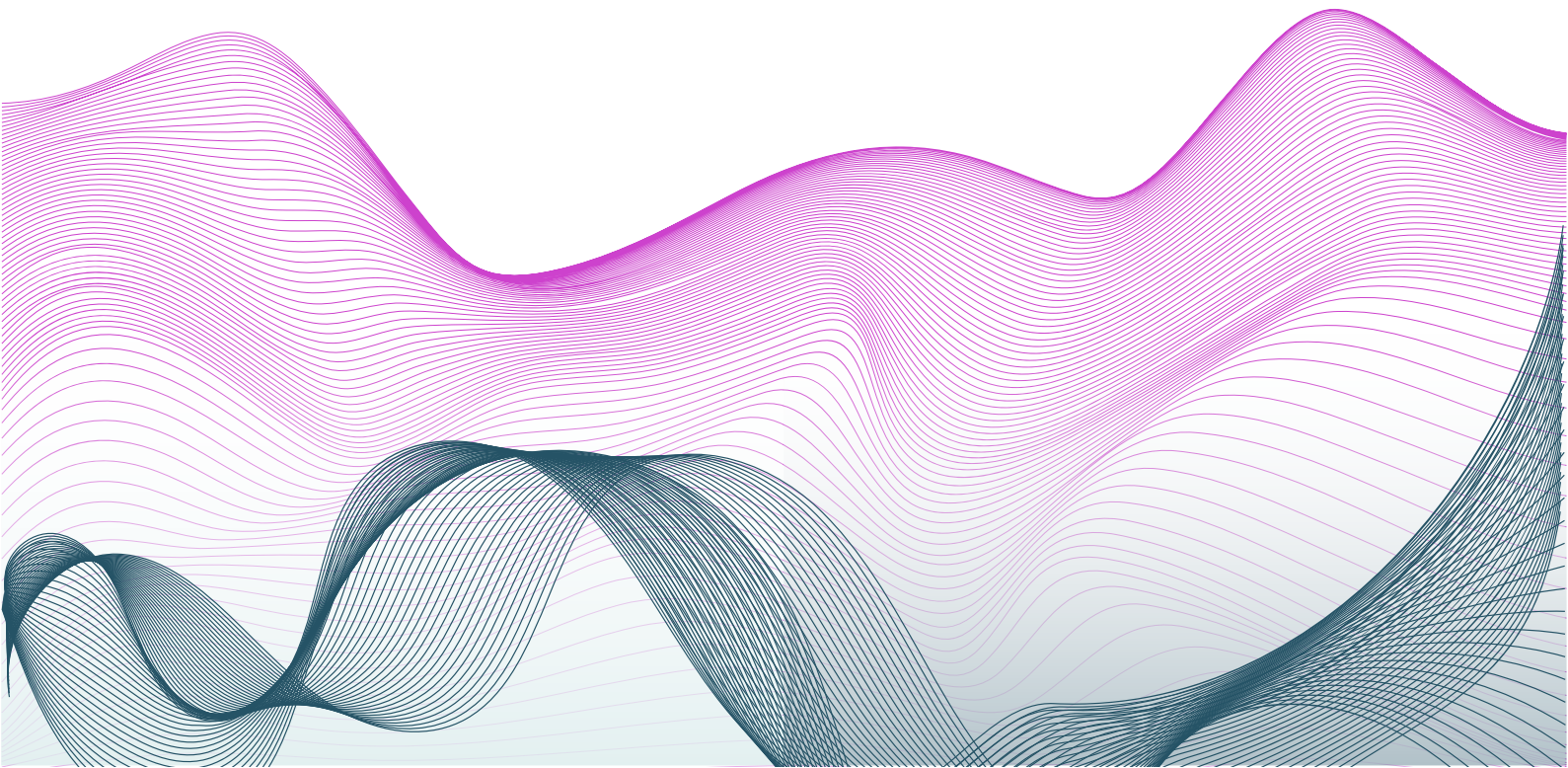

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Article

In Stallion Spermatozoa, Superoxide Dismutase (Cu−Zn) (SOD1) and the Aldo-Keto-Reductase Family 1 Member b (AKR1B1) Are the Proteins Most Significantly Reduced by Cryopreservation

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

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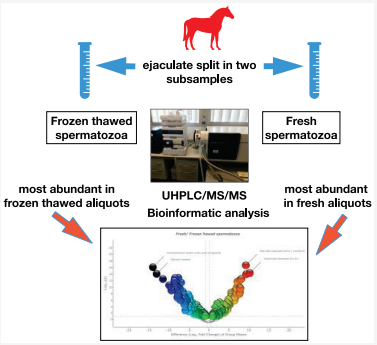
ABSTRACT: Although cryopreservation is widely used in animal breeding, the technique is still suboptimal. The population of spermatozoa surviving the procedure experiences changes attributed to alteration in their redox regulation. In order to expand our knowledge regarding this particular aspect, the proteome in fresh and frozen thawed aliquots of equine spermatozoa was studied to identify the proteins most severely affected by the procedure. If alteration of redox regulation is a major factor explaining cryodamage, proteins participating in redox regulation should be principally affected. Using a split sample design, 30 ejaculates from 10 different stallions were analyzed as fresh spermatozoa, and another aliquot from the same ejaculate was analyzed as a frozen thawed sample. The proteome was studied under both conditions using UHPLC-MS/MS and bioinformatic analysis conducted to identify discriminant variables between both conditions. Data are available through the ProteomeXchange Consortium with identifier PXD022236. The proteins most significantly reduced were *Aldo-keto reductase family 1 member B* ($p = 2.2 \times 10^{-17}$) and *Superoxide dismutase (Cu−Zn)* ($p = 4.7 \times 10^{-14}$). This is the first time that SOD1 has been identified as a discriminating variable using bioinformatic analysis, where it was one of the most highly significantly different proteins seen between fresh and frozen thawed semen. This finding strongly supports the theory that alteration in redox regulation and oxidative stress is a major factor involved in cryodamage and suggests that control of redox regulation should be a major target to improve current cryopreservation procedures.



KEYWORDS: *spermatozoa, cryopreservation, redox, proteomics, equids*

INTRODUCTION

In comparison with other domestic species, artificial insemination (AI) with frozen thawed spermatozoa is not as widely used in equine breeding. There are a number of factors that can explain this situation, among them past restrictions in the use of reproductive technologies by most stud books. As a result of these restrictions, in most breeds artificial insemination has been introduced in the past two decades. In comparison, artificial insemination has been widely used in bovines since the 1950s. As a consequence, research in equine reproductive biotechnologies is lagging a few decades behind. Perhaps the major constraint for the development of AI using equine frozen thawed semen is the stallion to stallion variability in cryotolerance.[1,2] However, in spite of recent research,[3,4] the reasons behind this variability remain largely unknown. Cryopreservation causes osmotic stress during freezing and especially during thawing, damaging all the sperm structures, including plasma and mitochondrial membranes.[4] Osmotic effects account for sperm death due to acute necrosis; however, an important percentage of the surviving population experience accelerated aging leading to premature cell death. It is believed this process is due to redox imbalance and oxidative stress originating in the mitochondria. Advances in the identification of all the factors that control cryotolerance could be of significant importance for the equine industry. These are likely to serve as powerful tools for definition of specific targets which can be used to improve existing protocols used for cryopreservation. The introduction of the omics in spermatology is facilitating a rapid progression of knowledge of the biology of spermatozoa,[5−9] and these technologies have recently been introduced as a tool for the study of the stallion spermatozoa.[10,11] Proteomic technologies have been applied to investigate the changes induced by cryopreservation in different species,[11−18] but to date, only one study[11] has addressed the impact of cryopreservation in equine spermatozoa, revealing an important impact on proteins involved

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J. Proteome Res. 2021, 20, 2435−2446

in both oxidative phosphorylation and redox regulation. This study focused on enrichment analysis of groups of proteins based on gene ontology terms and pathways analysis. The present study aimed to define the effects of cryopreservation on the equine sperm proteome.

MATERIALS AND METHODS

Reagents and Media

Hoechst 33342 [(Ex: 350 nm, Em: 461 nm), (ref: H3570)] was purchased from Molecular Probes (Leiden, The Netherlands). Anti-4 hydroxynonenal (4-HNE) antibody [HNEJ-2] (ref: ab48506) and goat antimouse IgG (H&L) Alexa Fluor 647 [(Ex: 652 nm, Em: 668 nm) (ref: ab150115)] were purchased from Abcam (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated.

Semen Collection and Processing

A sample of 10 stallions of different breeds was used to obtain semen. Animals were housed as required by specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). The study was approved by the University ethics committee. Collection of ejaculates was performed using a Missouri model artificial vagina, which was warmed and lubricated. An inline filter was used for removal of the gel fraction of the ejaculate. Upon collection semen was subsequently transferred straight to the laboratory where it was evaluated and processed. A split sample approach was used for experimental design, by dividing single ejaculates into two subsamples (fresh and frozen thawed experimental groups). Semen was processed in the laboratory using colloidal centrifugation[19,20] to remove both dead spermatozoa and seminal plasma, and then either resuspended in Tyrodes media (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM $NaHCO_3$, 1 mM Na-Pyruvate, 21.6 Na-Lactate, 2 mM $CaCl_2{\cdot}2H_2O$, 3.1 mM KCl, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.3 mM $NaH_2PO_4{\cdot}H_2O$, 0.3% BSA) 285 and 315 mOsm/kg at pH 7.4[21] (fresh extended semen), or resuspended in cryopreservation media using standard procedures and protocols, as described in previous research by our group used for freezing (frozen thawed semen).[22] Briefly, dilution of the aliquot was performed using the Cáceres freezing medium (University of Extremadura Cáceres, Spain), which is formulated from 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100×10^6 spermatozoa/mL. Extended semen was loaded into 0.5 mL straws (IMV, L'Aigle, France), which were then sealed ultrasonically using an UltraSeal 21 (Minitube of America MOFA, Verona, Wisconsin, USA) machine, after which they were immediately transferred to an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The freezing curve used followed the subsequent steps. Straws were first kept at 20 °C for 15 min, after which they were then cooled slowly from 20 to 5 °C at a cooling rate of 0.1 °C/min. The freezing rate was then increased to −40 °C/min to take the temperature from 5 °C to −140 °C. After completion of the freezing curve straws were then plunged into liquid nitrogen and stored until further analysis. Thawing of frozen straws was performed using a water bath at 37 °C for at least 30 s.

Experimental Design

Ejaculates were collected from 10 different stallions on 3 different occasions and collection and processing was as follows. Ejaculates were divided into two, and half of each ejaculate was frozen using standard protocols previously described in our laboratory (frozen thawed), while the other half was processed as for fresh spermatozoa (fresh).

Sperm Preparation

Both fresh and frozen thawed (FT) samples of spermatozoa were washed three times using PBS (600g × 10 min). After this, samples were subsequently pelleted and kept frozen at −80 °C until further analysis. Phase contrast microscopy was used to ensure purity of the samples.

Protein Solubilization

Lysis buffer (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7 M urea, 2 M thiourea, and 40 mM Tris (pH 10.4)) was used to solubilize isolated spermatozoa (200×10^6 spermatozoa). Twenty microliters of lysis buffer were added for every 10×10^6 spermatozoa, which were then vortexed and incubated while under constant rotation at −4 °C for 1 h.

Protein Quantification

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

In-Solution Trypsin Digestion

100 μL of 25 mM ammonium bicarbonate buffer pH 8.5 (100 μg of protein in 300 μL of solution) was mixed with 200 μL of solution obtained from the previous protein solubilization stage. Proteins were reduced in this solution by the addition of 30 μL of 10 mM DTT after which they were incubated at 56 °C for 20 min. Alkylation of proteins was then performed by adding 30 μL of 20 mM IAA with subsequent incubation for 30 min at room temperature (r.t.) in the dark. Lastly, 1 μL of Trypsin Proteomics grade (Sigma) (Trypsin solution: 1 μg/μL in 1 mM HCl) was added for digestion of proteins for at least 3 h to overnight at 37 °C. Ten μL of 0.1% formic acid was used to stop the reaction and samples were filtered through 0.2 μm (hydrophilic PTFE) into 2 mL dark glass vials. To complete the process, samples were dehydrated using a nitrogen current with the vial placed in a heating block at 35 °C. Dry samples were then resuspended in 20 μL of buffer A, consisting of water/acetonitrile/formic acid (94.9:5:0.1)

UHPLC-MS/MS Analysis

A UHPLC-MS system consisting of an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) fitted with an automated multisampler module and a high speed binary pump, coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface was used to separate and analyze the samples. MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01) was used to control the HPLC and Q-TOF. Samples were injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 μm, 150 × 2.1 mm, Agilent technologies), appropriate for peptide separation and analysis, at a flow rate of 0.4 mL/min and thermostatted at 55 °C. Operation of the mass spectrometer was in positive mode and a gradient program was used starting with 2% of B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) remaining in isocratic mode for 5 min, increasing linearly up to 45% B over a period of 40 min, after which it was further increased up to

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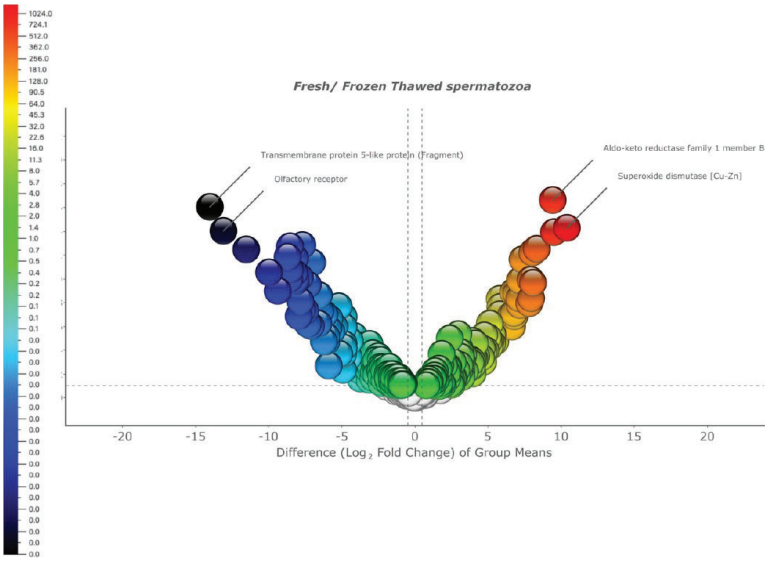

Figure 1. Volcano plot showing changes in the stallion sperm proteome as a consequence of cryopreservation. Proteins which were more abundant in fresh samples (and less abundant in frozen thawed samples) are presented on the right-hand side of the volcano plot, proteins most abundant in frozen and thawed samples (and less abundant in fresh samples) are presented on the left-hand side of the volcano plot. The two most significantly different proteins between the two conditions (higher fold change and higher p and q values) are depicted. The difference of protein content ($\log_2$ fold change) is plotted against the significance of the difference $-\log_{10}(p)$ between the two conditions (fresh and frozen thawed spermatozoa) (3 independent ejaculates from 10 different stallions in addition to two technical replicates n = 60 samples were used to derive results from).

95% B over a time frame of 15 min and then kept constant for 5 min. The initial condition for column conditioning was used for 5 min of post-time after this 65 min run prior to the next run. Nebulizer gas pressure was set to 35 psi, and drying gas flow was set to 10 L/min at a temperature of 250 °C, with sheath gas flow set to 12 L/min at 300 °C. Capillary spray, fragmentor and octopole RF Vpp voltages were 3500 V, 340 and 750 V respectively. Profile data were acquired for both MS and MS/MS scans in Extended dynamic range mode[23,24] was used for acquisition of profile data for both MS and MS/MS scans to eradicate potential contaminants. MS and MS/MS scan rates were 8 spectra/sec and 3 spectra/sec respectively with a mass range of 50−1700 m/z. Precursor selection by abundance and a maximum of 20 precursors selected per cycle were used in auto MS/MS mode. A ramped collision energy was used with a slope of 3.6 and an offset of −4.8. The same ion was rejected after two consecutive scans.

Data Processing

Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) was used for processing and analysis of data. In summary, default conditions were used for extraction of raw data as follows: nonfixed or variable modifications were selected; $[MH]^+$ 50−10 000 m/z; maximum precursor charge +5; retention time and m/z tolerance ±60 s; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The following criteria were used for the MS/MS search against the appropriate and updated protein database (in this case: Uniprot/Horse): selection of nonfixed modifications with the following selected as variable modifications: carbamidomethylated cysteines and tryptic digestion with 5 maximum missed cleavages; ESI-Q-TOF instrument, minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation

Figure 2. Venn diagram showing the overall impact of cryopreservation on the proteome of stallion spermatozoa. The number of proteins present in significantly different amounts (either increased or decreased) in both conditions are presented in the diagram. On the left, the number of proteins present in significantly different amounts in fresh semen, on the right the number of proteins present in significantly different amounts in frozen thawed samples.

of reversed database scores. Validation of peptide and protein data was performed using the autovalidation algorithm. This is completely automated and used to validate the highest-scoring results; those which do not require manual review are considered high-quality results. The autovalidation strategy used was autothreshold, in which the peptide score is automatically optimized for a target % FDR (1.2%). Protein polishing validation was then performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0%).

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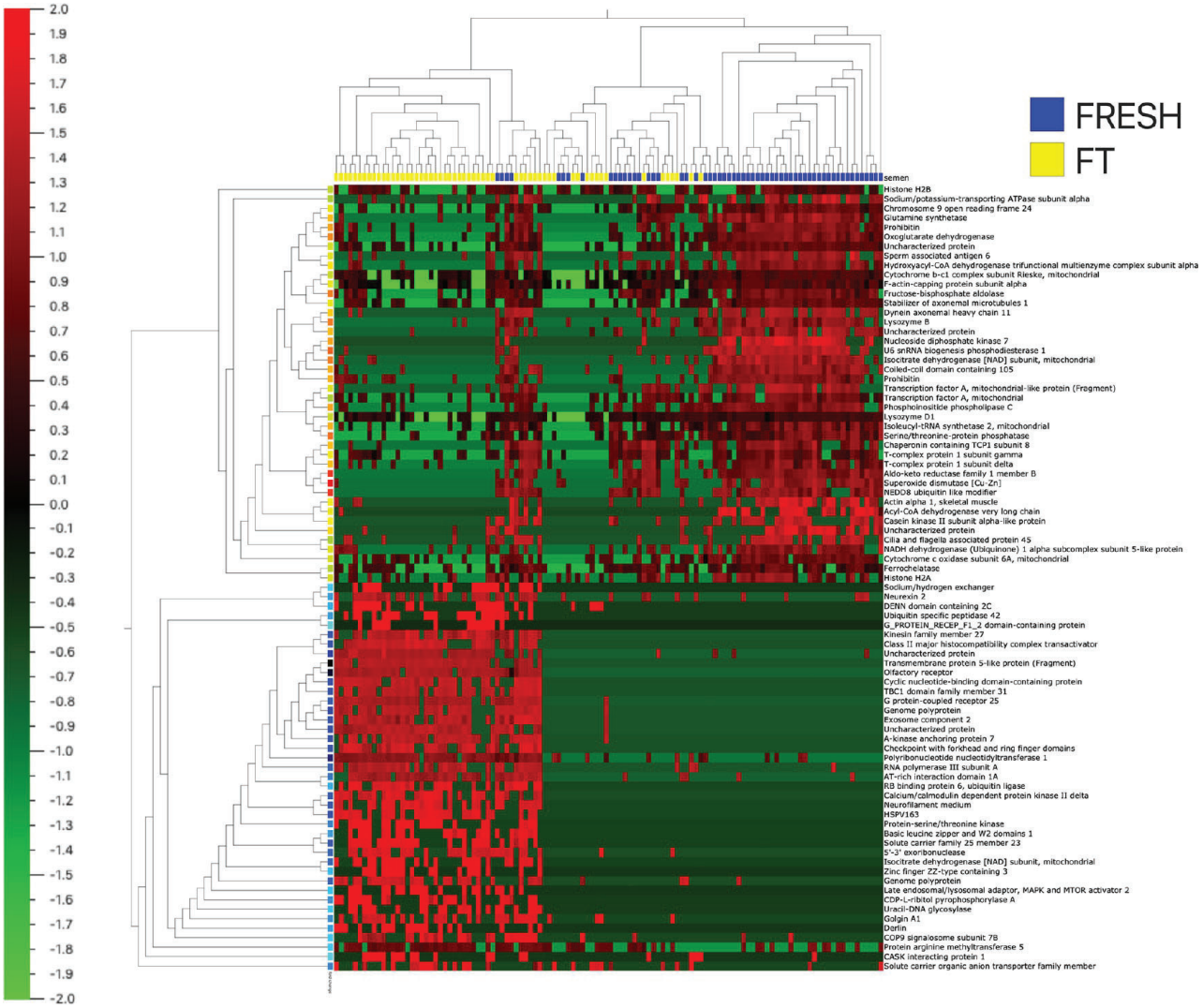

Figure 3. Heat map showing the impact of cryopreservation on the proteome of stallion spermatozoa. Proteins are classified following hierarchical clustering. Blue marks correspond with fresh samples, yellow marks correspond with frozen thawed samples. The heat map code is present with red areas representing greater amounts of protein and green areas lesser amount of protein. Proteins were normalized and filtered by a fold change >3 with $p = 0.04$ and $q = 0.1$.

Bioinformatics

Variance Filtering and PCA. Data were normalized and log transformed. Variables with low overall variance were filtered out to reduce the impact of noise, and the remaining variables were then centered and scaled to zero mean and unit variance, after which Principal Component Analysis (PCA) was used for visualization of the data set in a three-dimensional space. The optimal filter threshold was established using the projection score.[25,26] Qlucore Omics Explorer version 3.6 Lund Sweden (https://qlucore.com) bioinformatics software was used for analysis. Hierarchical clustering and heat maps were used to display protein expression patterns[27] and t-SNE (t-statistic Stochastic Neighbor Embedding) maps of standardized samples were used to identify relations between samples.[28,29]

Identification of Discriminating Variables. Qlucore Omics Explorer (https://qlucore.com) was used for identification of discriminating variables that are most highly significantly different between the two subgroups, fresh and frozen thawed spermatozoa. Identification of significantly different variables between the subgroups of fresh and frozen thawed spermatozoa from every single ejaculate was undertaken by fitting a linear model for each variable with each condition as a predictor and including the stallion nuisance covariate. The Benjamini−Hochberg method[30,31] was used for multiple testing with adjusted p-values, and variables with adjusted p-values below 0.1 were considered significant.

Enrichment Analysis. Enrichment analysis was performed using the g:Profiler web server (https://biit.cs.ut.ee/gprofiler/gost)[32] of the most abundant proteins in fresh or frozen thawed ejaculates. Electronically inferred annotations were excluded from enrichment analysis and focus on annotations with stronger evidence when higher confidence is wanted for the enrichment results. Only annotated genes were used and the g:SCS threshold was set at $P < 0.01$.

Spermatozoa Motility

A Computer Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) was used to assess sperm motility and

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J. Proteome Res. 2021, 20, 2435−2446

kinematic parameters as previously described.[33−35] In brief, a Leja chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) was placed on a warmed stage, at 38 °C and loaded with semen. Evaluation of 60 consecutive digitalized images obtained using a 10× negative phase-contrast objective (Olympus CX41) was used for analysis. A minimum of three different fields were captured, ensuring that at least 500 spermatozoa were analyzed per sample. Spermatozoa with a VAP (average velocity) < 15 μm/s were considered immotile, while spermatozoa with VAP > 15 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

Measurement of Lipid Peroxidation. Two μL/mL of a stock solution of 0.1 mg/mL of anti 4-HNE primary antibody was used to stain spermatozoa (5×10^6/mL) in 1 mL of PBS and these were then incubated at r.t. in the dark for 30 min. PBS was then used to wash cells and these were stained with 2 μL/ml of secondary Anti mouse Alexa Fluor 647 antibody (Excitation 650 nm, Emission 665 nm) and 0.2 μM Hoechst 33342 (Excitation 350 nm, Emission 461 nm) and incubated for a further 30 min in the dark at r.t.. Cells were then washed in PBS and samples were run immediately through the flow cytometer (Cytoflex LX flow cytometer Beckman Coulter, Brea, CA, USA), equipped with ultraviolet, violet, blue, yellow, red, and infrared lasers. Daily calibration of the instrument was performed using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowjoV 10.7.1 Software for Mac (Ashland, OR, USA). Controls consisted of unstained, single stained, secondary only antibody staining, and fluorescence minus one (FMO) controls, to ensure gates and compensations were properly set. Positive controls for 4-HNE were samples supplemented with 800 μM SO_4Fe and 1.7 M of H_2O_2 (Sigma) to induce the Fenton reaction. Debris were gated out based on DNA content of the events after H33342 staining.[36,37]

Statistical Analysis

GraphPad Prism version 7.00 for Mac, La Jolla, CA, USA, www.graphpad.com was used for statistical analysis. Fresh and frozen thawed samples were compared using a two tailed Mann−Whitney test. Differences were considered significant when $p < 0.05$, and results are displayed as means ± SEM.

RESULTS

Cryopreservation Impacts the Stallion Sperm Proteome

Identification and quantification of a total of 910 different proteins was performed. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE[38] partner repository with data set identifier PXD022236.

Cryopreservation caused an important impact on the stallion sperm proteome (Figure 1). A Volcano plot revealed that numerous proteins experienced either increases or decreases in levels as a result of cryopreservation. Venn diagrams were used to identify the number of proteins varying between both conditions (Figure 2). Following this a two group (fresh vs frozen thawed) analysis was performed and changes identified in the proteome with a fold change >2 with $p = 0.04$ and $q = 0.1$, obtaining 102 proteins affected by the procedure. In the heat map in Figure 3, changes in the stallion proteome as a consequence of cryopreservation are represented. Another Venn diagram was then built to identify proteins changing during the procedure and proteins which were abundant under both fresh and frozen thawed conditions (Figure 4). In order to better identify changes caused by cryopreservation, enrichment analysis in both groups using g profiler was performed. In fresh semen, the proteins identified were enriched with the GO terms and KEGG pathways related to *oxidoreductase activity*, *cellular respiration*, *mitochondria*, *chaperone complex*, and *metabolic pathways* (Figure 5). In proteins which were most abundant in frozen thawed samples, the Kyoto encyclopedia of genes and genomes (KEGG) pathway RNA degradation (KEGG:03018) was significantly enriched (Figure 5).

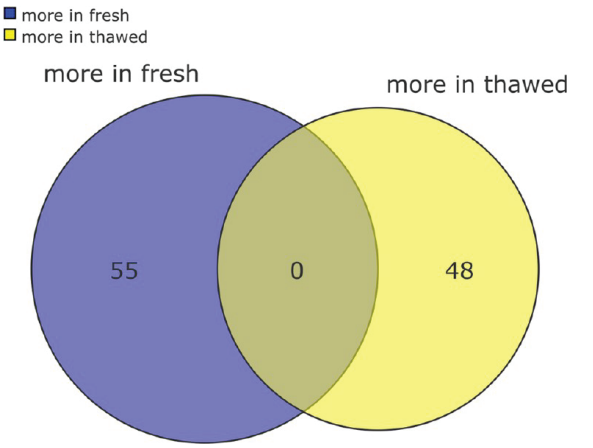

Figure 4. Venn diagram showing changes in the stallion sperm proteome as a consequence of cryopreservation. The number of proteins most abundant in each of the conditions are shown. On the left 55 proteins were more abundant in fresh spermatozoa, on the right 48 proteins were more abundant in frozen and thawed spermatozoa.

In order to reduce the number of proteins retrieved, the threshold for the fold change was further increased and the q value reduced, leading to identification of 33 proteins in which amounts in the stallion spermatozoa were significantly affected by the cryopreservation procedure with a fold change >4 with $p = 1.15 \times 10^{-18}$ and $q = 3.6 \times 10^{-8}$. These included proteins whose levels either increased or decreased as consequence of cryopreservation (Figure 6 and 7). A separate analysis for proteins whose level increased or decreased after cryopreservation was also performed. A 3D principal component analysis (PCA) was then conducted of the variables most significantly affected by the procedure revealing two highly differentiated groups of proteins (Figure 8). A t-SNE analysis was also applied to the individual ejaculates, revealing two distinct populations, with the majority corresponding to either fresh or frozen thawed aliquots (Figure 9).

Cryopreservation Reduces the Amount of Nine Sperm Proteins

The amount of nine sperm proteins was significantly reduced as a consequence of cryopreservation (fold change >4, $p = 6.40 \times 10^{-10}$ and $q = 7.51 \times 10^{-9}$). These proteins were *superoxide dismutase (Cu−Zn)*, *serine/threonine-protein phosphatase*, *an uncharacterized protein (A0A3Q2HAZ2)* belonging to the actin family, *aldo-keto reductase family 1 member B*, *lysozyme B*, *U6 snRNA biogenesis phosphodiesterase 1*, *isocitrate dehydrogenase (NAD) subunit, mitochondrial*, *nucleoside diphosphate kinase 7*, and *NEDD8 ubiquitin like modifier*. The proteins most significantly reduced were *aldo-keto reductase family 1 member B* ($p = 2.2 \times 10^{-17}$) and *superoxide dismutase (Cu−Zn)* ($p = 4.7 \times 10^{-14}$) (Figure 10).

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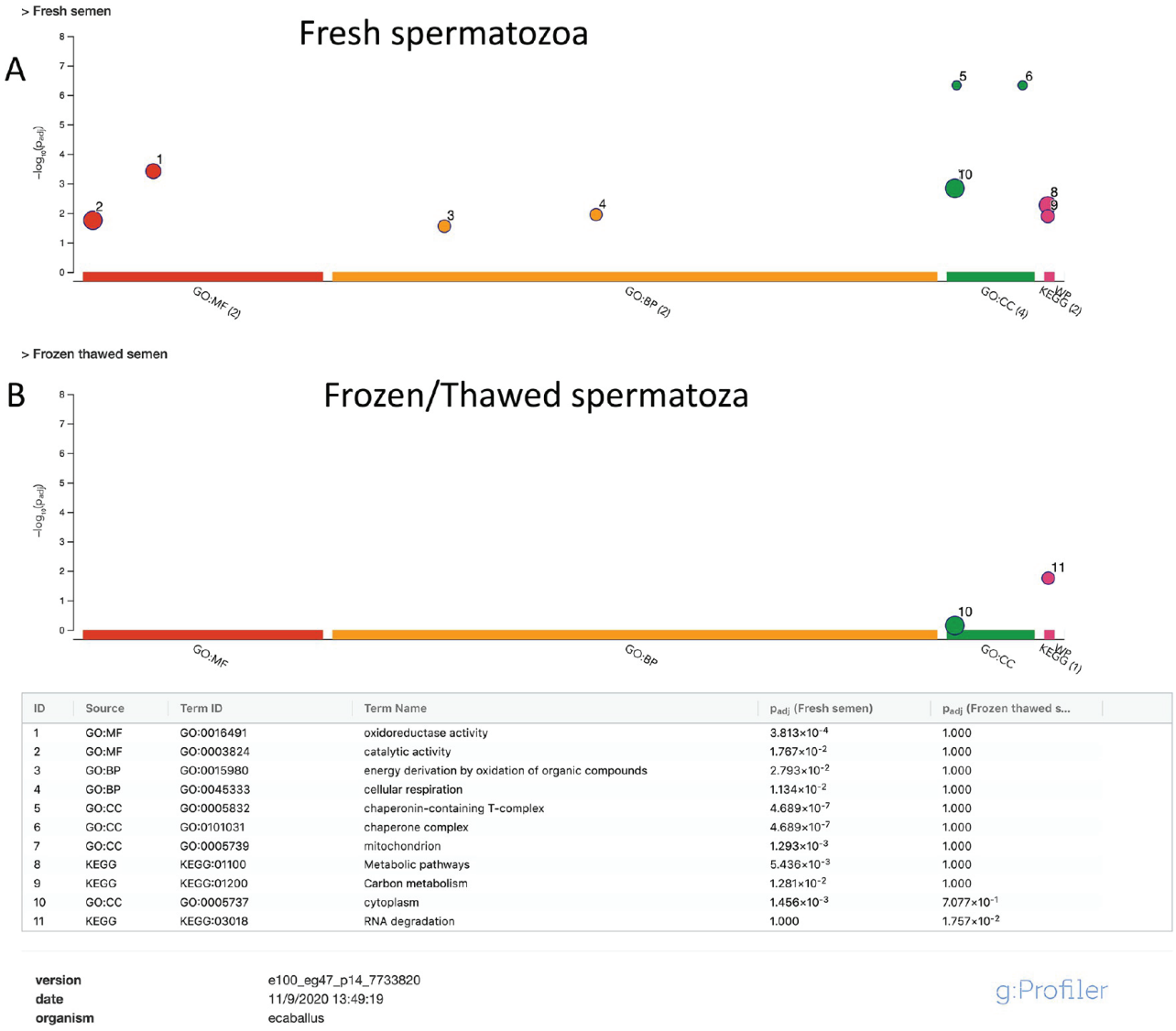
J. Proteome Res. 2021, 20, 2435−2446



ID	Source	Term ID	Term Name	p_{adj} (Fresh semen)	p_{adj} (Frozen thawed s...
1	GO:MF	GO:0016491	oxidoreductase activity	3.813×10^{-4}	1.000
2	GO:MF	GO:0003824	catalytic activity	1.767×10^{-2}	1.000
3	GO:BP	GO:0015980	energy derivation by oxidation of organic compounds	2.793×10^{-2}	1.000
4	GO:BP	GO:0045333	cellular respiration	1.134×10^{-2}	1.000
5	GO:CC	GO:0005832	chaperonin-containing T-complex	4.689×10^{-7}	1.000
6	GO:CC	GO:0101031	chaperone complex	4.689×10^{-7}	1.000
7	GO:CC	GO:0005739	mitochondrion	1.293×10^{-3}	1.000
8	KEGG	KEGG:01100	Metabolic pathways	5.436×10^{-3}	1.000
9	KEGG	KEGG:01200	Carbon metabolism	1.281×10^{-2}	1.000
10	GO:CC	GO:0005737	cytoplasm	1.456×10^{-3}	7.077×10^{-1}
11	KEGG	KEGG:03018	RNA degradation	1.000	1.757×10^{-2}

version	e100_eg47_p14_7733820
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organism	ecaballus

g:Profiler

Figure 5. g:GOST multiquery Manhattan plot showing enrichment analysis of proteins present in higher amounts in fresh (A) and frozen thawed samples (B). The sperm proteome under each condition was queried against the equine proteome database. Gene Ontology (GO) terms for molecular function (MF) are in red, those for biological process (BP) in orange, and those for cellular component (CC) in green. KEGG pathways are depicted in red. The *p* values are depicted on the *y* axis and in more detail in the results table below the image.

Cryopreservation Increases the Amount of 16 Proteins

Amounts of numerous proteins were increased as a result of cryopreservation (Figure 11). These were the *RNA polymerase III subunit A, polyribonucleotide nucleotidyltransferase 1, an uncharacterized transmembrane protein similar to a putative spermatogenesis-associated protein 31C1 in humans (66.7% similarity), an uncharacterized protein, kinesin family member 27, class II major histocompatibility complex transactivator,G-protein coupled receptor 25, transmembrane protein 5 like protein, olfactory receptor, exosome component 2, HSPV163, cyclic nucleotide binding domain-containing protein, A kinase anchoring protein 7, TBC1 domain family member, checkpoint with forkhead and ring finger domain protein,* and *genome polyprotein*. The most statistically significant changes observed were in the amounts of *transmembrane protein 5 like protein* ($p = 8.4 \times 10^{-17}$) and the *olfactory receptor* ($p = 8.5 \times 10^{-15}$) (Figure 11).

Cryopreservation Impairs Sperm Functionality and Causes Oxidative Stress

Freezing and thawing caused a major impact on sperm functionality. The percentage of total and linear motile spermatozoa dropped after cryopreservation from 84.6 ± 1.7 and 62.5 ± 2.2 in fresh samples to 32.8 ± 2.9 and 22.5 ± 1.1%, respectively, in frozen thawed samples ($P < 0.0001$) (Figure 12A,B). Significant reductions in sperm velocities were seen after cryopreservation; circular velocity (VCL) dropped from 186.5 ± 5.6 μm/s in fresh samples to 118.3 ± 14.6 μm/s in thawed samples ($P < 0.0001$) (Figure 12D). Also, straight line (VSL) and average path velocities (VAP) were equally affected by cryopreservation (Figure 12E,F). The percentage of spermatozoa showing detectable levels of the α,β-unsaturated hydroxyalkenal, 4-hydroxynonenal (4-HNE) that is produced by lipid peroxidation in cells, increased in cryopreserved samples, from 9.6 ± 1.2%

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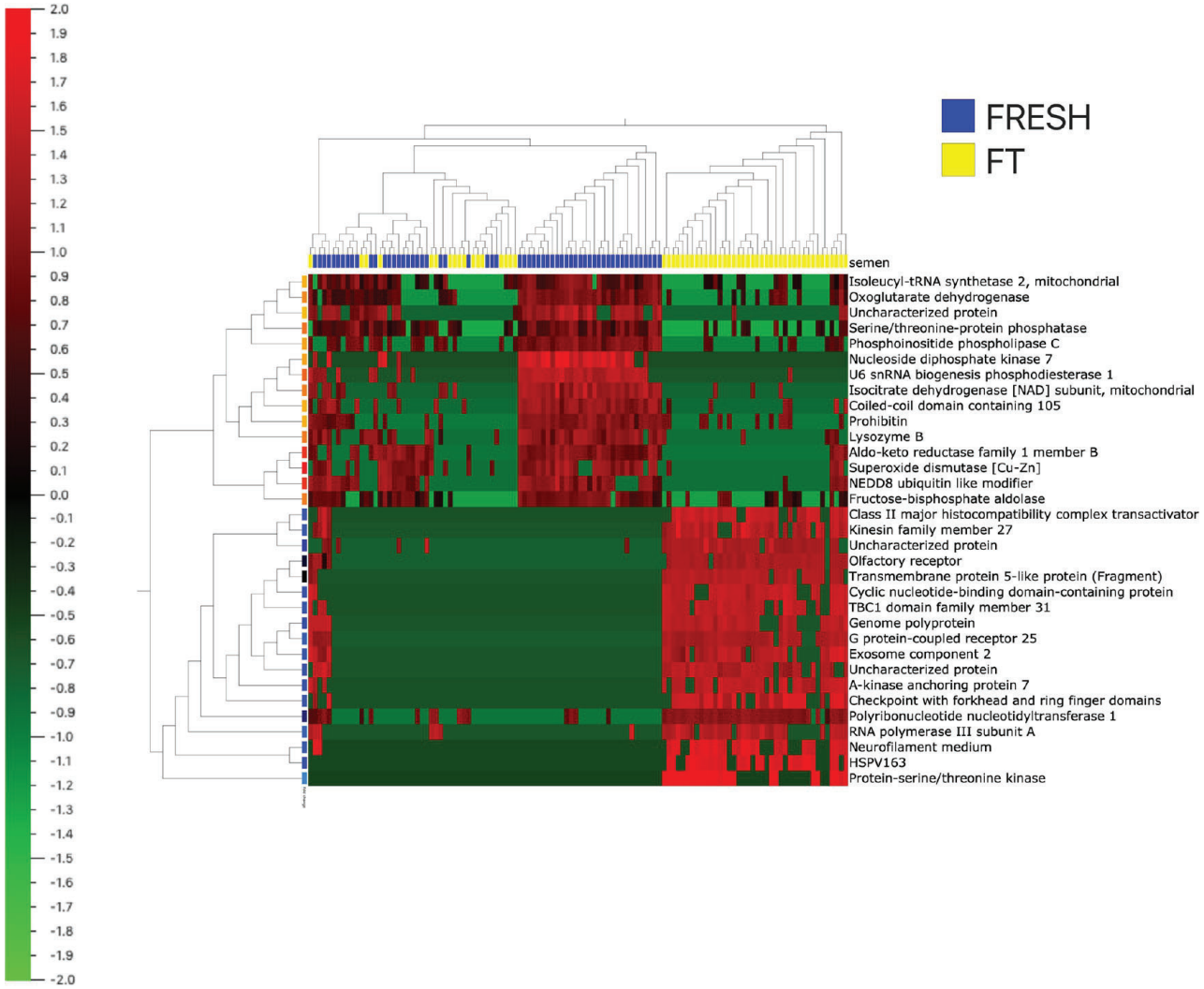

Figure 6. Heat map showing discriminant variables between fresh and frozen thawed spermatozoa. Proteins are classified following hierarchical clustering. Blue marks correspond with fresh samples, yellow marks correspond with frozen thawed samples. The heat map code is present with red areas representing greater amounts of protein and green areas lesser amounts of protein. Proteins were normalized and filtered by a fold change >3.75 with $p = 1.15 \times 10^{-18}$ and $q = 3.6 \times 10^{-8}$.

in fresh spermatozoa to 27.6 ± 3.5% after thawing ($p < 0.001$) (Figure 12C).

DISCUSSION

Cryopreservation inflicts a major insult on stallion spermatozoa. Among other insults, spermatozoa suffer osmotic stress at freezing and then again during thawing. They also experience cryoprotectant toxicity. On average all these factors mean that 50% or more of the spermatozoa initially entering the process succumb to osmotic induced necrosis, mainly at thawing.[39,40] The surviving population also experiences changes arising due to the effect of osmotic stress in the mitochondria causing increased production of mitochondrial reactive oxygen species (ROS), exhaustion of antioxidants, and finally oxidative stress leading to accelerated senescence of the spermatozoa and premature death.[41] Major changes in the stallion sperm proteome as a consequence of cryopreservation were identified in line with previous reports in different species.[11,15,17,18,42] The gene ontology (GO) terms enriched in proteins in higher amounts in fresh spermatozoa reflected the importance of metabolism and redox reactions in spermatozoa and the well documented importance of mitochondria in these cells. To the contrary, in frozen thawed spermatozoa only the KEGG pathway for RNA degradation was enriched. The specific proteins that experienced highly significant changes as a result of cryopreservation were also evaluated and thus can be discriminants for the major consequences of stress imposed by the procedure. Cryopreservation caused a marked and highly significant decrease in the amount of Superoxide dismutase (Cu−Zn) (SOD1), an enzyme which forms part of the first line of defense against oxidative stress in most organisms.[43−45] Although it has previously been reported that this enzyme is affected by cryopreservation,[11,46] this is the first time that SOD1 is identified as a discriminating variable using bioinformatic analysis, being one of the most highly significantly different proteins between fresh and frozen thawed semen. This finding strongly supports the theory that alterations in redox regulation and oxidative stress are a major factor involved in cryodamage as also seen in the present study, indicated by the increased level of 4-HNE in cryopreserved spermatozoa. Other proteins with oxidoreductase

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J. Proteome Res. 2021, 20, 2435−2446

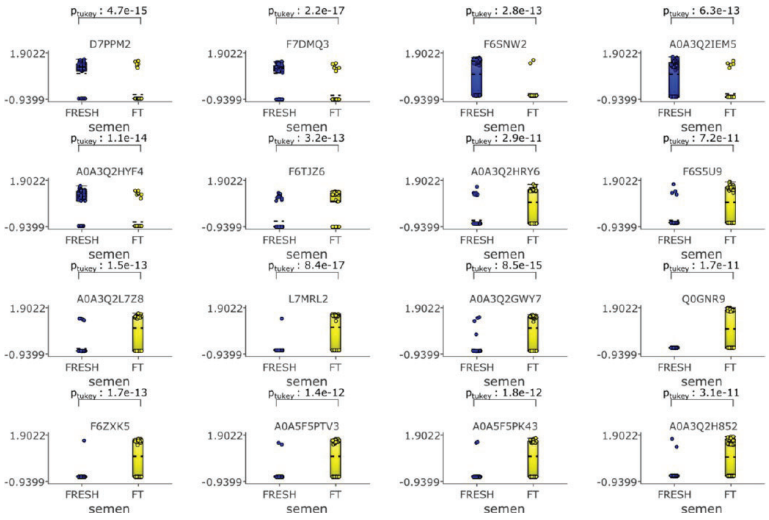

Figure 7. Differences in the amount of specific representative proteins under two conditions, fresh and frozen thawed spermatozoa. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts between fresh and frozen thawed spermatozoa. Proteins in fresh spermatozoa are represented by blue circles, proteins in frozen thawed spermatozoa are represented by yellow circles. Proteins were normalized and filtered by a fold change >4 with $p = 1.15 \times 10^{-18}$ and $q = 3.6 \times 10^{-8}$.

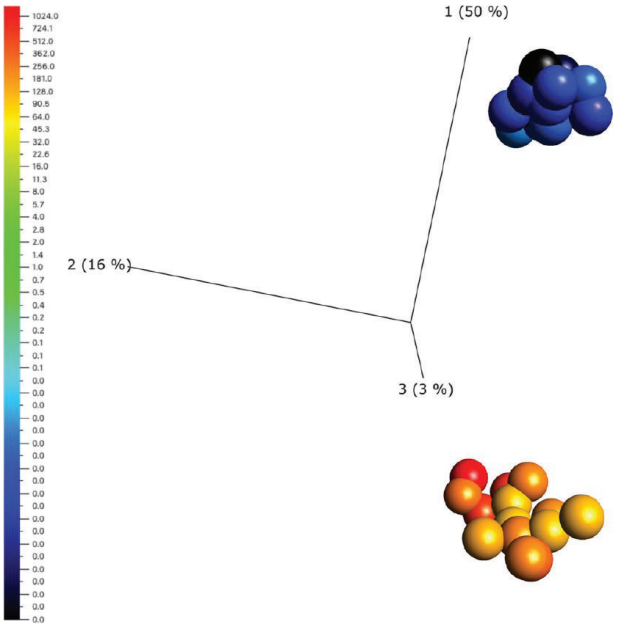

Figure 8. 3D principal component analysis (PCA) of the variables affected by cryopreservation. Two groups are clearly seen representing proteins in which amounts increase after cryopreservation (yellow-orange) and those in which amounts are reduced as a consequence of cryopreservation (blue-black). Variables were prefiltered by standard deviation (S/S_{max}) 0.6 and were then normalized and filtered by a fold change >4 with $p = 1.15 \times 10^{-18}$ and $q = 3.6 \times 10^{-8}$.

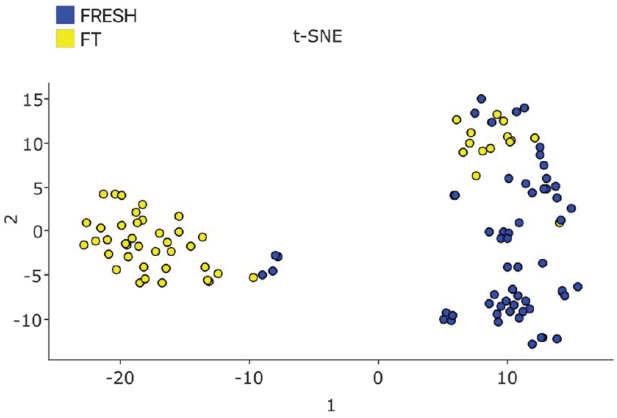

Figure 9. t-Distributed stochastic neighbor embedding (t-SNE), machine learning algorithm for visualization based on Stochastic Neighbor Embedding. The t-SNE map shows the distribution of the samples based on their proteome. After t-SNE clustering most of the fresh samples and the frozen thawed samples are classified in the same cluster. Fresh spermatozoa are shown in blue and frozen thawed in yellow.

activity were also identified as discriminant variables. The aldo-keto-reductase family 1 member b (AKR1B1) was also identified using bioinformatic analysis as discriminant for frozen thawed semen. Among the activities of this protein, there are some with special importance in the context of sperm biotechnologies. These include catalysis of the NADPH-dependent reduction of carbonyls, detoxifying lipid-derived unsaturated carbonyls, such as crotonaldehyde, 4-hydroxynonenal, trans-2-hexenal, trans-2,4-hexadienal and glutathione-conjugates of carbonyls (GS-carbonyls) as well as catalysis of the reduction of diverse phospholipid aldehydes such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphoethanolamin (POVPC) and related phospholipid aldehydes that are generated from the oxidation of phosphatidylcholine and phosphatidylethanolamines.[47] In this context the role of this enzyme in detoxifying 4-HNE, a compound that has been characterized as extremely toxic for the spermatozoa is particularly interesting,[48] and cryopreservation has been demonstrated to cause significant increases in the content of 4-HNE in spermatozoa,[49] as also were evident in the present study. Thus, results presented here establish a plausible molecular explanation to much of the molecular damage occurring after cryopreservation, that is the reduction of the amount of key antioxidant proteins. In view of these findings it seems logical that spermatozoa containing higher amounts of (AKR1B1) may survive better after cryopreservation. Lysozyme B (LYZLB) was also present in higher

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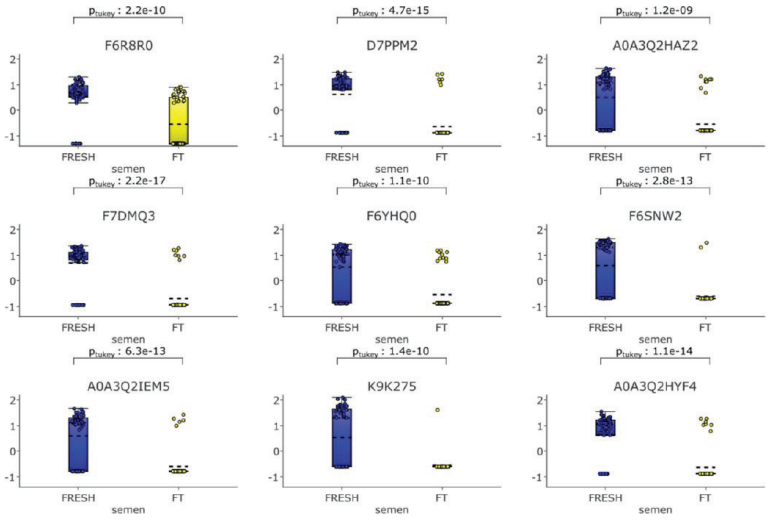

Figure 10. Proteins which are more abundant in fresh samples filtered by a fold change >4 with $p = 6.4 \times 10^{-10}$ and $q = 7.51 \times 10^{-9}$. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts between fresh and frozen thawed spermatozoa. Proteins in fresh spermatozoa are represented by blue circles, proteins in frozen thawed spermatozoa are represented by yellow.

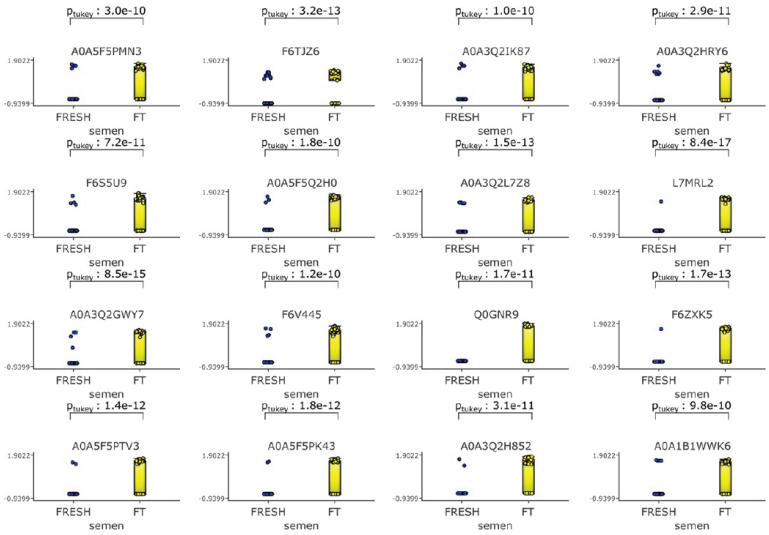

Figure 11. Direct comparison showing proteins which are more abundant in frozen thawed samples $p = 8.00 \times 10^{-10}$, $q = 3.39 \times 10^{-9}$ fold change >4. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins based on spectral counts between fresh and frozen thawed spermatozoa. Proteins in fresh spermatozoa are represented by blue circles, proteins in frozen thawed spermatozoa are represented by yellow circles.

amounts in fresh samples. According to geneontology.org this protein is involved in three biological processes: defense response to Gram negative and positive bacterium and fusion of sperm to egg plasma membrane involved in single fertilization.[50] This finding may constitute another factor that explains the reduced fertility observed with cryopreserved spermatozoa.[51] A serine/threonine-protein phosphatase was also more abundant in fresh sperm; BLAST revealed that this equine protein has a 100% homology with the human serine/threonine-protein phosphatase PP1- alpha catalytic subunit, a protein which is essential for spermatogenesis and spermatozoa motility.[52] Reduction in the amount of proteins after cryopreservation can be explained by the well reported effects of freezing and thawing in the spermatozoa, reduction of antioxidant proteins can be explained by exhaustion due to the oxidative stress occurring during the procedure,[53] and reduction in the amounts of other proteins can be explained due to protein degradation due to the osmotic stress occurring during the freeze−thaw cycle.[13,15,18,54]

Cryopreservation caused a highly significant increase in the amounts of 12 proteins. Two proteins were the most highly significantly enriched in thawed samples. *Transmembrane protein like 5* was increased in thawed samples, probably due to the intense stress that the plasma membrane of the spermatozoa experiences during cryopreservation.[39,55] The increase in the amount of the *olfactory receptor* in cryopreserved samples is also noteworthy. This is also a membrane protein, a member of the class A rhodopsin-like family of G proteins coupled receptors.[56,57] The most likely reason for the increase in this membrane receptor in cryopreserved samples is the intense

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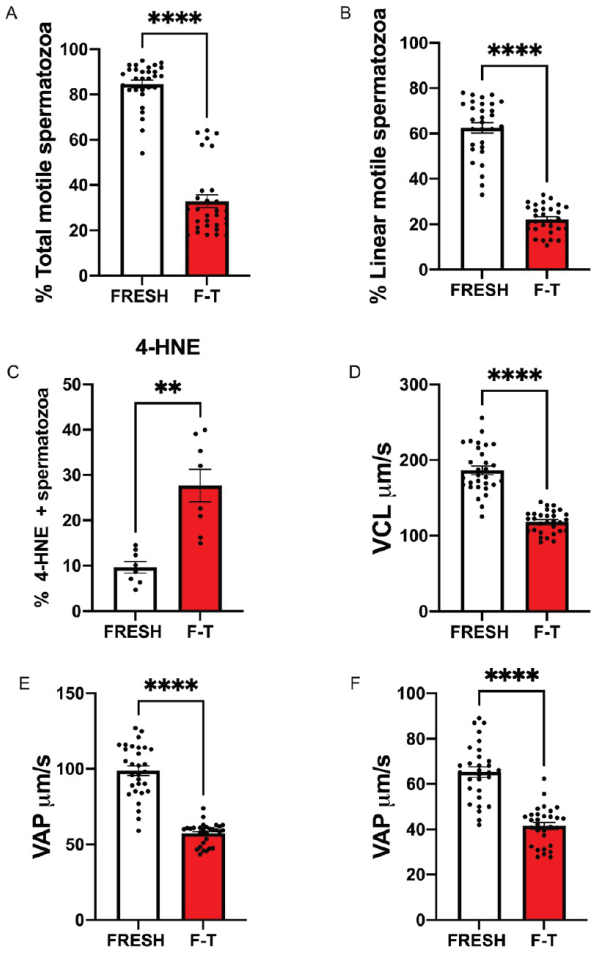

Figure 12. Impact of cryopreservation on sperm functionality. Stallions ejaculates were processed and analyzed as described in the Materials and Methods. Computer assisted sperm analysis (CASA) was used to assess sperm motility (% total and linear motile spermatozoa) and velocities, VCL (circular velocity) μm/s, VAP (average path velocity) μm/s, and straight line velocity (VSL) μm/s. Flow cytometry was used to determine spermatozoa experiencing lipid peroxidation (4-hydroxynonenal 4-HNE). Data are presented as means ± SEM. **** $P < 0.0001$, ** $P < 0.01$.

disruption of the sperm membrane caused by the procedure. Since spermatozoa are translationally and transcriptionally silent cells, increases in the amounts of proteins are difficult to explain. As previously indicated, the stress of cryopreservation may have facilitated the exclusion of some proteins from the membrane and/or proteins that have experienced post translational modifications such as phosphorylation. Changes in the secondary and/or tertiary structure of the proteins have been proposed as an explanation for the increase in amounts of specific proteins after cryopreservation;[18] however, this issue warrants further research.

In conclusion, cryopreservation imposes a major change on the stallion sperm proteome. These changes provide a molecular explanation for the reduced fertility observed with the use of frozen thawed spermatozoa, and provide molecular targets to be explored, with the aim of improving current cryopreservation procedures. Cryopreservation impacts the major antioxidant protein SOD1. Particular attention should be paid to the energetic metabolism of the spermatozoa and the relation with redox regulation in these cells in order to improve current cryopreservation procedures.

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Notes

The authors declare no competing financial interest.

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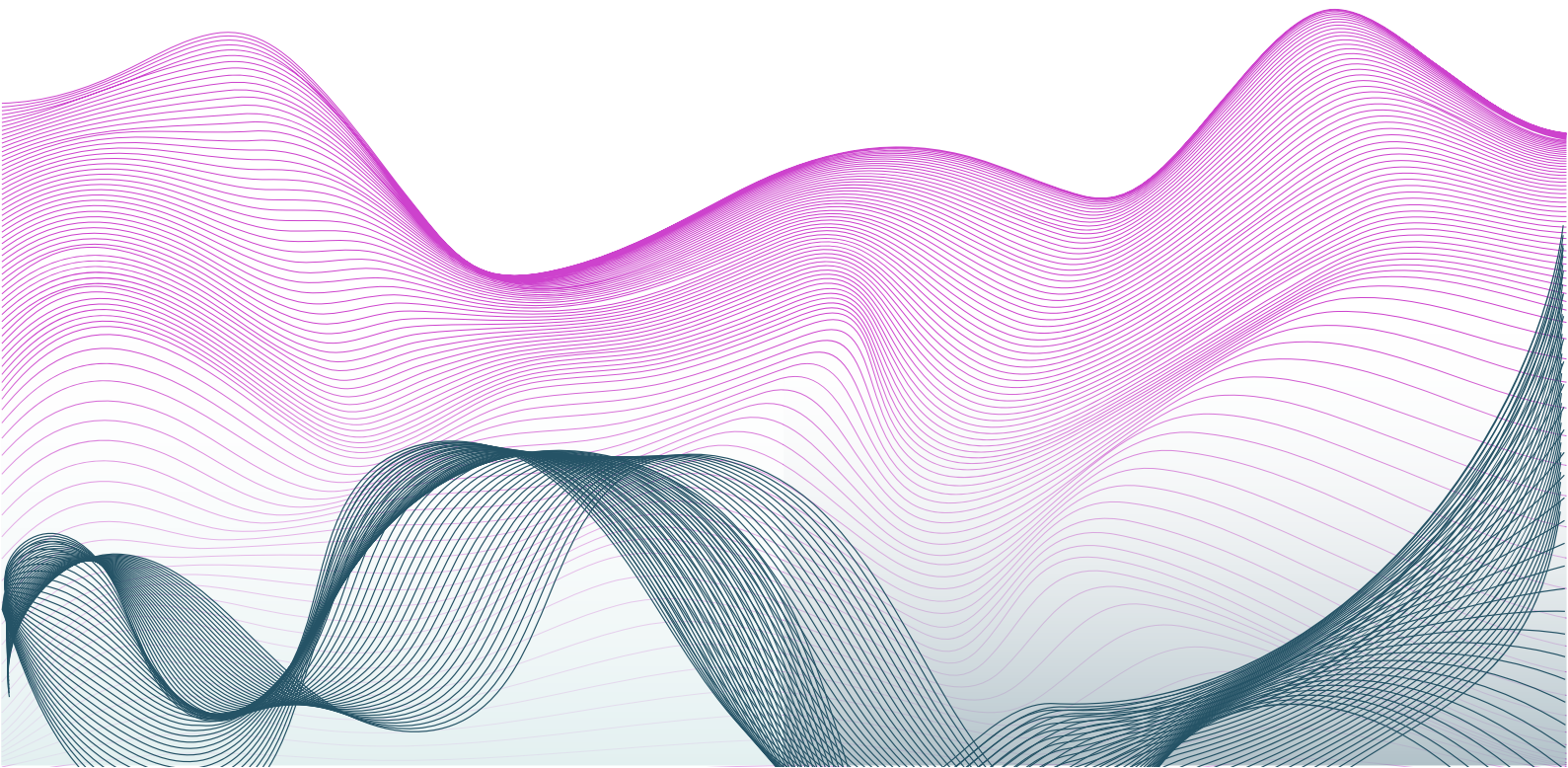

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Research Article

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Research Article

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

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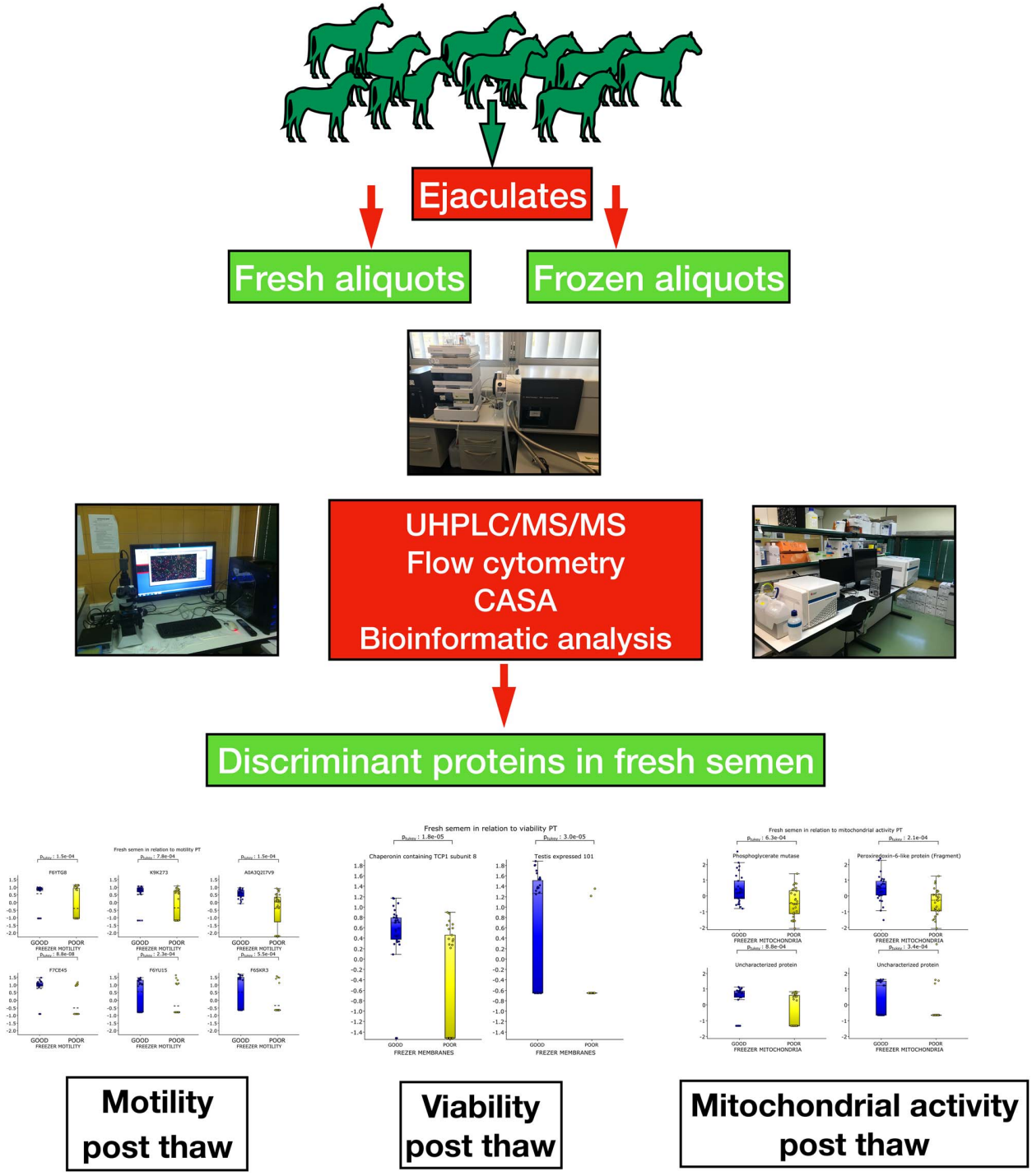
Abstract

The identification of stallions and or ejaculates that will provide commercially acceptable quality post-thaw before cryopreservation is of great interest, avoiding wasting time and resources freezing ejaculates that will not achieve sufficient quality to be marketed. Our hypothesis was that after bioinformatic analysis, the study of the stallion sperm proteome can provide discriminant variables able to predict the post-thaw quality of the ejaculate. At least three ejaculates from 10 different stallions were frozen following a split sample design. Half of the ejaculate was analyzed as a fresh aliquot and the other half was frozen and then analyzed as a frozen-thawed aliquot. Computer-assisted sperm analysis and flow cytometry were used to analyze sperm quality. Detailed proteomic analysis was performed on fresh and frozen and thawed aliquots, and bioinformatic analysis was used to identify discriminant variables in fresh samples able to predict the outcome of cryopreservation. Those with a fold change > 3, a $P = 8.2e\text{-}04$, and a $q = 0.074$ (equivalent to False discovery rate (FDR)) were selected, and the following proteins were identified in fresh samples as discriminant variables of good motility post-thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15, and F6SKR3. Other discriminant variables were also identified as predictors of good mitochondrial membrane potential and viability post-thaw. We concluded that proteomic approaches are a powerful tool to improve current sperm biotechnologies.

Graphical Abstract



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

Introduction

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

Materials and methods

Reagents and media

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

Semen collection and processing

Semen was collected from 10 stallions of various breeds maintained as indicated in specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). All stallions were of proven fertility, with a median age of 10.8 years old (range: 5–19 years old), including five Andalusians, one Spanish sport horse, one Lusitano, one Arab, one Anglo-Arabian, and one Spanish-Arabian horse. Semen was collected from all stallions on a regular basis (2–3 times per week), and the ejaculates used in this study were collected after depletion of the extragonadal sperm reserves. The ethical committee of the university approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina. The gel was removed with an inline filter. Semen was immediately transported to the laboratory after collection for evaluation and processing. The experimental design employed a split sample approach, with single ejaculates divided in two subsamples (fresh and frozen-thawed [FT] experimental groups). Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [21, 22] to remove dead spermatozoa, seminal plasma, and contaminating cells and then one aliquot was re-suspended in Tyrodes medium (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM $NaHCO_3$, 1 mM Na-Pyruvate, 21.6 mM Na-Lactate, 2 mM $CaCl_2$*2H2O, 3.1 mM KCl, 0.4 mM $MgSO_4$*7H2O, 0.3 mM NaH_2PO4*H2O, 0.3% BSA), 315 mOsm/kg, and pH 7.4 [23] (fresh extended semen), and the other aliquot was re-suspended in freezing media and frozen using standard procedures that have been previously described by our laboratory (FT semen) [24]. In brief, the aliquot was diluted in the freezing medium, Cáceres (University of Extremadura, Cáceres, Spain), containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 $\times$ 10^6 spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were sealed ultrasonically with UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) and were immediately placed in an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The following freezing curve was used. Straws were kept for 15 min at 20 °C, and they were then slowly cooled from 20 °C to 5 °C at a cooling rate of 0.1 °C/min. Thereafter, the freezing rate was increased to −40 °C/min from 5 °C to −140 °C. The straws were then plunged into liquid nitrogen and were stored until analysis. Frozen samples were thawed in a water bath at 37 °C for at least 30 s.

Experimental design

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

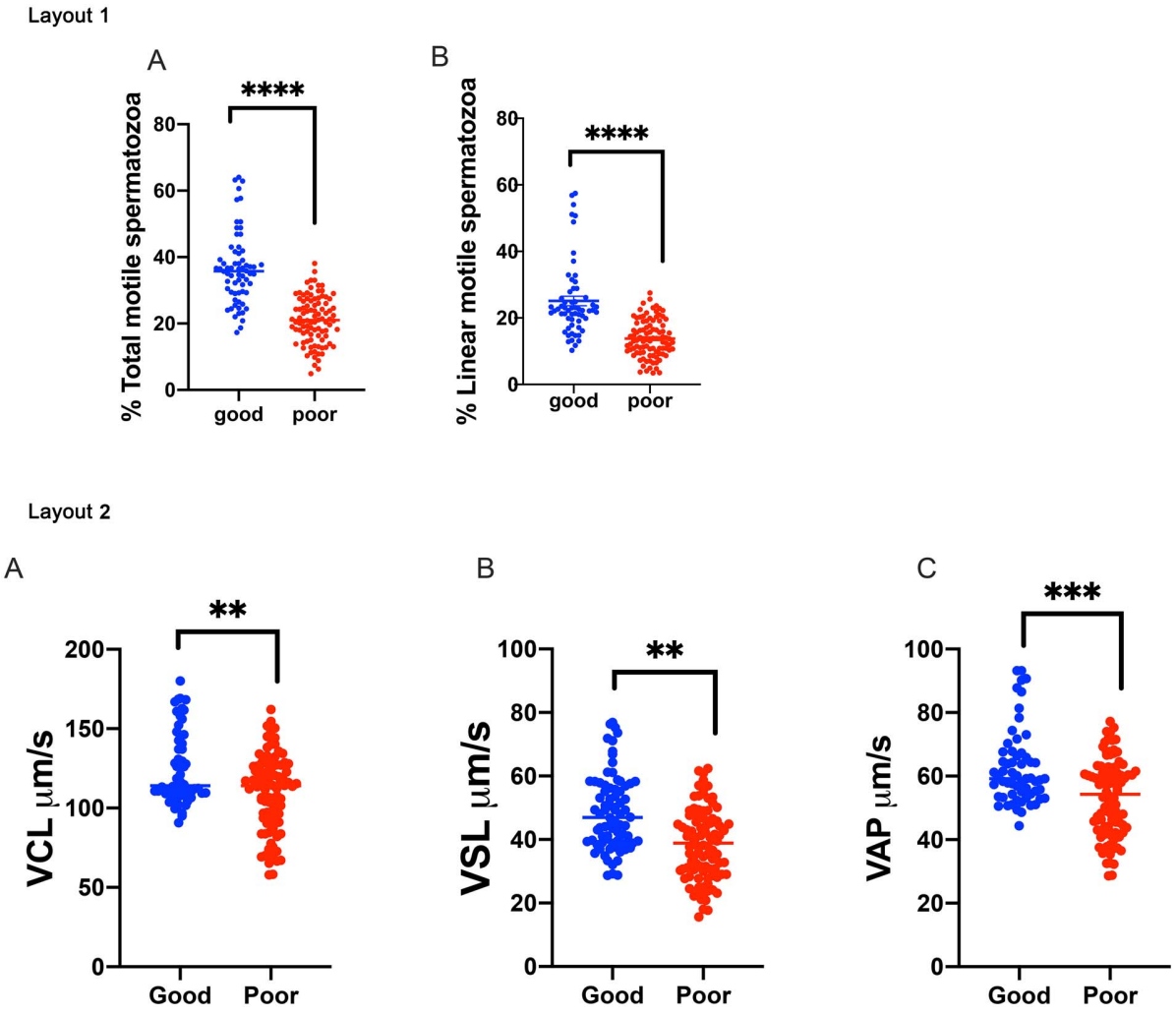


Figure 1. (Layout 1) Thirty stallion ejaculates were frozen and thawed as described in Material and Methods. Based in their post-thaw motility, they were classified as good (>35% total motility) or poor freezers (<35% total motility post-thaw). Computer-Assisted Sperm Analysis was used to analyze sperm quality after thawing. (A) Percentage of total motile spermatozoa in good and poor freezers. (B) Percentage of linear motile spermatozoa in good and poor freezers. Data are means +/− s.e.m ***P (*in italics*) <0.0001 in this figure 1.1. (Layout 2) (A) Circular velocity (VCL). (B) Straight line velocity (VSL). (C) Average path velocity (VAP) in good and poor freezers. Data are means ± s.e.m. ****$P < 0.001$ and **$P < 0.01$.

Sperm preparation

The spermatozoa (fresh and FT samples) were washed three times in PBS (600 g × 10′), and the fresh and FT samples were pelleted and kept frozen at −80 °C until analysis.

Protein solubilization

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

Protein quantification

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

In-solution trypsin digestion

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

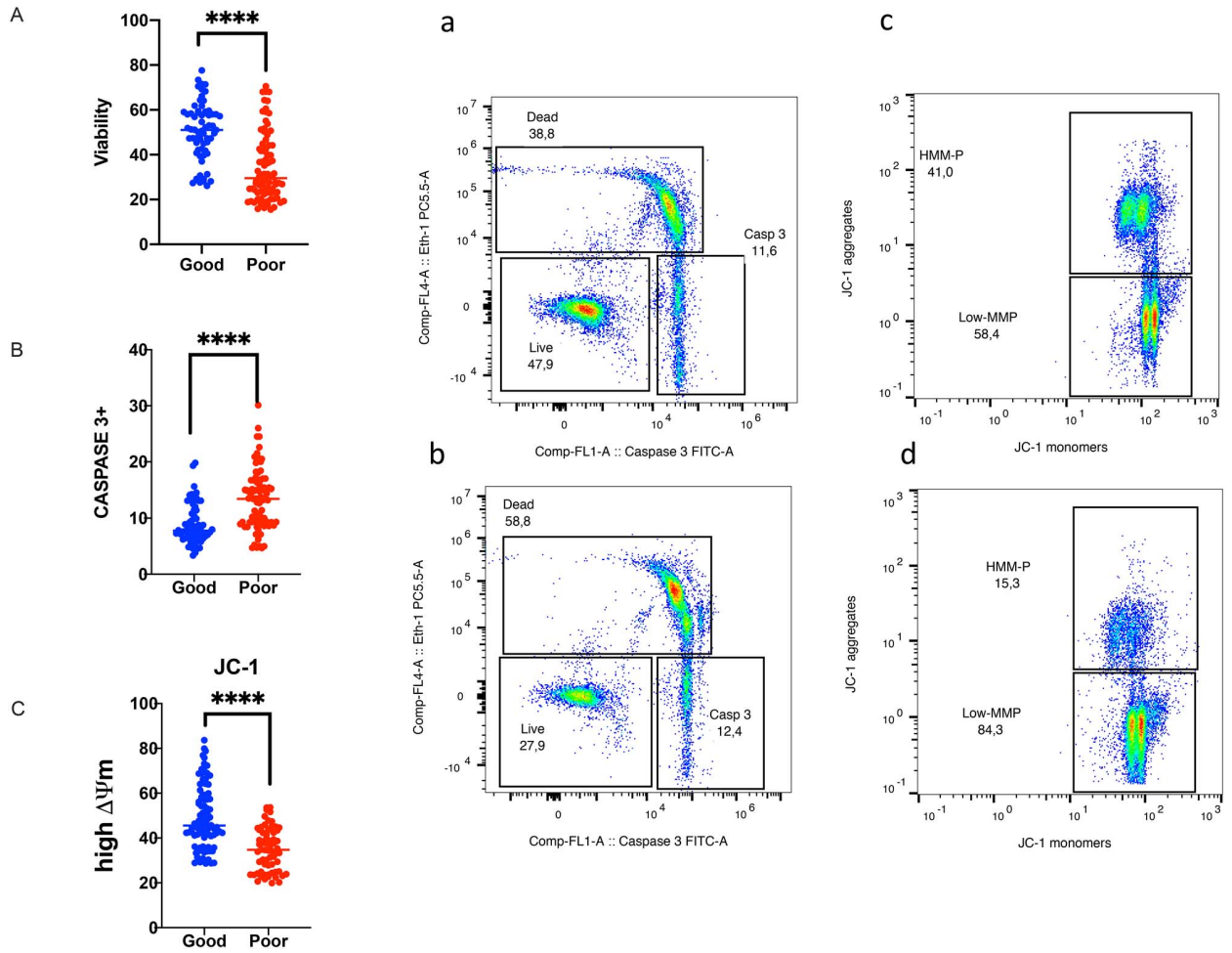


Figure 2. Viability and mitochondrial membrane potential in stallions showing good and poor viability and mitochondrial activity post-thaw. Thirty stallion ejaculates were frozen and thawed as described in Material and Methods. Samples were processed for flow cytometry analysis as described in material and methods. (A) Viability (intact membranes and negative for caspase 3 spermatozoa). (B) Caspase 3-positive spermatozoa. (C) Spermatozoa depicting high mitochondrial membrane potential in good and poor freezers. (a, b) Representative cytograms of viability analysis, (c, d) representative cytograms of mitochondrial membrane potential analysis. Data are means ± s.e.m. $^{****}P < 0.0001$.

UHPLC–MS/MS analysis

The separation and analysis of the samples were performed with a UHPLC/MS system consisting of an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high speed binary pump and coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the UHPLC and Q-TOF was by using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). The sample was injected onto an Agilent AdvanceBio Peptide Mapping UHPLC column (2.7 µM, 150 × 2.1 mm, Agilent technologies), thermostatted at 55 °C, at a flow rate of 0.4 mL/min. The gradient program started with 2% of B (buffer B:water/acetonitrile/formic acid, 10:89.9:0.1) that remained in isocratic mode for 5 min and then increased linearly up to 45% B over 40 min, increasing up to 95% B over 15 min and remaining constant for 5 min. After this 65 min of run, 5 min of post-time followed using the initial condition for conditioning of the column for the next run. The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 l/min at a temperature of 250 °C, and the sheath gas flow was set to 12 l/min at a temperature of 300 °C. The capillary spray, fragmentor, and octopole RF Vpp voltages were 3500 V, 340 V, and 750 V, respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range were 50–1700 *m/z*, and scan rates were 8 spectra/s for MS and 3 spectra/s for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors were selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of −4.8. The same ion was rejected after two consecutive scans.

Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies). Briefly, raw data were extracted under default conditions as follows: non-fixed or variable modifications were selected; [MH] + 50–10000 *m/z*; maximum precursor charge, +5; retention time and m/z tolerance, ±60 s; minimum signal-to-noise (S/N) MS, 25; and finding ^{12}C signals. The MS/MS search against the appropriate and updated

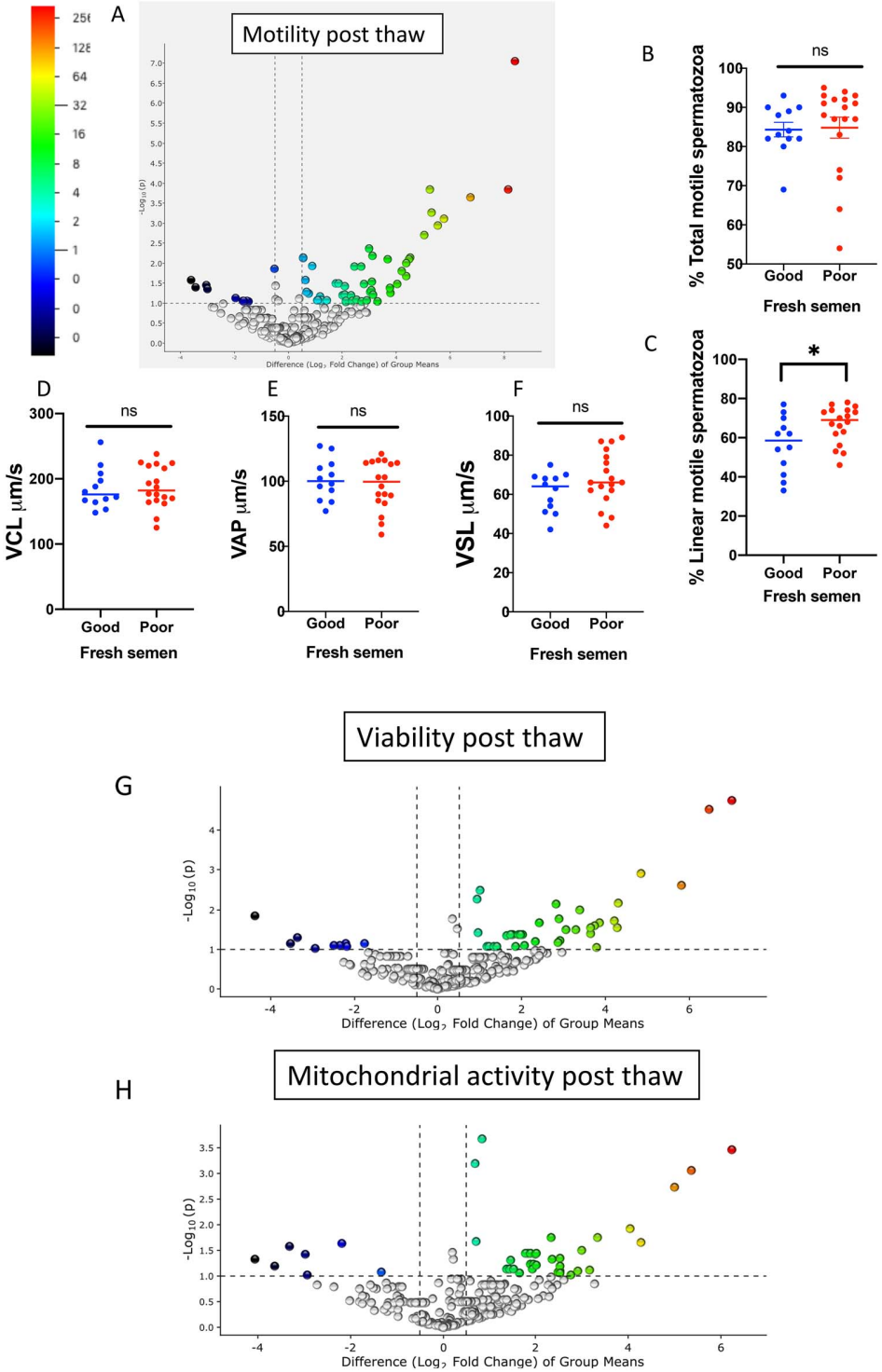


Figure 3. Computer-assisted sperm analysis and shot gun proteomic analysis in pre-freezing samples. (A) Volcano plot showing differences in protein expression in fresh samples in stallions showing good and poor motility post-thaw. (B) Percentages of total motile spermatozoa in fresh samples of good and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

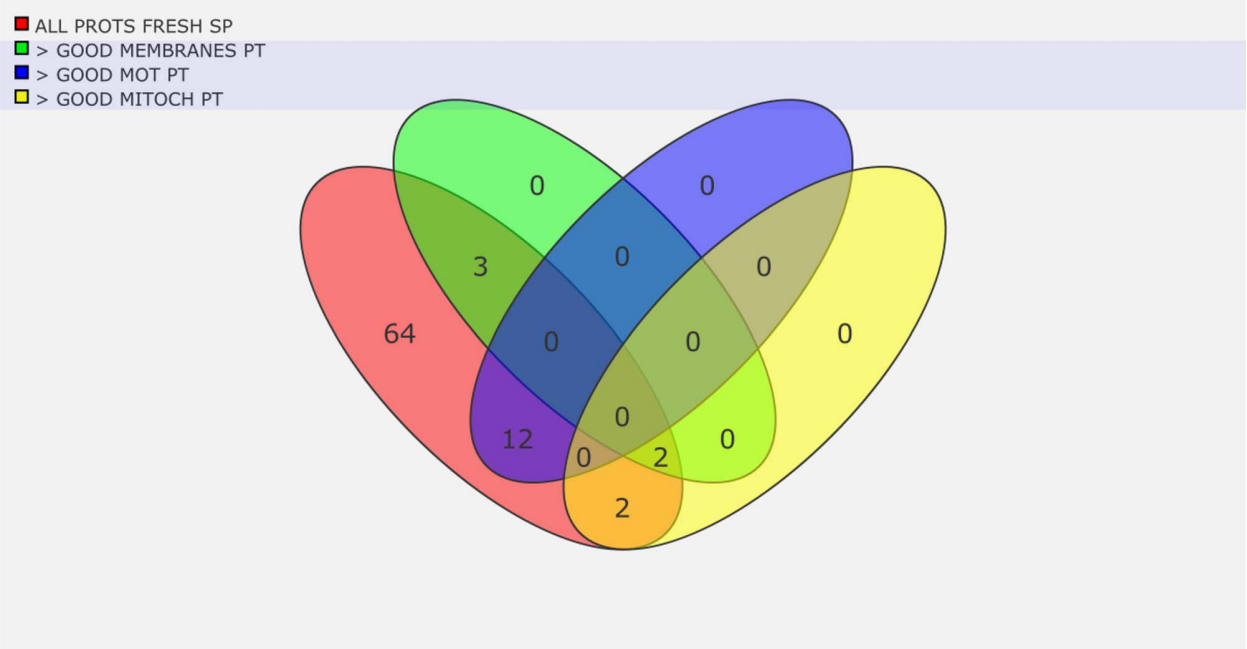


Figure 4. Venn diagram showing proteins significantly enriched (discriminant variables) in fresh spermatozoa and those in each category for stallions showing good motility, good mitochondrial membrane potential, and good viability post-thaw.

protein database (in this case: Uniprot/Horse) was performed using the following criteria: non-fixed modifications were selected and as a variable modification, carbamidomethylated cysteines and tryptic digestion, with five maximum missed cleavages were selected. ESI-Q-TOF instrument was selected with a minimum matched peak intensity of 50%, maximum ambiguous precursor charge of +5, monoisotopic masses, peptide precursor mass tolerance of 20 ppm, product ion mass tolerance of 50 ppm, and calculation of reversed database scores. The autovalidation strategy used was auto-threshold, in which, the peptide score is automatically optimized for a target % FDR (1.2%). Then the protein polishing validation was performed in order to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0%).

Computer-assisted sperm analysis

Sperm motility and velocity were assessed in fresh and FT samples using a Computer-Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to the standard protocols used at our center [25]. Semen samples were loaded into a Leja® chamber with a depth of 20 µM (Leja, Amsterdam, The Netherlands) and were placed on a stage warmed at 37 °C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10× negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with a VAP > 35 µM/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

Flow cytometry

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

Measurement viability and mitochondrial membrane potential in stallion spermatozoa

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

Assessment of viability and caspase 3 activity

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

Bioinformatic analysis

Variance filtering and PCA. Data were normalized and $\log_2$-transformed using Qlucore Omics Explorer (https://qlucore.com). Principal component analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with a low overall variance, to reduce the impact of noise and for centering and scaling the remaining variables to zero mean and unit variance.

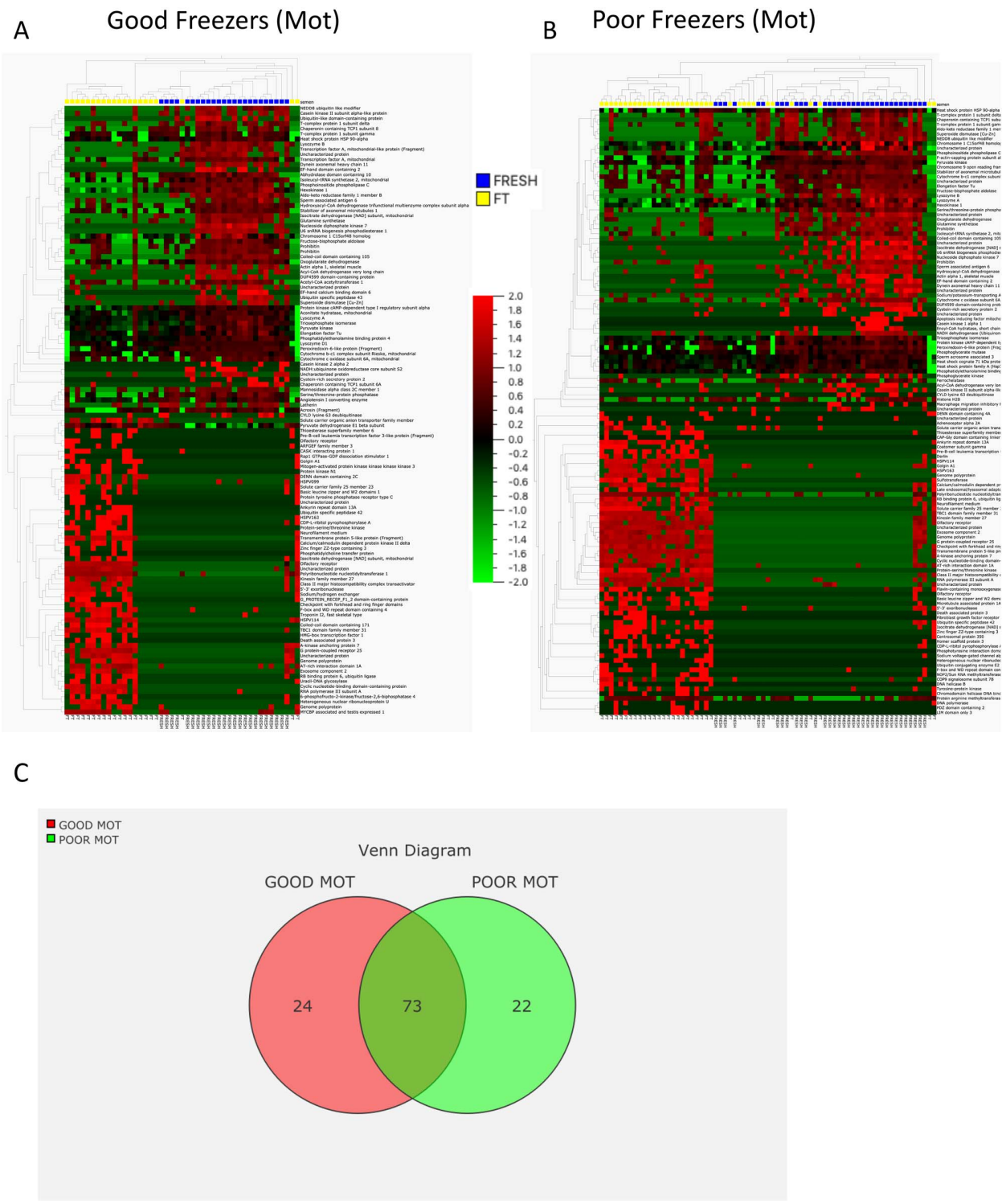


Figure 5. Heat map showing the impact of cryopreservation on the proteome of stallions showing good or poor motility post-thaw. Proteins are classified following a hierarchical clustering. Fresh samples correspond to the blue marks, FT samples correspond to the yellow marks. The heat map code is present,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

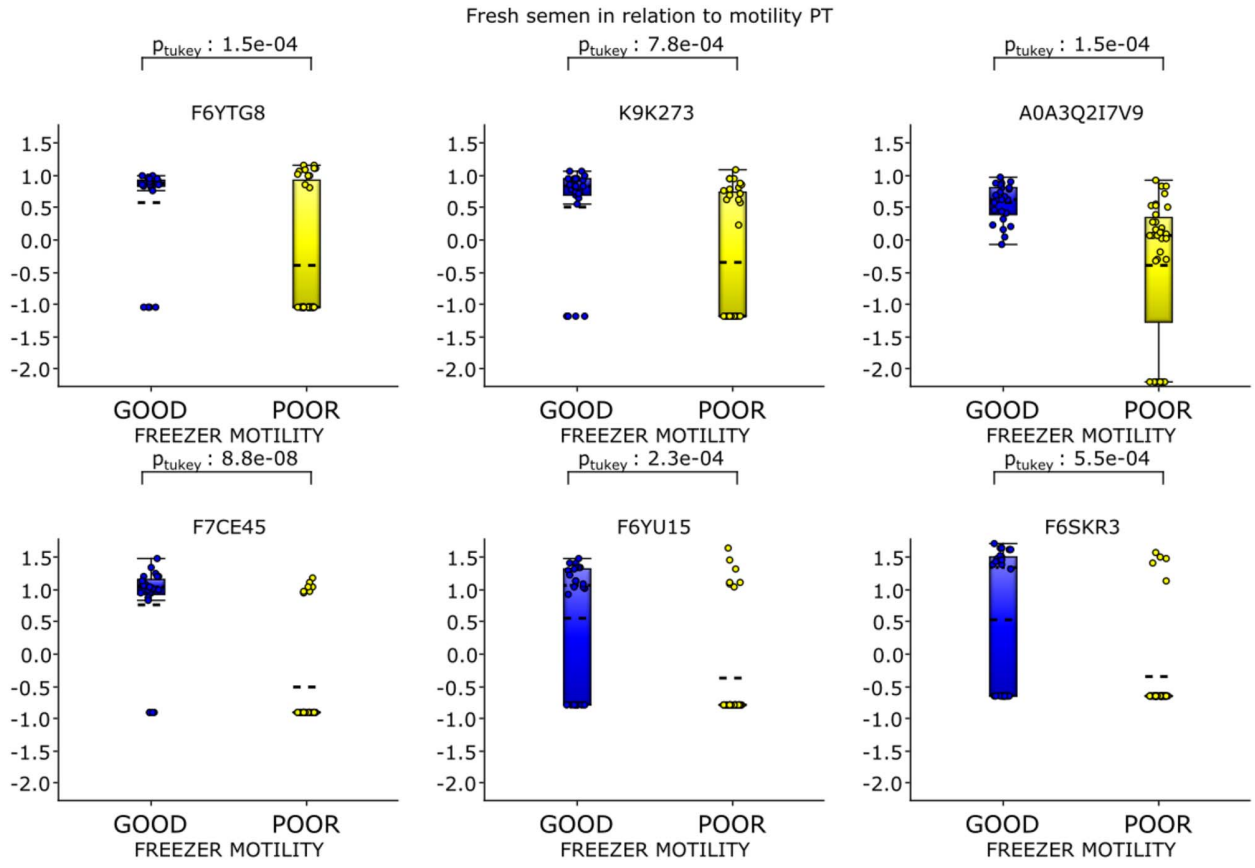


Figure 6. Discriminant variables between good and poor stallions in terms of motility post-thaw F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15, and F6SKR3 corresponding to mannosidase alpha class 2C member 1, mitochondrial NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9-like protein, isoleucyl-tRNA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

The projection score [29] was used to determine the optimal filtering threshold.

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

Statistical analysis

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

Results

Sperm characteristics in good and poor freezers

Stallions were classified according to the total motility (CASA) post-thaw, with those showing values for total motility > 35% considered as good and those showing values <35% considered as poor (Figure 1). This threshold was based on current recommendations for minimum quality for commercial doses of equine semen (http://www.wbfsh.org/GB/Other%20activities/Semen%20standards.aspx).

Highly significant differences were observed between stallions showing good and poor motility post-thaw in all motility and velocity parameters (Figure 1). Four stallions were classified as good and six as poor, in terms of motility post-thaw. Additionally, two further categories were established [32–34]. The first depended on the viability at thawing, with good showing >40% intact membranes post-thaw and poor <40% intact membranes, with five stallions in each category. The third category considered mitochondrial activity at thawing, with good freezers showing >40% of spermatozoa displaying high mitochondrial membrane potential at thawing and poor freezers displaying <40% of spermatozoa with high mitochondrial membrane potential at thawing. In this category, five stallions were classified as good and five as poor. Only one stallion was classified as good in all three categories. Average values for groups of good and poor freezers in these categories are given in Figure 2.

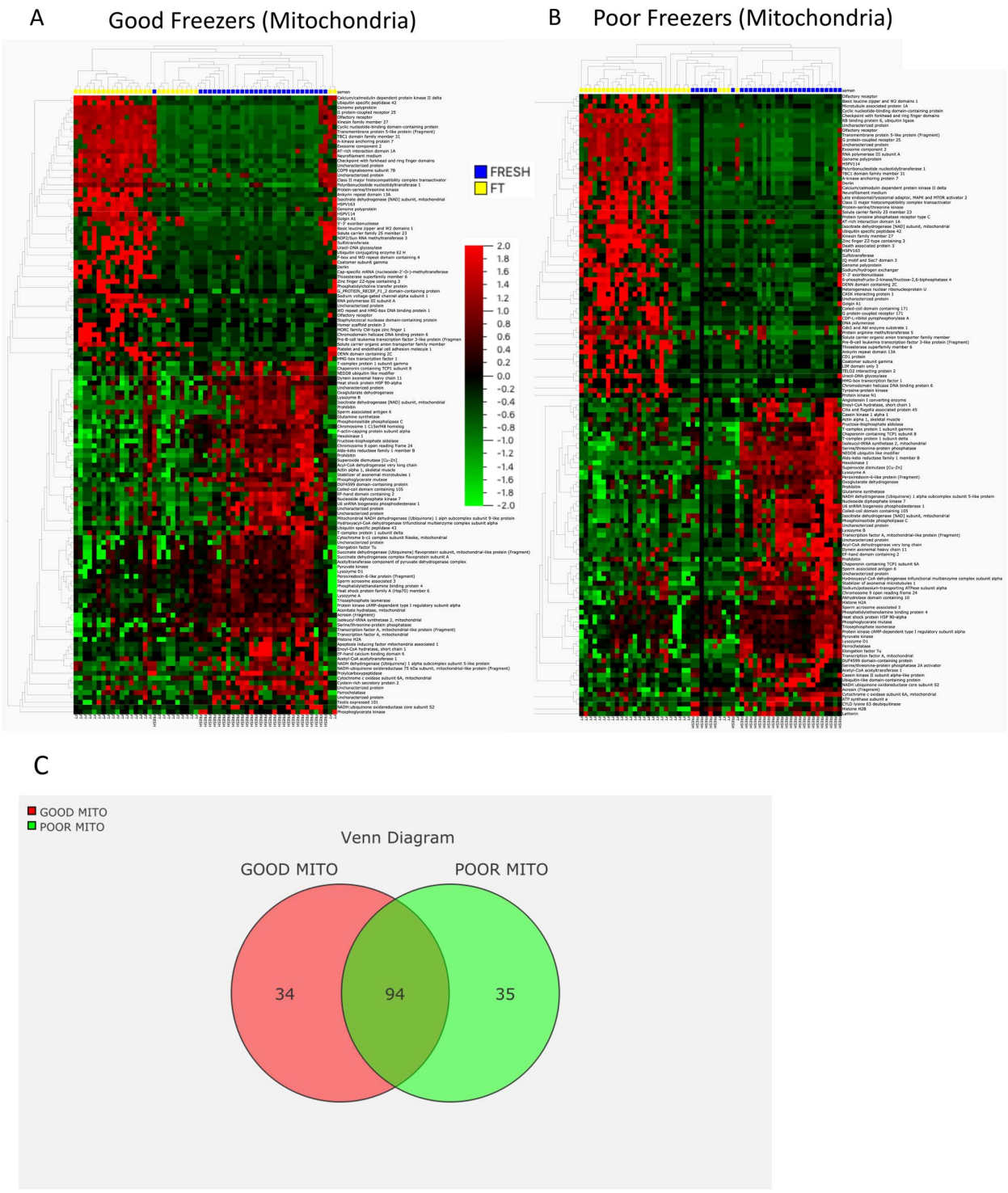


Figure 7. Heat map showing the impact of cryopreservation in the proteome of stallions showing good or poor mitochondrial membrane potential post-thaw. Proteins are classified following hierarchical clustering. Fresh samples correspond to the blue marks, FT samples correspond to the yellow marks. The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

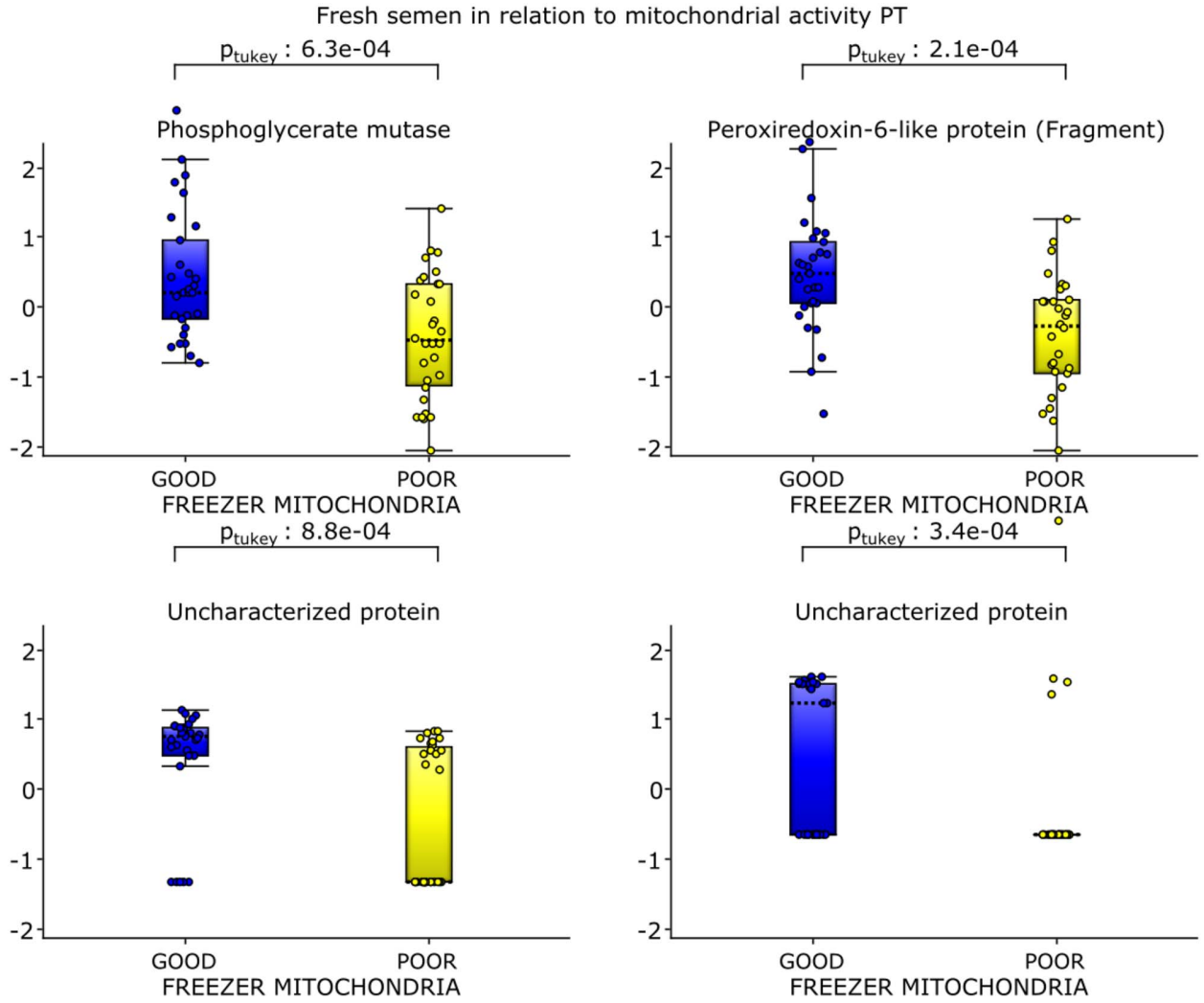


Figure 8. Discriminant variables between good and poor freezers in terms of mitochondrial membrane potential post-thaw. The discriminant proteins were F7A616, K9KDP8, A0A3Q2HAZ2, and A0A3Q2L2U8, corresponding to PGAM, peroxiredoxin 6-like protein, an uncharacterized protein similar to actin-1 and an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

Motility in fresh samples does not predict the outcome after cryopreservation

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

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

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

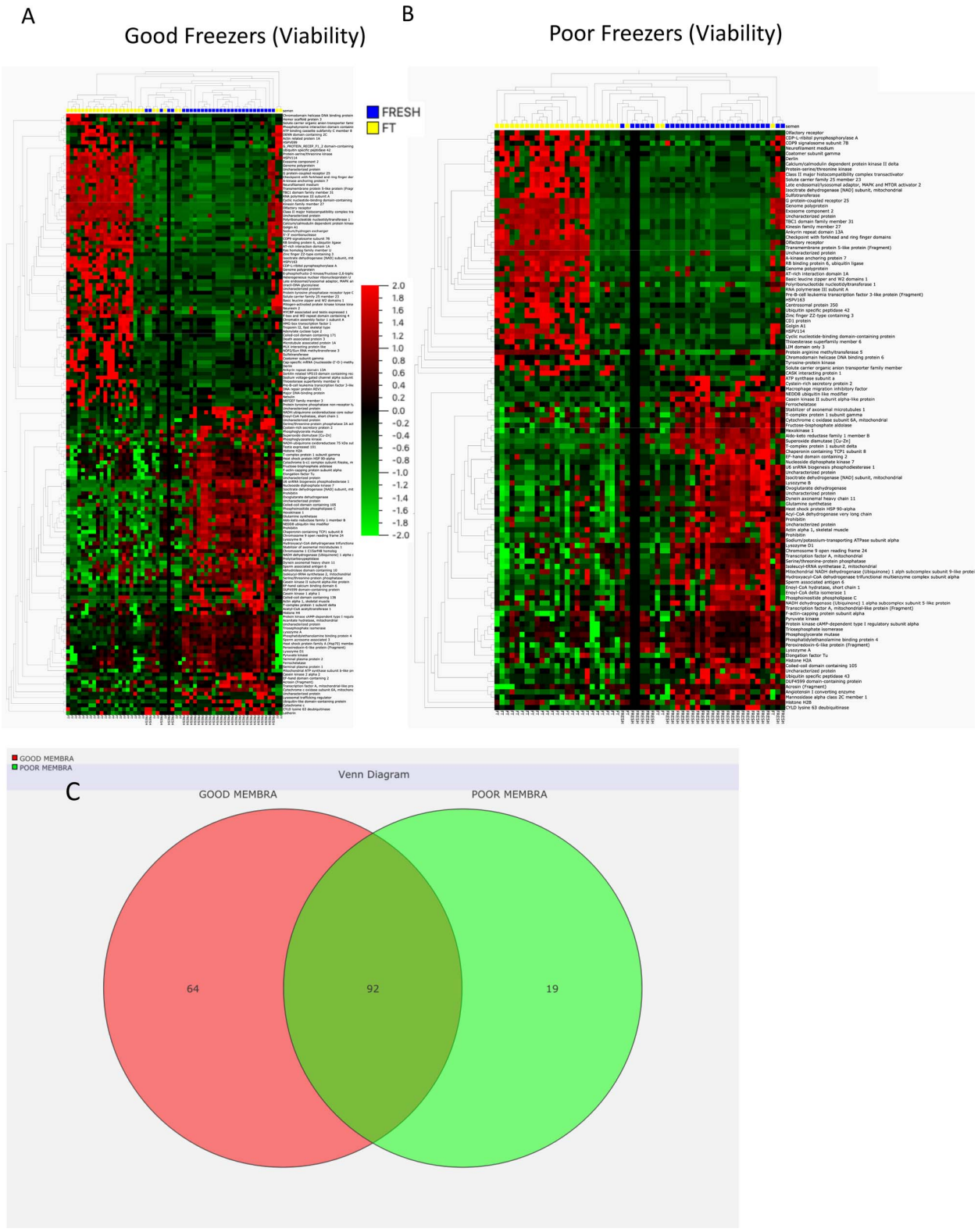


Figure 9. Heat map showing the impact of cryopreservation on the proteome of stallions showing good and poor membrane integrity post-thaw. Proteins are classified following hierarchical clustering. Fresh samples correspond to the blue marks, FT samples correspond to the yellow marks. The heat map code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

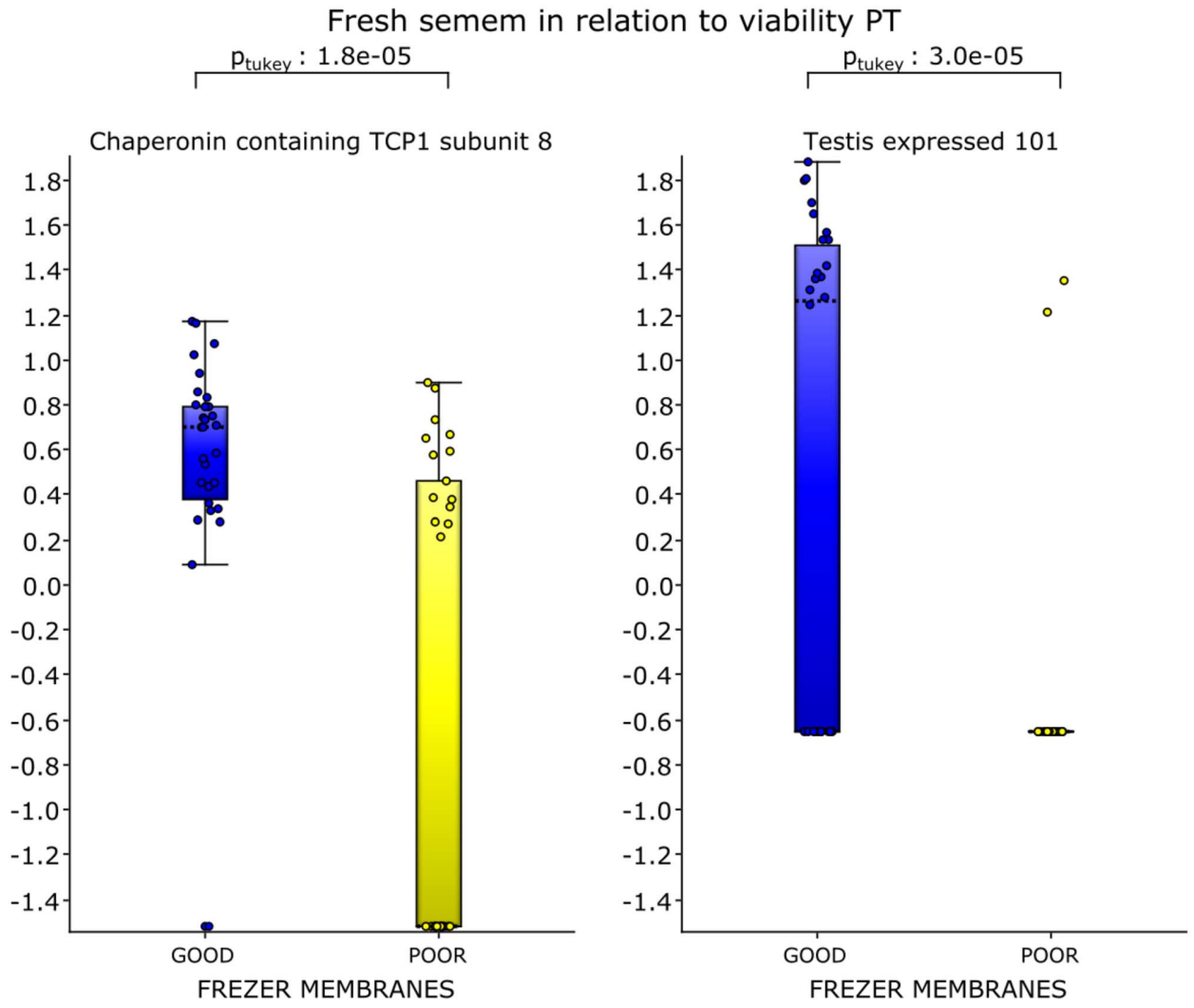


Figure 10. Discriminant variables between good and poor freezers in terms of viability post-thaw, chaperonin containing TCP1 subunit 8 and testis expressed 101. Fold change > 3, $P = 0.001$ and $q = 0.01$.

from fresh samples were selected in order to identify predictive discriminant variables that were able to distinguish stallions showing good motility post-thaw. Those variables with a fold change > 3, a $P = 8.2e\text{-}04$, and a $q = 0.074$ (equivalent to FDR) were selected, and the following proteins were identified as discriminant variables able to identify stallions showing good motility post-thaw: F6YTG8, K9K273, A0A3Q2I7V9, F7CE45, F6YU15, and F6SKR3 (Figure 6), corresponding to mannosidase alpha class 2C member 1, mitochondrial NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9-like protein, isoleucyl-tRNA synthetase 2, mitochondrial, acetyl-CoA acetyltransferase 1, latherin, and ubiquitin-specific peptidase 43.

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

Viability post-thaw. Samples showing percentages over or below 40% intact membranes post-thaw showed different changes in the sperm proteome as a consequence of cryopreservation. Differences in the fresh and FT samples in both groups were observed with a

fold change > 2, P = 0.01, and q = 0.1 (Figure 9A and B). Venn diagrams revealed 92 proteins present in stallions showing good and poor viability post-thaw, with 19 proteins only present in poor freezers and 64 which were present only in good freezers (Figure 9C). Following this, a search was performed for discriminant variables in fresh semen that were able to identify stallions likely to show good viability post-thaw (>40% intact membranes at thawing). Variables with a fold change > 3, P = 0.001, and q = 0.01 were selected, and two proteins were retrieved, serving as discriminant variables, chaperonin containing TCP1 subunit 8 and testis expressed 101 (Figure 10).

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

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

Discussion

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

Although cryopreservation had a similar impact in both groups, causing reduction of the levels of some proteins and increases in others, there were specific proteins associated with stallions producing ejaculates with better motility post-thaw. Six proteins were identified in fresh samples, capable of identifying the group of stallions showing better motility post-thaw. Three of them were mitochondrial proteins (K9K273, A0A3Q2I7V9, and F7CE45), stressing the importance of these organelles for sperm function [35, 36], and in particular, producing ATP for sperm motility through oxidative phosphorylation. The F6YTG8 (alpha mannosidase) is a protein with a role in the catabolism of oligosaccharides [37]. Alpha mannosidase activity prevents the accumulation of oligosaccharides. More recently, a role in preventing mitochondrial dependent apoptosis has been proposed [38]. Since an important proportion of the damage occurring during cryopreservation involves a mitochondrial apoptotic pathway [39, 40], the aforementioned function of this protein provides an explanation for our findings. Moreover, a study in ovine semen found a positive correlation between alpha mannosidase and a positive outcome after cryopreservation [41]. F7CE45, acetyl-CoA acetyltransferase 1, catalyzes the last step in the mitochondrial beta oxidation pathway [42] and also plays a major role in ketone body metabolism [43]. Spermatozoa are able to obtain energy for motility using the beta oxidation pathway [10, 14], providing an explanation for the link between a major presence of this protein in fresh samples and better motility post-thaw. Finally, the presence of latherin (F6YU15) was described for the first time in the spermatozoa. This is present in the saliva and sweat of horses and has strong surfactant properties [44, 45]. Its activity is responsible for the foam formed in the skin of horses during vigorous exercise. It is not clear what the possible function of this protein is in the spermatozoa, although antibacterial properties inhibiting the growth of biofilms [46] have been attributed to latherin. A potential contribution of sperm latherin to endometrial health is a tempting possibility that warrants further research.

Cryopreservation also caused a different impact in ejaculates showing good and poor mitochondrial activity post-thaw. The Venn diagram revealed 94 common proteins, 34 proteins specific to the group showing high mitochondrial membrane potential post-thaw and 35 to the group showing poor mitochondrial membrane post-thaw. Next, a search was performed for discriminant variables in fresh semen able to predict mitochondrial activity post-thaw, and four proteins were found to be potent discriminant variables for the prediction of good mitochondrial membrane potential post-thaw. The peroxiredoxin-like 6 protein was more abundant in the ejaculates showing better mitochondrial activity post-thaw. Peroxiredoxin 6 is considered as one of the major antioxidant defenses of the spermatozoa [47, 48], and taking into account that a high percentage of cryodamage come from oxidative stress [49, 50], it is not surprising that samples richer in this antioxidant protein are able to better withstand the cryopreservation process. A glycolytic enzyme, PGAM, was also more abundant in good freezers. This enzyme is upregulated in many cancer cells [51] and catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) during glycolysis. In cancer cells that overexpress this protein, there is an increase in 2-PG and a decrease in 3-PG. Also, these cells express higher levels of lactate and increased flux through the pentose phosphate pathway [51], thus producing more reducing power in the form of NADPH. This mechanism may also explain the enhanced cryo-survival of ejaculates with higher levels of PGAM in stallions and warrants further research on the interaction between redox metabolism and redox regulation in the spermatozoa. The GRAM domain containing 1A was also more abundant in the spermatozoa of stallions showing good mitochondrial membrane potential post-thaw; this is a cholesterol transfer protein, with a role in the early stages of autophagosome formation [52]. These functions may explain the major presence of this protein in good freezers since mitophagy has recently been related to sperm quality [53]. Finally, an uncharacterized protein similar to actin-1 was also more abundant in good freezers; a tempting possibility is that this protein is also related to mitophagy. Actin structures cage damaged mitochondria during mitophagy [54], however, further research is warranted to characterize this protein and to identify its role in the spermatozoa.

Cryopreservation also had a different impact in the groups showing good and poor membrane integrity post-thaw. The Venn diagram revealed 92 common proteins in both groups, 64 specific

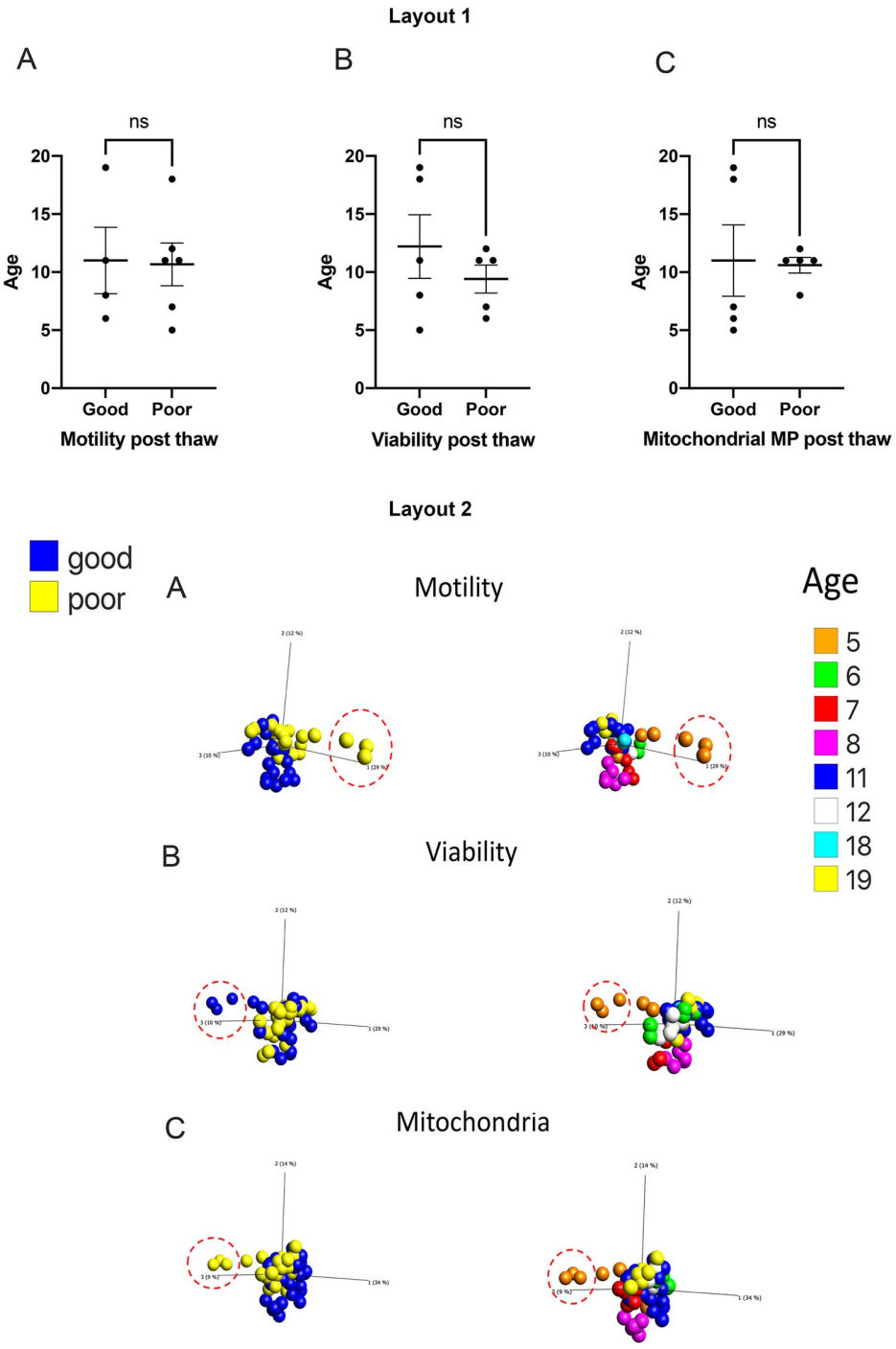


Figure 11. (Layout 1) Effect of age on the outcome of cryopreservation; stallion ejaculates were collected and processed as described in Material and Methods. The effect of age on motility (A), viability (B), and mitochondrial membrane potential (C) post-thaw was analyzed. No significant differences were observed (two-sided *t*-test). (Layout 2) Three-dimensional PCA of the stallions' outcome after cryopreservation (left) in the three categories considered and of the stallion's age (right). Color codes identify both the outcome of cryopreservation and the age of the stallions.

to the group showing high viability post-thaw and 19 specific to the group showing poor motility post-thaw. Bioinformatic analysis to reveal discriminant variables in fresh semen that were able to predict viability after thawing was then performed.

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

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

Conflict of interest

The authors have declared that no conflict of interest exists.

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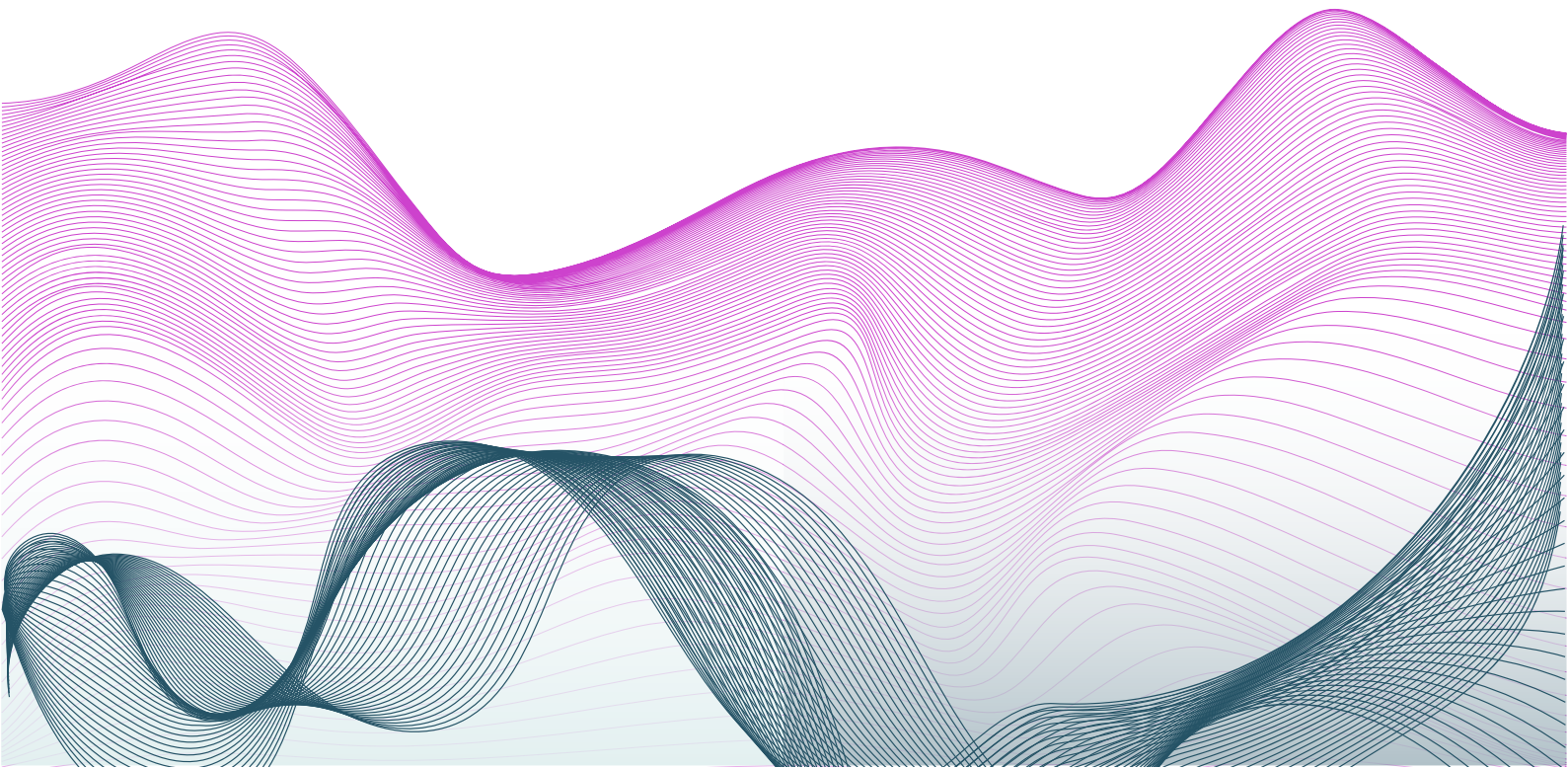

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Research Article

OXFORD

Phosphoproteomics for the identification of new mechanisms of cryodamage: the role of SPATA18 in the control of stallion sperm function†

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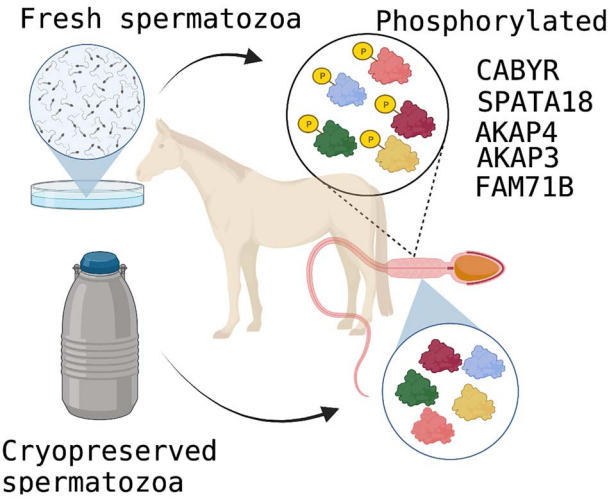
Abstract

Although recent research has addressed the impact of cryopreservation on the stallion sperm proteome, studies addressing the stallion sperm phosphoproteome are lacking. In the present study, the data set of proteomes of fresh and cryopreserved spermatozoa were reanalyzed, showing that cryopreservation caused significant changes in the phosphoproteome. The phosphoproteins reduced most significantly by cryopreservation were Ca^{2+} *binding tyrosine phosphorylation regulated, protein kinase cAMP-activated catalytic subunit beta* (CABYR), *mitochondria eating protein* (SPATA18), *A kinase anchoring protein 4* (AKAP4), *A-kinase anchoring protein 3* (AKAP3) and the *Family with sequence similarity 71 member B* (FAM71B). These proteins belong to the gene ontology (GO) terms sperm fibrous sheath (GO: 0035686), and sperm principal piece (GO: 0097228). The regulatory interactions between kinases and phosphorylation sites on the proteins that were affected most were also investigated, and the potential kinases (based on human orthologs) involved in the regulation of these phosphoproteins identified were: PKCß for SPATA18 and GSK3ß for CABYR. Kinase inhibition assays were also conducted showing that kinases phosphorylating the above-mentioned proteins play an important role in their activity and thus, phosphorylation controls the activity of these proteins and their role in the regulation of the functionality and viability of stallion spermatozoa. In conclusion, the data reported here contribute to the understanding of the fact that the dephosphorylation of certain proteins is a molecular lesion induced by cryopreservation in the stallion spermatozoa.

Graphical Abstract



Keywords: horses, reproduction, spermatozoa, cryopreservation, phosphoproteomics

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Introduction

Cryopreservation is a reproductive technology widely used in animal breeding and the equine industry. Despite the recent progress made, this biotechnology is still suboptimal, and considerable stallion to stallion variability constrains wider use [1]. Freezing and thawing cause osmotic-induced necrosis in a notable proportion of spermatozoa undergoing the process, and the surviving spermatozoa experience important modifications that reduce their functionality and lifespan in the mare genital tract [2]. These modifications have been termed capacitation like, apoptosis like, or spermptosis, and originate from the osmotic effect on the mitochondria, causing alterations in the electron transport chain (ETC), and increased the production of the superoxide anion ($O_2^{\bullet-}$), which finally surpasses the sperm antioxidant defenses causing oxidative stress, inducing lipid peroxidation, and oxidative damage to DNA and proteins [3, 4]. Since redox regulation is an essential mechanism controlling sperm functionality, irreversible redox modifications of key proteins may induce major alterations in sperm functionality, fertilizing ability, and capability to sustain early phases of embryo development [5, 6]. Spermatozoa are terminal and highly differentiated cells with very little capacity for transcription of new proteins. Because of these characteristics, spermatozoa are highly dependent on the incorporation of new proteins through microvesicle trafficking and posttranslational modifications (PTM) of existing proteins. Protein phosphorylation is a PTM involved in regulating most biological processes [7]. Phosphorylation of sperm proteins plays a major role in many important functions such as the acquisition of motility upon ejaculation, capacitation, and fertilization [8–11]. Interestingly, many phosphorylation events in the spermatozoa are redox regulated, including the phosphorylation of proteins essential for motility, capacitation, and viability [12]. Spermatozoa are cells that are particularly suited to proteomic studies, and in the last decade, an increasing amount of research has been published in the field of sperm proteomics, revealing new and interesting aspects of sperm biology [13–19]. Despite this, studies involving the investigation of changes in the phosphoproteome of the spermatozoa are scarce [20, 21], and to the authors' knowledge, there are no previous studies investigating the stallion sperm phosphoproteome. Since cryopreservation induces oxidative stress and redox deregulation in the spermatozoa potentially altering the phosphorylation of sperm proteins, the hypothesis for this study is that alterations in the phosphoproteome may explain damage induced by cryopreservation, thus the study of the phosphoproteome in fresh and frozen-thawed spermatozoa may reveal novel mechanisms involved in cryodamage, that may provide targets that can be used to improve the biotechnology and shed light on mechanisms of sperm malfunction related to infertility.

Material and methods

Reagents and media

The PKCß II/EGFR inhibitor (inhibitor of EGFR and PKC isozymes α, βI and βII, CG), bisindolylmaleimide I (Protein Kinase C inhibitor GF), and GSK3-ß inhibitor XIX (IM) were purchased from Sigma Aldrich (Madrid, Spain). All other reagents for flow cytometry (FC), chicken anti-SPATA18 polyclonal antibody (Invitrogen Catalog# PA5-72739) and goat anti-chicken IgY (H + L) Alexa Fluor 488 (Invitrogen Catalog# A-11039) were purchased from Thermo Fisher (Carlsbad, CA, USA). ViaKrome 808 Fixable Viability was purchased from Beckman Coulter (Miami, FL, USA). Ultrapure deionized water (>18.2 MΩ·cm) was produced from a Millipore Milli-Q Gradient system (Millipore, Bedford, MA, USA).

Experimental design

Reanalysis of data set of proteins from fresh and cryopreserved stallion spermatozoa

Data from our laboratory consisting of sperm proteins from ejaculates collected from 10 different stallions on three different occasions were reanalyzed; in brief, each ejaculate was divided into two identical aliquots, and half of each ejaculate was cryopreserved using standard protocols previously described in our laboratory [22] (cryopreserved, frozen-thawed), whereas the other half was processed as for fresh spermatozoa (fresh). In brief, the aliquot to be cryopreserved was diluted in the freezing medium, Cáceres (University of Extremadura, Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100×10^6 spermatozoa/mL. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were sealed ultrasonically with UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) and immediately placed in an IceCube 14S programmable freezer (SY-LAB Neupurkersdorf, Austria). Straws were kept at 20°C for 15 min, and they were then slowly cooled from 20 to 5°C at a cooling rate of 0.1°C/min. Thereafter, the freezing rate was increased to −40°C/min from 5 to −140°C. The straws were then plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a water bath at 37°C for at least 30 s.

The samples used to obtain proteomes were from stallions of proven fertility routinely used in a commercial AI program, belonging to various Iberian breeds (Andalusian, Lusitano, and Spanish Sport Horse), Arabs, and cross breeds, maintained as indicated in institutional and European regulations for animal care (Law 6/2913 11 June and European Directive 2010/63/EU). All stallions were of proven fertility, with a median age of 10.8 years old. Detailed information on the characteristics of these ejaculates, fresh and cryopreserved are described in a previous publication by our laboratory [3] and the original spectra can be found in the ProteomeXchange Consortium via the PRIDE [23] partner repository with the data set identifier PXD022236. Additional details are provided in Supplementary Table 1; stallions showing average post-thaw motility >35%, membrane integrity >40%, and the percentage of active mitochondria >40% were considered good freezers.

Phosphoproteins reanalysis

We reanalyzed the data set of proteins from stallion spermatozoa obtained as described in previous publications [2, 24]. In brief, proteins were extracted from stallion spermatozoa and analyzed using UHPLC/MS/MS (Agilent 1290 Infinity II Series UHPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high-speed binary pump coupled to an Agilent 6550 Q-TOF mass spectrometer.

Data processing reanalysis

Data sets of fresh and cryopreserved stallion spermatozoa were reanalyzed using Spectrum Mill MS Proteomics

Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). In summary, default conditions were used for the extraction of raw data as follows: non-fixed or variable modifications were selected, [MH] + 50–10,000 *m/z*, maximum precursor charge +5, retention time and *m/z* tolerance ± 60 s, minimum signal-to-noise MS 25, and finding ^{12}C signals. The following criteria were used for the MS/MS search against the appropriate and updated protein database (in this case Uniprot/Horse): selection of non-fixed modifications with the following selected as variable modifications: carbamidomethylated cysteines, phosphorylated Serine (S), phosphorylated Threonine (T), and phosphorylated Tyrosine (Y), and tryptic digestion with five maximum missed cleavages; ESI-Q-TOF instrument, minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation of reversed database scores. Validation of peptide and protein data was performed using the auto-validation algorithm for validation of peptide and protein data. This is completely automated and used to validate the highest-scoring results; those that do not require manual review and are considered high-quality results. The auto-validation strategy used was auto-threshold, in which the peptide score is automatically optimized for a target % false discovery rate (FDR) (1.2%). Protein polishing validation was then performed to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0%).

Bioinformatics of reanalyzed data

Variance filtering and PCA

QlucoreOmics Explorer version 3.7.21 Lund Sweden (https://qlucore.com) bioinformatics software was used for the analysis. Data were normalized, and log transformed. Variables with low overall variance were filtered out to reduce the impact of noise and the remaining variables were then centered and scaled to 0 mean and unit variance. Principal component analysis (PCA) was used for the visualization of the data set in a 3D space. The optimal filter threshold was established using the projection score [25, 26]. The Benjamini–Hochberg method [27, 28] was used for multiple testing with adjusted *P*-values, and variables with adjusted *P*-values (*q*-value) below 0.1 were considered significant. The Biomarker Workbench in Qlucore version 3.7.21 was also used. Using this algorithm, variables (phosphoproteins) differentially expressed were considered potential biomarkers only if *q*-values were below 0.05, in order to establish a stronger statistical threshold to consider a phosphoprotein as especially modified in cryopreserved samples; this approach has been previously applied in our laboratory [29].

Enrichment analysis

Enrichment analysis was performed using the g:Profiler web server (https://biit.cs.ut.ee/gprofiler/gost) [30] of the most abundant phosphoproteins in fresh or frozen-thawed ejaculates. Only annotated genes were used, and the Benjamini–Hochberg FDR threshold was set at $P < 0.05$. The Metascape platform (https://metascape.org) [31] was also used to search for enrichment of specific terms. In this case, all the phosphoproteins were identified, with a specific search for the term "mitochondria".

Identification and analysis of the most relevant phosphoproteins identified to be affected by cryopreservation

The following analyses were conducted on independent ejaculates from three different stallions. The experiments were conducted to validate the data obtained once changes in the phosphoproteome were identified and aimed to study the role of the phosphorylation of the proteins identified in the functionality of the spermatozoa.

Western blotting

SDS-PAGE was performed to separate the proteins according to their apparent molecular masses, as previously described [32, 33]. Briefly, proteins were extracted and denatured by boiling for 10 min at 70°C in a loading buffer supplemented with 5% mercaptoethanol. The protein content was calculated using the Bradford assay [34]. Ten micrograms of sperm protein extract were loaded on a 10% polyacrylamide gel and resolved using SDS-PAGE. Immunoblotting was performed by incubating the membranes with a blocking buffer containing primary antibody chicken anti-SPATA18 polyclonal antibody (Invitrogen Catalog# PA5-72739) 1:1500 for 2.5 h at r.t. The secondary antibody was goat anti-chicken IgY (H + L) Alexa Fluor 488 (Invitrogen Catalog# A-11039) 1:5000 and incubated for 1 h at r.t. Positive controls used were liver and lung lysates. Western blotting was measured in a Biorad Chemidoc MP imaging system.

Immunocytochemistry

Indirect immunofluorescence was performed as previously described [35]. After blocking, the spermatozoa were incubated with primary antibody anti-SPATA18, overnight at 4°C diluted 1/200 in PBS containing 5% BSA (w/v). The following day, cells were washed with PBS and further incubated for 45 min at r.t. with goat anti-chicken IgG antibody conjugated with Alexa Fluor 488 diluted to 1/500 in PBS containing 5% BSA (w/v). Finally, cells were thoroughly washed with PBS. A total of 5000 cells were analyzed in the ImageStream X Mark II Imaging Flow Cytometer (Merck Millipore) using a 488 nm line laser with intensity set to 100 mW, at ×60 magnification. Data analysis of the raw images was accomplished using IDEAS1software (version 6.0.309). The absence of nonspecific staining was measured by processing the samples without a primary antibody (secondary antibody only).

Prediction of regulatory kinases

NetworKIN 3.0 (http://networkin.science) was used to investigate the regulatory interactions between kinases and phosphorylation sites, providing annotation of the relationships between predicted kinases and phosphorylation sites [21, 36, 37]. After the identification of phosphorylated proteins, and transformation to the correspondent human orthologs, all the phosphorylation sites were submitted to NetworKIN 3.0 for prediction of in vivo kinase–substrate relationships. To further determine kinases with major roles in sperm functions, we focused on the phosphoproteins and phosphorylation sites most significantly affected by the cryopreservation procedures, with an FDR below 0.05.

Kinase inhibition assays

Inhibitors of the kinases identified in the previous step were used in an inhibition assay in six independent experiments

(three different stallions and three ejaculates each). Fresh stallion spermatozoa were incubated in Tyrode's medium (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$.2H2O, 0.4 mM $MgSO_4.7H_2O$, 0.3 mM KH_2PO4, 20 mM HEPES, 5 mM glucose, 21.7 mM NaLactate, 1 mM Na Pyruvate, 15 mM $NaHCO_3$, 3 mg/mL BSA, 50 mg/mL Kanamicin) pH 7.4 [38] in the presence of a PKCßII/EGFR inhibitor (CG), Bisindolylmaleimide I, a PKC inhibitor (GF), and GSK3ß inhibitor XIX (IM). Controls consisted of spermatozoa incubated in Tyrode's medium containing 1% DMSO (v/v). After incubation for 0 and 3 h at 37°C, motility and sperm kinematics were assessed using computer-assisted sperm analysis (CASA), whereas sperm viability was investigated using FC.

Computer-assisted sperm analysis

Sperm motility and velocity were assessed using a CASA system (ISAS Proiser, Valencia, Spain) in fresh and frozen-thawed spermatozoa according to standard protocols used at our center [33]. Semen samples were loaded into a Leja® chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed to 37°C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with VAP > 35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile.

Flow cytometry

FC analysis was conducted using a Cytoflex LX® flow cytometer (Beckman Coulter, Miami, FL, USA) equipped with ultraviolet, violet, blue, yellow, red, and infrared lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. The combination of probes used required minimal compensation for spectral overlap. Files were exported as FCS files and analyzed using Cytobank® (Beckman Coulter, Miami, FL, USA). Unstained, single-stained, and fluorescence minus one controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications from our laboratory [39–41].

Assessment of viability, Caspase 3 activity, and phosphatidylserine translocation

Annexin-V 647 conjugated and CellEvent® Caspase 3/7 Green Detection Reagent were combined in a multiparametric test and evaluated by FC [42]. Samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent (2 μM) and incubated at room temperature for 15 min. Following this, the samples were washed using a short centrifugation spin for 12″ and suspended in 200 μL of annexin binding-buffer (solution in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Five microliters of Annexin-V was added to 200 μL of sample. After 15 min of incubation in the dark at room temperature, 400 μL of 1 × Annexin binding buffer was added before reading in the flow cytometer. To gate dead spermatozoa, samples were stained with Viakrome 808 (2.5 μL of the reconstructed solution as indicated by the manufacturer). Samples were evaluated in a Cytoflex LX® flow cytometer (Beckman Coulter). CellEvent staining was validated as previously described [43].

Statistical analysis

GraphPad Prism version 7.00 for Mac, La Jolla, California, USA, www.graphpad.com and Cytobank (Beckman Coulter, Miami, FL, USA) were used for statistical analysis. The effect of different kinase inhibitors was compared after incubation up to 3 h at 37°C with its controls using a one-way ANOVA. The analysis compared motility and velocities, and the FC-derived data, viability, apoptosis, mitochondrial membrane potential, and GSH content in controls and treated aliquots. Differences were considered significant when $P < 0.05$ and results are displayed as means ± SEM.

Results

Identification of phosphoproteins in stallion spermatozoa

A total of 633 phosphoproteins (Supplementary Table 2) were identified in stallion spermatozoa, both in fresh and cryopreserved spermatozoa. Enrichment analysis of this set of proteins revealed the gene ontology (GO) terms sperm fibrous sheath ($P = 2.005 \times 10^{-6}$), sperm principal piece ($P = 8.168 \times 10^{-5}$), sperm flagellum ($P = 5.830 \times 10^{-3}$), sperm midpiece ($P = 8.872 \times 10^{-3}$), and acrosomal membranes ($P = 2.102 \times 10–^{2}$), among others (Figure 1A). Because of the deeper annotation of the human proteome, equine proteins were transformed into their human orthologs, and new enrichment analysis was conducted, in addition to the terms related to sperm midpiece and acrosome, previously disclosed, annotated terms related to immune functions and interestingly, transcription factors, were present (Figure 1B). Furthermore, a specific search in Metascape for the term "mitochondria" revealed significant enrichment in phosphoproteins related to this term ($P = 7.1e{-}05$, Figure 1C).

Cryopreservation modifies the stallion sperm phosphoproteome

Cryopreservation causes major damage to the spermatozoa because of the intense osmotic stress that these cells experience at freezing and, especially at thawing. On average, 50% of spermatozoa succumb to osmotic-induced necrosis, whereas the surviving population experience altered functionality. Thus, we investigated if the process of cryopreservation modifies the phosphoproteome of the stallion spermatozoa. The biomarker workbench (Table 1) in Qlucore Omics Explorer was used to identify phosphoproteins differentially expressed in fresh and frozen-thawed spermatozoa, and six proteins presented a *q*-value (FDR) equal or below 0.05 meaning highly significant differences in the expression of these phosphoproteins between fresh and cryopreserved aliquots were present.

Cryopreservation reduces specific phosphoproteins

The effect of freezing and thawing on the stallion sperm phosphoproteome was investigated; cryopreservation significantly reduced the presence of the phosphoproteins; *Ca^{2+} binding tyrosine phosphorylation regulated, protein kinase cAMP-activated catalytic subunit beta* (CABYR) ($P = 1.6e^{-03}$), *mitochondria eating protein* (SPATA18) ($P = 5.0e^{-03}$), *A kinase anchoring protein 4* (AKAP4) ($P = 7.8e^{-22}$), *A kinase anchoring protein 3* (AKAP3) ($P = 6.3e^{-08}$), and *family with sequence similarity 71 member B* (FAM71B) ($P = 2.3e^{-07}$; Figure 2A).

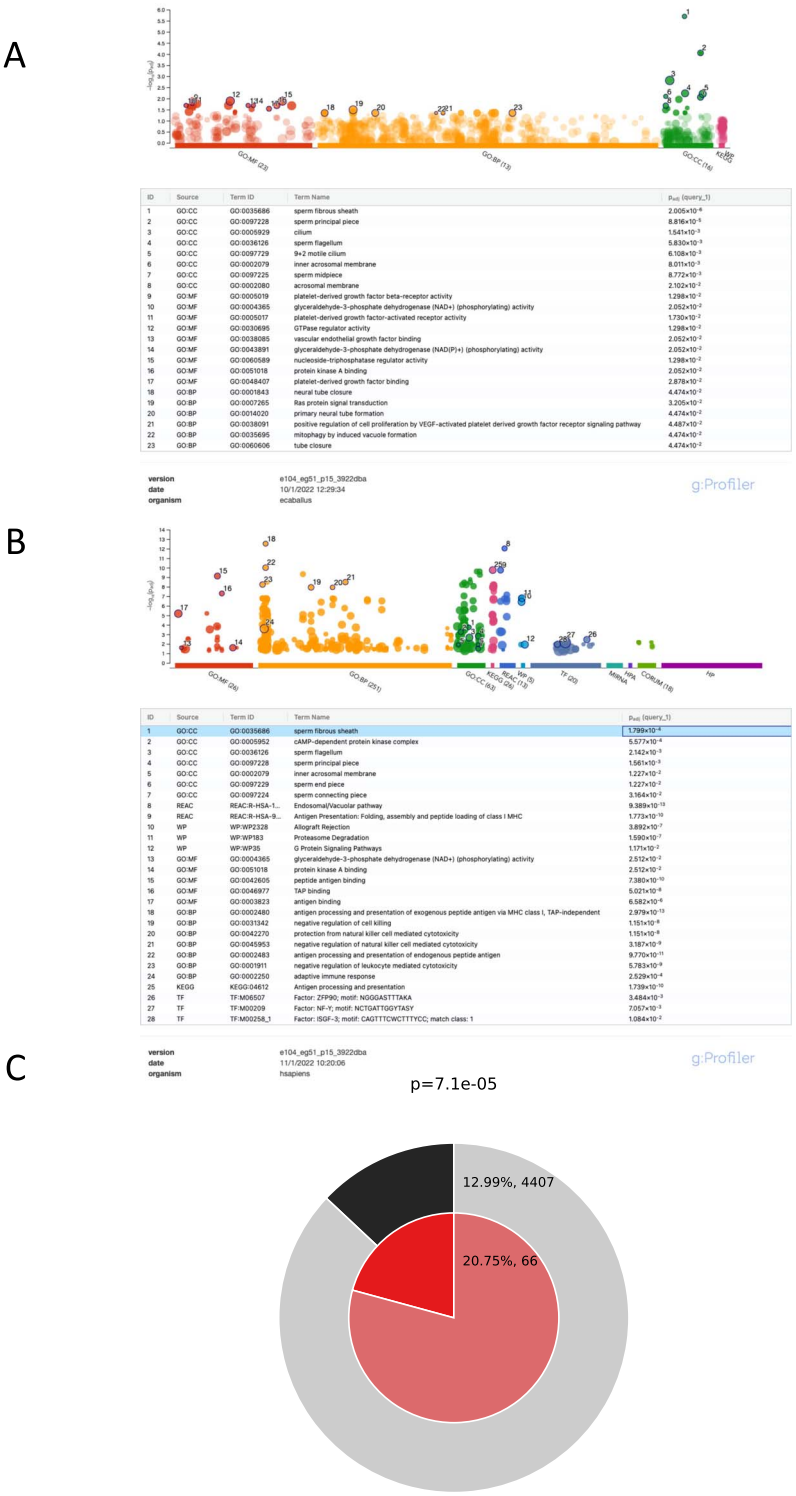

Figure 1. Manhattan plots obtained after g:Profiler enrichment analysis using equine (A) and human orthologs (B). (A) GO terms for biological process (BP) are in orange and those for cellular component (CC) in green. (B) KEGG pathways are depicted in red, and Reactome pathways are depicted in blue (using human orthologs). The *P*-values are depicted on the *y*-axis and in more detail in the results table below the image. (C) Enrichment of genes matching membership term: mitochondria. The outer pie shows the number and the percentage of genes in the background that are associated with the membership (in black); the inner pie shows the number and the percentage of genes in the individual input gene list that are associated with the membership ($P = 7.1e^{-5}$).

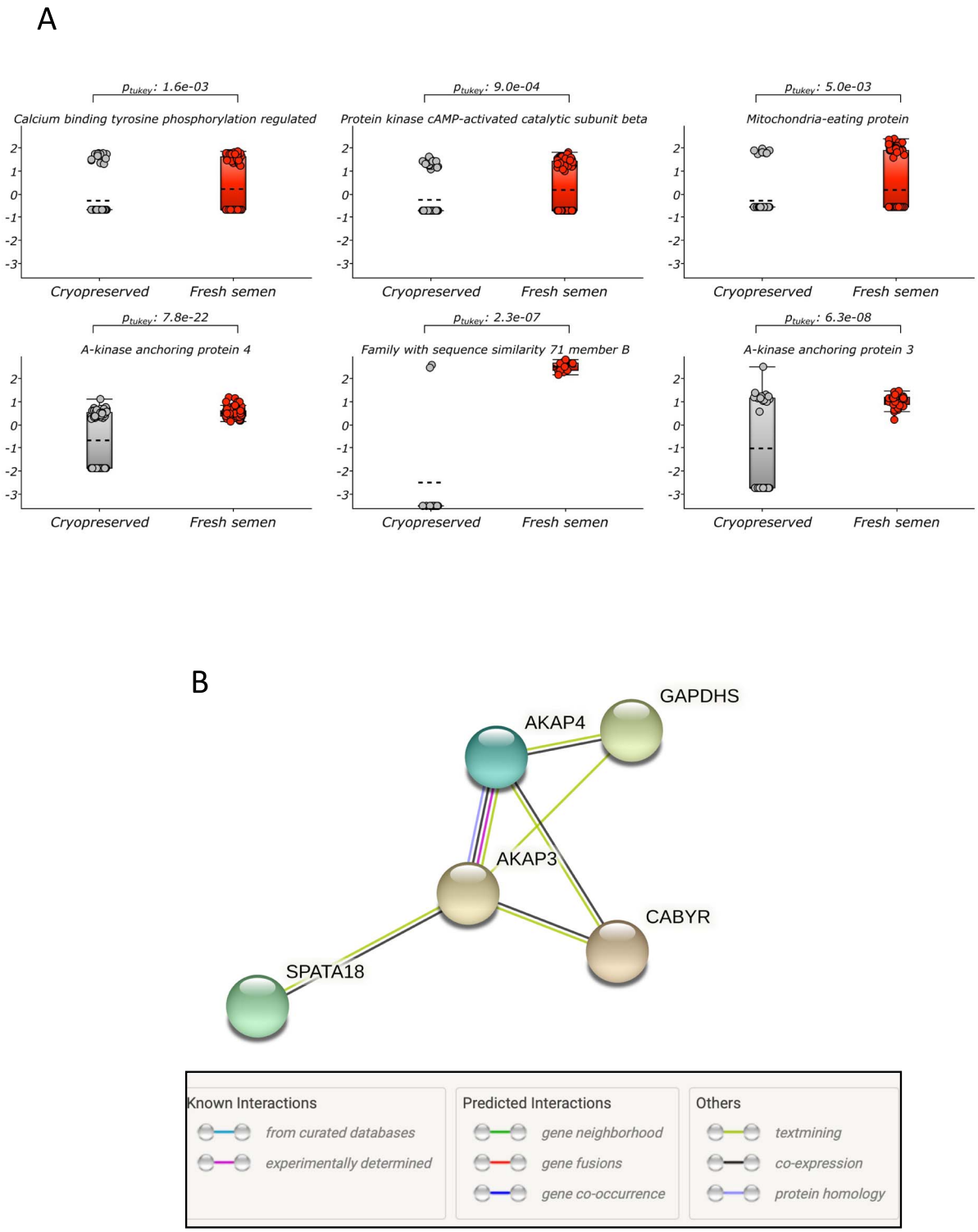

Figure 2. (A) Phosphoproteins reduced during the cryopreservation of stallion spermatozoa. These data were obtained after the reanalysis of data from our laboratory corresponding to fresh and cryopreserved aliquots of stallion semen (10 stallions, three ejaculates each) for the identification of phosphorylated proteins in Serine (S), Threonine (T), and Tyrosine (Y) conducted and published by our laboratory. Detailed information on the characteristics of these ejaculates, both fresh and cryopreserved, is described in a previous publication by our laboratory [3] and the original spectra can be found in the ProteomeXchange Consortium via the PRIDE [23] partner repository with the data set identifier PXD022236. Bioinformatics was conducted using Qlucore Omics Explorer (https://qlucore.com) as described in Material and Methods. (B) Protein–protein interaction among phosphoproteins downregulated after cryopreservation obtained in STRING. https://string-db.org

Table 1. Biomarker workbench in Qlucore Omics Explorer (https://www.qlucore.com) to identify phosphoproteins differentially expressed in fresh and frozen-thawed spermatozoa

Response variable	Response variable	Two group	Two group	Two group
Type	Details	Sample annotation 1[Cryopreseved ≠ Fresh semen] *P*-value	Sample annotation 1[Cryopreseved ≠ Fresh semen] *q*-value	Sample annotation 1[Cryopreseved ≠ Fresh semen] Fold change
Variable	A-kinase anchoring protein 4	7.780864190783239e−22	2.1008333315114747e−20	0.0022945646645022395
Variable	Protein kinase cAMP-activated catalytic subunit beta	0.0009014203415419723	0.0036193792318403605	0.08936035825564884
Variable	Calcium binding tyrosine phosphorylation regulated	0.0016813932048714774	0.005674702066441236	0.10110228242543928
Variable	A-kinase anchoring protein 3	2.739783368783472e−07	3.698707547857687e−06	0.11555111747080885
Variable	Mitochondria-eating protein	0.005667224931912356	0.013213390796474048	0.1927533211750831
Variable	Family with sequence similarity 71 member B	2.2616004527921588e−05	0.0002035440407512943	0.33605952211990753

Table 2. Panther overrepresentation test of phosphoproteins differentially expressed in fresh and frozen-thawed stallion spermatozoa

Analysis type:	**PANTHER Overrepresentation Test (Released 20,210,224)**						
Annotation Version and Release Date:	GO Ontology database DOI: 10.5281/zenodo.5228828 Released 2021-08-18						
Analyzed List:	upload_1 (*Equus caballus*)						
Reference List:	*E. caballus* (all genes in database)						
Test Type:	FISHER						
Correction:	FDR						
GO CC complete	*E. caballus*—REFLIST (20774)	upload_1 (99)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw *P*-value)	upload_1 (FDR)
sperm fibrous sheath (GO:0035686)	8	4	0.04	+	>100	2.29E−07	4.11E−04
sperm principal piece (GO:0097228)	23	4	0.11	+	36.49	7.68E−06	6.90E−03

Enrichment analysis and protein–protein interaction network

An enrichment test was performed in Panther (http://www.pantherdb.org). The GO terms sperm fibrous sheath (GO:0035686) and sperm principal piece (GO:0097228) were enriched in the phosphoproteins differentially expressed between fresh and cryopreserved spermatozoa (Table 2). Functional protein–protein interactions were investigated in STRING (https://string-db.org) showing functional interactions among the phosphoproteins dephosphorylated in cryopreserved spermatozoa (Figure 2B).

Identification of SPATA18 in stallion spermatozoa

Most of the downregulated proteins have been previously reported in stallion spermatozoa; however, to the best of our knowledge, SPATA18 has not been previously reported in horses. Thus, we used WB and image FC to confirm the presence of this protein in the stallion spermatozoa (Figure 3, Panel A). The distribution of the proteins agrees with the previously described distribution in the cytoplasm that relocates to the mitochondrion outer membrane following cellular stress (Figure 3, Panel B) [44, 45].

Prediction of regulatory kinases in stallion spermatozoa

It is well known that cryopreservation causes reduced sperm functionality, thus changes in the phosphorylation of specific proteins, and identification of potential kinases involved in the phosphorylation of these sites in proteins may shed light on new mechanisms involved in cryodamage. NetworKIN 3.0 analysis [36, 37, 46] was applied to identify candidate kinases involved in the regulation of sperm functionality. The equine proteins were converted to the equivalent human orthologs, and attention was focused on identifying potential kinases regulating the phosphoproteins reduced after cryopreservation. These phosphorylated proteins were AKAP3, AKAP4, CABYR, FAM71B, and SPATA18 (Figure 2A). Potential kinases (based on human orthologs) involved in the

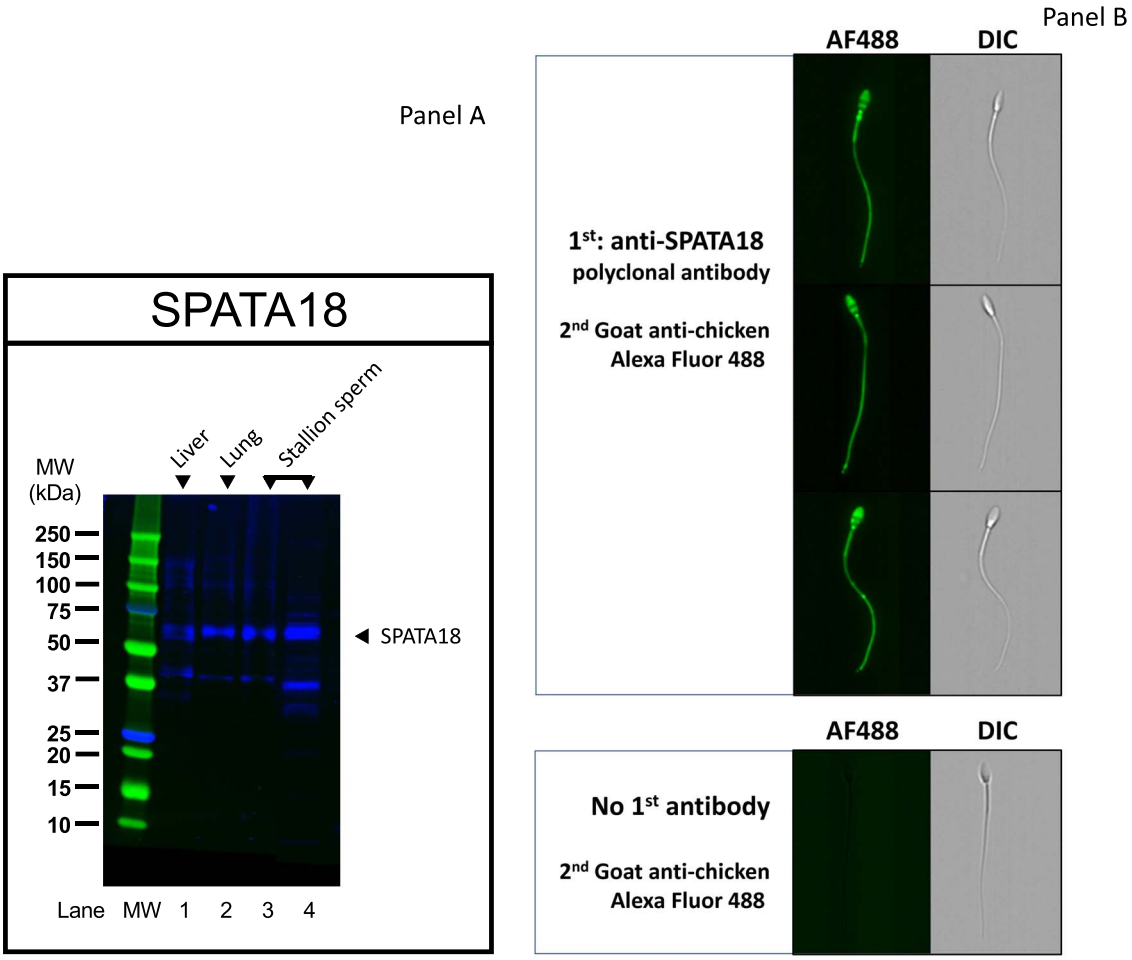

Figure 3. Panel A. Detection of SPATA18 in stallion spermatozoa Lane MW: precision plus protein plus dual standards, 5 μL (BioRad #1610374), Lane 1: rat liver extract (20 μg), Lane 2: rat lung extract (15 μg), Lanes 3 and 4: stallion sperm lysates (20 μg). Panel B. Immunolocalization of SPATA18 in stallion spermatozoa. Representative images of 5000 cells were analyzed in the ImageStream X Mark II Imaging Flow Cytometer (Merck Millipore) using a 488 nm line laser with intensity set to 100 mW, at ×60 magnification. AF 488 (Alexa Fluor 488), Differential Interface Contrast Microscopy (DIC).

phosphorylation of these proteins were PKCß for SPATA18, GSK3ß for CABYR, PKCß, and PKAß for AKAP4 and CK1δ for AKAP3.

Kinase inhibition assay

A kinase inhibition assay was performed using inhibitors of the predicted kinases, PKCßII/EGFR inhibitor (CG, inhibiting the phosphorylation of SPATA18) bisindolylmaleimide I, a selective inhibitor of PKC (GF, inhibiting the phosphorylation of SPATA18), and GSK3ß inhibitor XIX (IM, Inhibiting CABYR). The inhibition of PKC, with the general inhibitor (GF) and the more specific inhibitor of the PKCß (CG) had no effect on the percentages of live spermatozoa after 3 h of incubation at 37°C (Figure 4A and B). In the same way, the inhibitor of GSK3ß had no effect on viability (Figure 4C). However, the inhibition of PKC and particularly PKCß, increased apoptosis (caspase 3+ and Annexin+). The PKCß inhibitor CG increased the percentages of caspase 3+ spermatozoa after 3 h of incubation at 37°C ($17.3 \pm 1.0\%$ in controls to 31.6 ± 1.2 at 5 μM, $P < 0.0001$ and to $27.6 \pm 2.5\%$ at 10 μM, $P < 0.01$; Figure 4D), no difference was observed between the time of incubation in untreated aliquots (Figure 4D). In a similar manner, whereas no differences were observed in the percentages of Annexin-V + spermatozoa after 3 h of incubation, aliquots incubated in the presence of the CG inhibitor showed increased percentages of Annexin-V positive spermatozoa with respect to initial controls (40.0 ± 6.9 in controls to $63.3 \pm 4.5\%$ in the presence of 5 μM GC, $P < 0.01$ and $67.3 \pm 3.8\%$ in presence of 10 μM GC, $P < 0.001$; Figure 4G). When the PKC (GF) inhibitor was present at 15 μM the percentage of caspase 3 + spermatozoa increased with respect to controls at the same time point of incubation ($17.3 \pm 1.8\%$ in controls to $27.1 \pm 3.0\%$ Figure 4E; $P < 0.01$), and at 30 μM the percentage of Annexin-V positive spermatozoa also increased with respect to the controls at the same time point (Figure 4H; $P < 0.0001$). The GSK3ß inhibitor IM increased the percentages of Caspase 3 positive spermatozoa with respect to the same time point controls at 15 and 30 μM (Figure 4F; $P < 0.001$ and $P < 0.01$, respectively). At both concentrations, the percentage of Annexin-V positive spermatozoa also increased (Figure 4I; $P < 0.01$ and $P < 0.05$, respectively). On the other hand, kinase inhibition had no major effect on motility (Figure 5A), but the inhibition of GSK3ß resulted in an increased percentage of linear motile spermatozoa (Figure 5B).

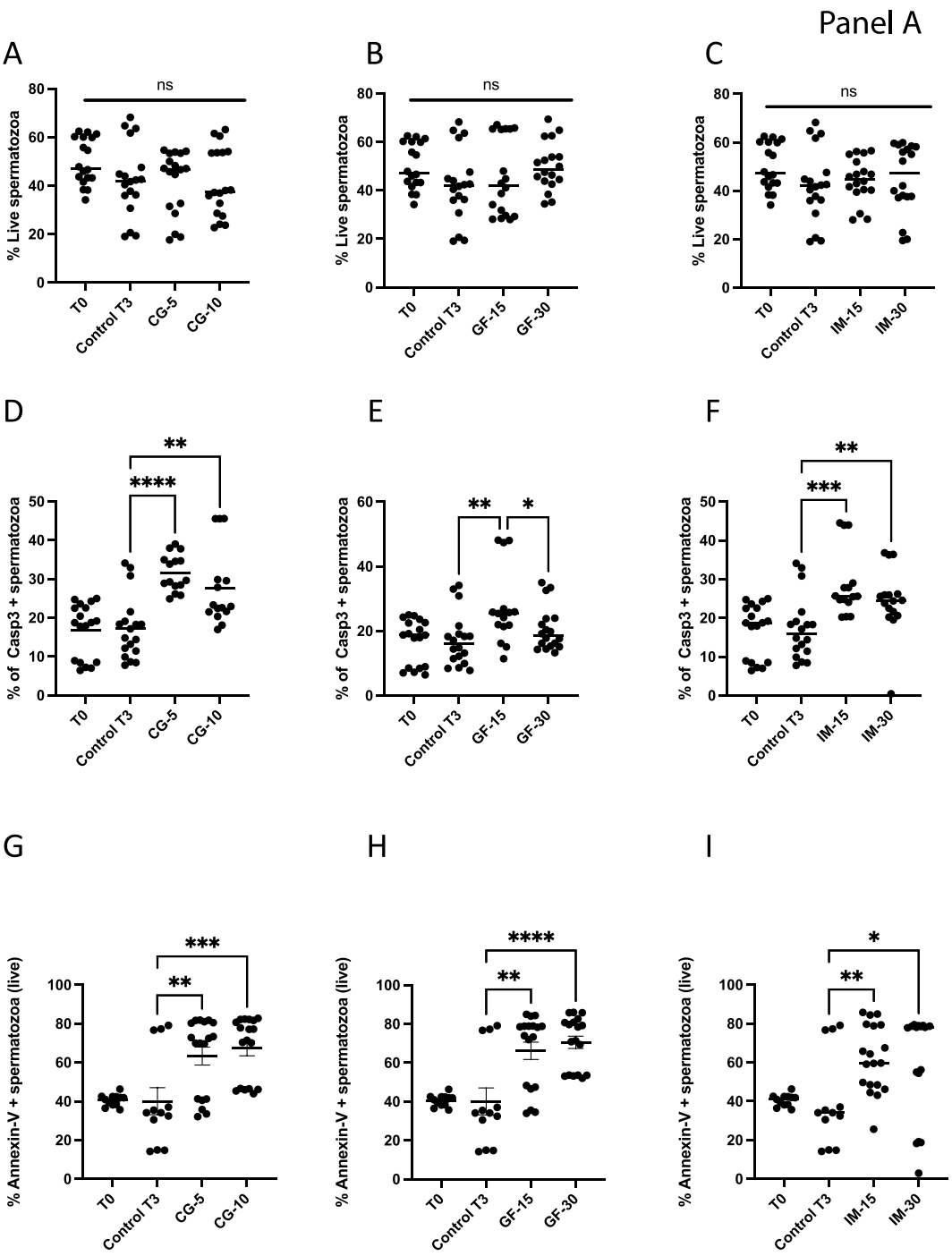

Figure 4. Effect of PKCß II/EGFR inhibitor (inhibitor of EGFR and PKC isozymes α, βI and βII, CG), bisindolylmaleimide I (Protein Kinase C inhibitor GF), and GSK3ß inhibitor XIX (IM) on stallion viability. Stallion spermatozoa were collected and processed as indicated in the Material and Methods, viability and apoptosis were measured at the beginning of the incubation period and after 3 h of incubation at 37°C. Panel A. (A–C) *Effect on the percentage of live spermatozoa;* none of the inhibitors tested reduced the percentage of live spermatozoa (D–F), *effect on the percentage of apoptotic spermatozoa (Caspase 3 +).* (D) Incubation in presence of CG (PKCβ inhibitor, the predicted kinase responsible for the phosphorylation of SPATA18) at 5 and 10 μM increased the percentages of apoptotic spermatozoa with respect to controls at the same time point of incubation. (E) GF increased the percentage of Caspase 3 positive spermatozoa only at a concentration of 15 μM. (F) Inhibition of GSK3ß, the predicted kinase responsible for the phosphorylation of CABYR, caused an increase in the percentage of Caspase 3 positive spermatozoa especially when IM was present at 15 μM. (G–I) *Effect on the percentage of Annexin-V spermatozoa.* (G) Incubation in the presence of CG increased the percentage of Annexin-V positive spermatozoa (apoptotic showing transposition of phosphatidylserine to the outer plasma membrane). (H) GF also increased the percentage of Annexin-V + spermatozoa at both concentrations tested. (I) IM increased the concentration of Annexin-V spermatozoa. Data are given as means ± SEM, and are derived from at least three independent ejaculates obtained from three different stallions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Panel B. Representative cytograms showing the gating strategy and changes in the percentages of live and apoptotic spermatozoa (Caspase 3+) in the kinase inhibition assay, spermatozoa were processed for FC analysis as described in Material and Methods. (A) Spermatozoa were gated based on side scatter (SCC) and forward scatter FSC; events with higher area in the FSC detector (clumps and doublets) were gated out. (B) Spermatozoa were then gated in Hoechst 33342 positive cells (DNA containing events) allowing debris and non-sperm events to be gated out. (C) Controls at the beginning of the incubation period, live and dead spermatozoa were detected using Viakrome 808™, apoptotic spermatozoa were detected using CellEvent™ (Caspase 3). (D) Controls after 3 h of incubation at 37°C. (E) Aliquot incubated in presence of CG (PKCβ inhibitor, the predicted kinase phosphorylating SPATA18) at 5 μM. (F) Aliquot incubated in the presence of CG (PKCβ inhibitor, the predicted kinase phosphorylating SPATA18) at 10 μM.

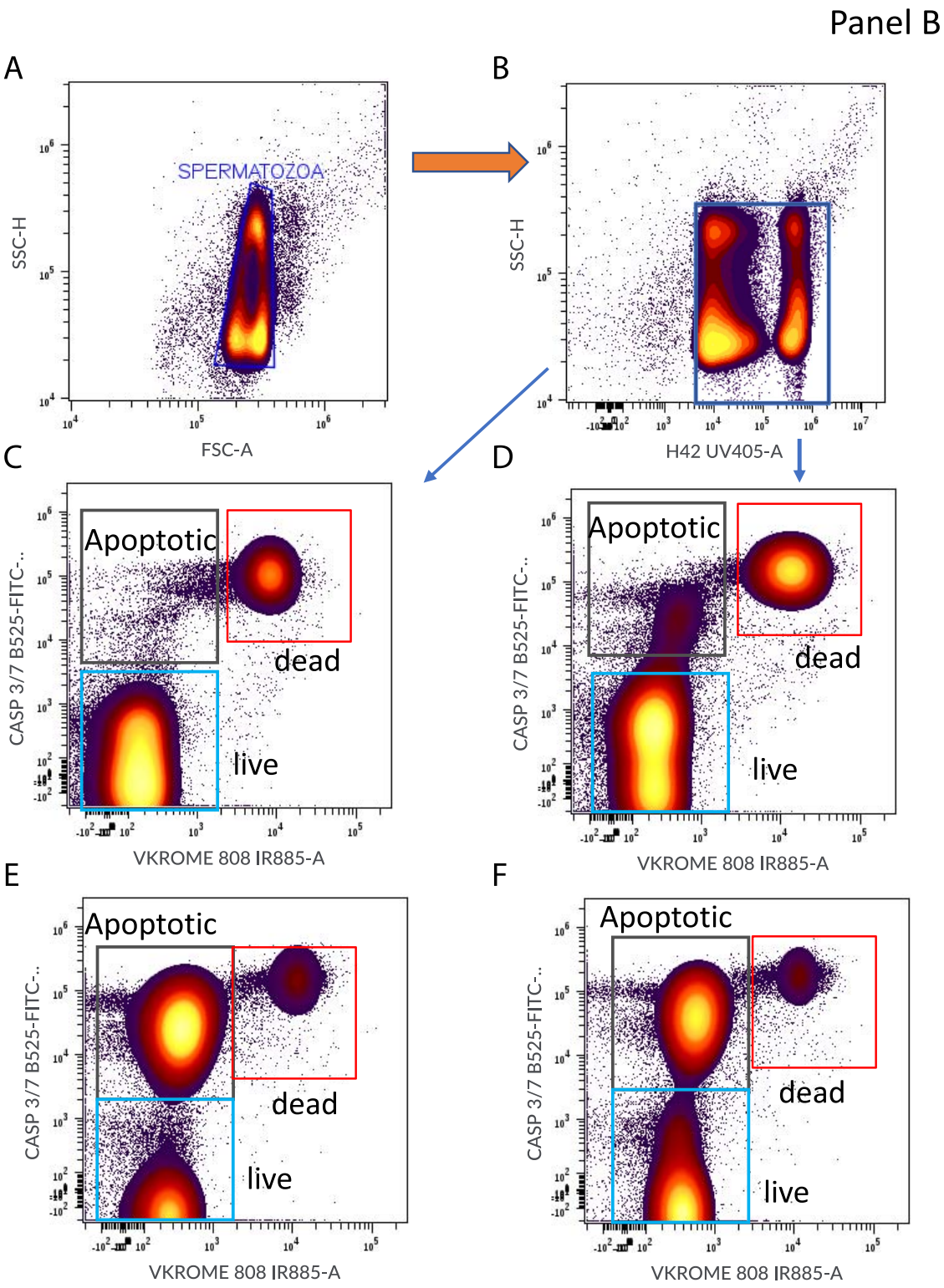

Figure 4. Continued

Discussion

In the present study, the phosphoproteome of the stallion spermatozoa was investigated; this, to the authors' knowledge, is the first study on this topic. We have provided evidence linking the presence of specific phosphoproteins to sperm functionality and investigated the effect of cryopreservation on the phosphoproteome; moreover, the functionality of phosphoproteins using kinase inhibition assays was studied. In this study, changes in the phosphoproteome provide a molecular explanation for the reduced functionality of the spermatozoa after thawing. One of the theories explaining the reduced fertility of cryopreserved spermatozoa claimed that after thawing spermatozoa experience accelerated capacitation and die rapidly in the female genitalia; this theory was

based mainly on changes in intracellular Ca^{2+} determined using the CTC assay [47, 48]. However, recent research using multiparametric FC and computational approaches disputes the theory of cryo-capacitation. On the one hand, there is no evident increase in tyrosine phosphorylation after thawing, and spermatozoa showing a phenotype compatible with capacitation are only evident in a small subpopulation of spermatozoa when a computational analysis is performed [42]. Cryopreservation is known to cause major impairments in sperm functionality, so we aimed to link changes in the phosphoproteome in the stallion spermatozoa to changes in sperm quality induced by the procedure. Since cryopreservation causes major damage to the spermatozoa, changes in phosphorylation may explain cryopreservation-induced damage. Cryopreservation significantly reduced phosphoproteins involved in mitochondrial maintenance like SPATA18. This protein has previously been described in rat, mouse, and boar spermatozoa [49–51]; however, to the best of our knowledge, the presence and the phosphorylation of this protein in the stallion spermatozoa have not been described. This protein showed a 79.9% homology with the human ortholog (Q8TC71). This is a recently described product of the *p53* gene [44, 45], with an important role in mitochondria quality control. This finding goes in line with the current consensus on the pivotal role of mitochondria in stallion sperm functionality. Mitophagy has been described in spermatozoa as a potential quality control system [52], the presence of SPATA18 in spermatozoa supports this previous finding, and in addition, is a good candidate for further support of the fundamental role of mitochondria and the development of new methods for sperm diagnosis. Furthermore, this protein was identified using WB and image FC. Phosphorylation of this protein appears to be necessary for its activation in stallion spermatozoa. SPATA18 or Mieap induces the accumulation of lysosomal proteins within mitochondria in response to mitochondrial damage and eliminates oxidized proteins to repair unhealthy mitochondria [44, 45, 53, 54]. In this context, it is well known that cryopreservation causes oxidative stress and that mitochondrial proteins are a preferent target for electrophilic aldehydes that originate after lipid peroxidation, like 4-hydroxynonenal (4-HNE) [22, 55]. It is likely that the stress of cryopreservation may lead to the dephosphorylation of this phosphoprotein, further contributing to mitochondrial damage. Potential kinases regulating the phosphorylation of this protein were identified, *in silico* analysis identified that this protein was phosphorylated by PKCß. Functional kinase assays using PKC beta inhibition showed that the inhibition of the phosphorylation sites of this protein leads to apoptotic changes in the stallion spermatozoa, interestingly it is well described that cryopreservation induces apoptotic changes [4, 56–58], as did PKCβ inhibition supporting the link between the dephosphorylation of this protein during cryopreservation and apoptotic changes.

Two phosphoproteins with important roles in sperm functions such as capacitation, the acrosome reaction, and motility were significantly reduced after cryopreservation, the *A-kinases anchoring proteins* (AKAP) 3 and 4. These proteins and particularly AKAP4 have been proposed as biomarkers of sperm quality in stallions [18]; moreover, AKAP4 has also been identified as highly susceptible to adduction by 4-HNE [18]; the changes observed in our experiment agree with this previous report and also suggest a role as a marker of sperm quality after cryopreservation.

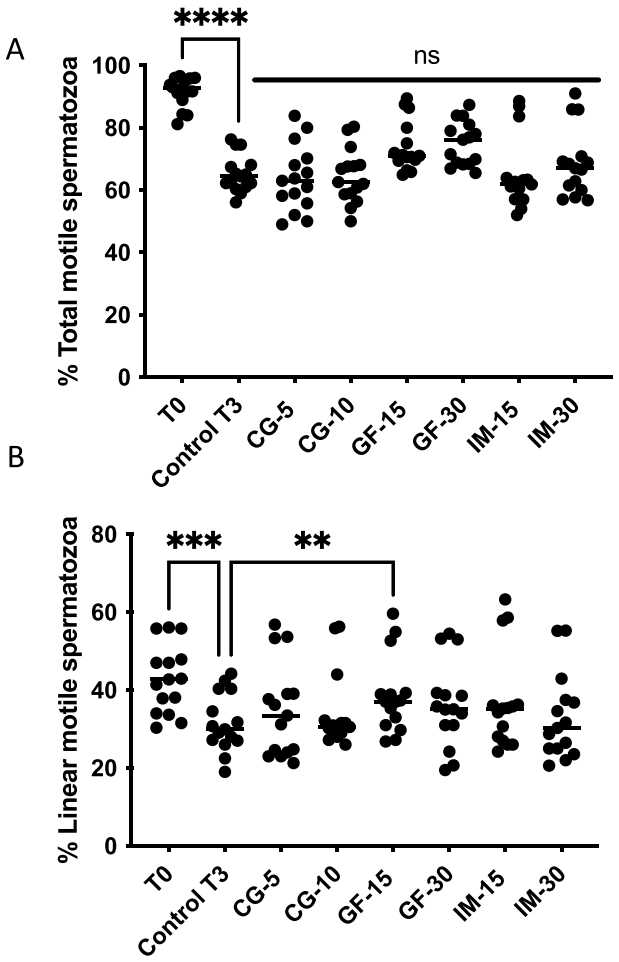

Figure 5. Effect of PKCß II/EGFR inhibitor (inhibitor of EGFR and PKC isozymes alpha, beta I and beta II, CG), bisindolylmaleimide I (ProteinKinase C inhibitor GF), and GSK3ß inhibitor XIX (IM) on motility. Stallion spermatozoa were collected and processed as indicated in the material and methods, motility (CASA) was measured at the beginning of the incubation period and after three hours of incubation at 37°C. Data are given as means ± SEM, and are derived from at least three independent ejaculates obtained from 3 different stallions. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

Ca^{2+} binding tyrosine phosphorylation regulated (CABYR) was another protein affected by cryopreservation. This protein has a major role in the regulation of capacitation and the acrosome reaction [59], and it is phosphorylated in tyrosine upon capacitation. In the present study, we observe a decrease in the general phosphorylation of this protein after cryopreservation, another finding that does not support the cryocapacitation theory. The predicted kinases revealed that this protein is regulated by GSK3ß, and the kinase inhibition assay performed supported the role of this protein in stallion sperm function since incubation in the presence of inhibitors of GSK3ß increased the percentage of apoptotic spermatozoa. Although the main known role of this protein so far is capacitation, our findings suggest important roles in other aspects of sperm functionality, which have also been proposed in mouse spermatozoa [60]. Interestingly, a recent report shows that in human spermatozoa, cryopreservation dephosphorylates the isoform GSK3α of this protein [61], a finding that falls well in line with our study.

In conclusion, the data reported here contribute to understanding molecular lesions induced by cryopreservation in the stallion spermatozoa and dispute the cryo-capacitation theory. In spite of the fact that previous enrichment on phosphoproteins was not performed (data based on reanalysis), noteworthy information was obtained, indicating that the study of the phosphoproteome is a powerful tool. Reduced motility after cryopreservation can be explained by modifications in key flagellar proteins such as AKAP3 and 4, and proteins involved in metabolism and redox regulation; moreover, molecular damage in key proteins with roles in the maintenance of mitochondrial health explains the reduced lifespan of cryopreserved spermatozoa and the activation of cell death mechanisms arising in the mitochondria. The data reported here may pave the way for further studies that could expand our knowledge of the biology of stallion spermatozoa in aspects directly related to sperm biotechnology such as cryopreservation.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Data availability

The underlying data for this article are available in the article and in its online supplementary material.

Conflict of interest

The authors have declared that no conflict of interest exists.

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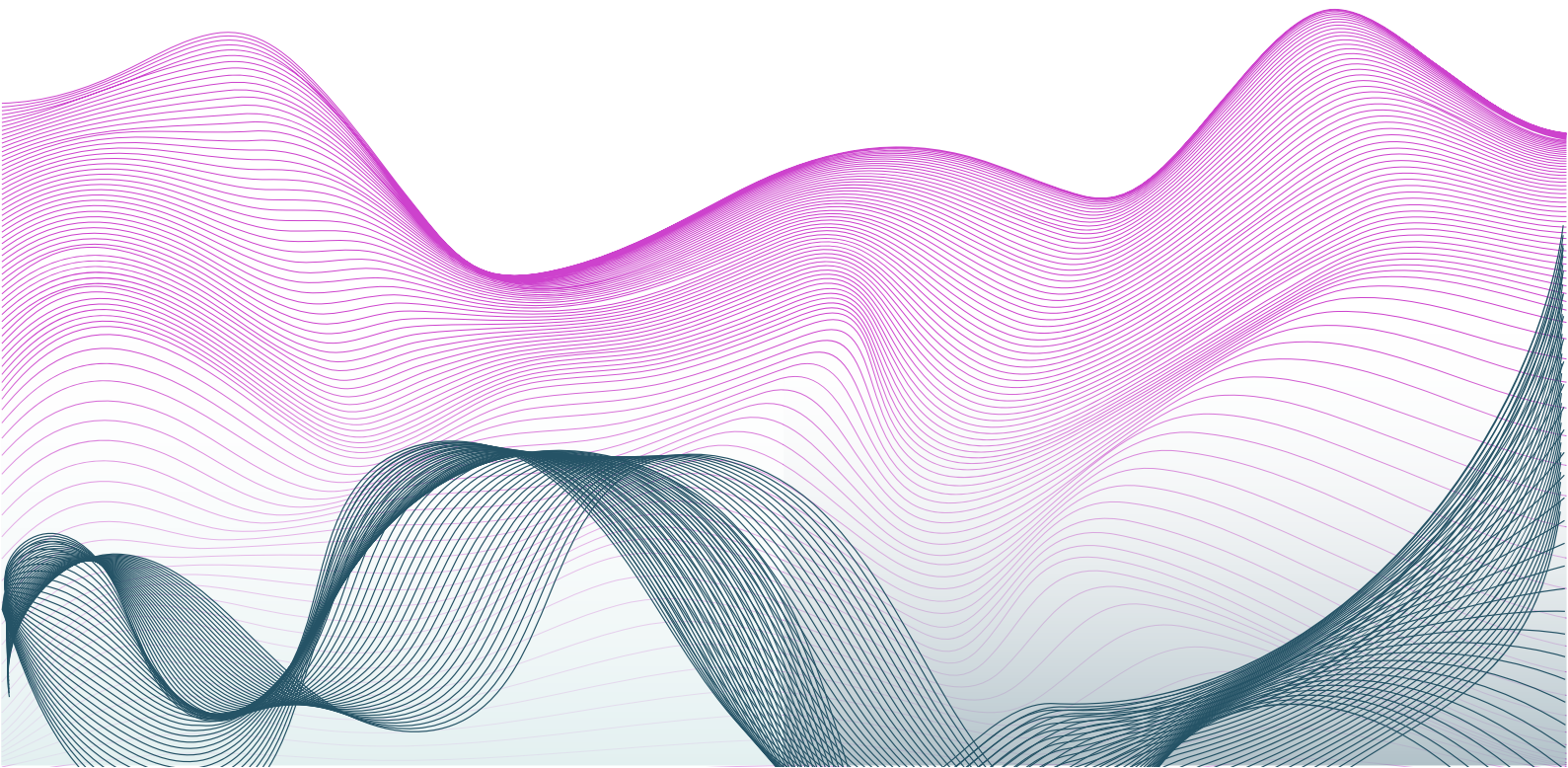
ARTICLE 8



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Data Article

Dataset of the sperm proteome of stallions with different motility

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ABSTRACT

This paper provides a detailed set of data on how the stallion sperm proteome differs among stallions with different sperm motilities, although within normal ranges. Findings distinguish proteins that may help to identify stallions of superior sperm motility. Sperm proteins were analyzed using a UHPLC/MS/MS system comprising of an Agilent 1290 infinity series UHPLC coupled to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). These data can be used to disclose potential targets to identify good sperm samples and to study specific pathways involved in the regulation of sperm motility. This data article is linked to the paper "Proteins involved in mitochondrial metabolic functions and fertilization predominate in stallions with better motility Journal of Proteomics 247:104335 doi: 10.1016/j.jprot.2021.104335".

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Specifications Table

Subject	Veterinary Sciences; Reproductive Biology.
Specific subject area	Proteomics of stallion spermatozoa.
Type of data	Raw data
How data were acquired	Samples were analyzed using a UHPLC/MS/MS system consisting of an Agilent 1290 infinity series UHPLC coupled to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA)
Data format	Processed Analyzed
Parameters for data collection	Equine spermatozoa from 10 fertile stallions with a median age of 10.8 years with different values of motility estimated using computer assisted sperm analysis (CASA). Proteins were extracted from the spermatozoa of these stallions and analyzed using UHPLC/MS/MS.
Description of data collection	Comparative mass spectrometry proteomic profiling of the sperm proteome of fertile stallions with different motility.
Data source location	Cáceres, Extremadura, Spain.
Data accessibility	ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD025807 ProteomeXchange title: Stallion sperm proteins in 10 stallions with different motility ProteomeXchange accession: PXD025807 PubMed ID: 34298182 ProjectWebpage: https://www.ebi.ac.uk/pride/archive/projects/PXD025807 FTP download: http://ftp.pride.ebi.ac.uk/pride/data/archive/2021/07/PXD025807/
Related research article	Gaitskell-Phillips G, Martín Cano FE, Ortiz Rosdríguez JM, Silva Rodríguez A, Rojo-Domínguez P, Tapia JA, Gil MC, Ortega Ferrusola C, Peña FJ (2021). Proteins involved in metabolic functions and fertilization predominate in stallions with better motility Journal of Proteomics 247:104335 https://doi.org/10.1016/j.jprot.2021.104335

Value of the Data

- The proteomic data reported here show that stallions with different sperm motility present notable differences in their proteome.
- Re-analysis of the data set may help to characterize traits related to sperm motility in stallions and the influence of factors such as breed or age in the motility.

1. Data Description

A data set of 903 proteins obtained from equine spermatozoa is provided (Supplementary Table 1). Proteins were extracted from spermatozoa from 10 fertile stallions, of a median age of 10.8 years old, of the following breeds; Arab, Andalusian, Andalusian-Arab, Spanish Sport Horse, Lusitanian and Anglo-Arabian. Relative abundance of proteins is also provided. These proteins were obtained from 10 stallions with different motility and classified as having good ($>$ 90% total motility), average (between 80 and 90% total motility), and poor $<$ 80% total motility), although all of them have sperm parameters within normal ranges [1] . Motility parameters were studied using Computer Assisted Sperm Analysis (CASA), including motility defined as the percentage of motile spermatozoa, linear motility, as the percentage of spermatozoa showing a linear pattern of movement, VCL defined as the actual velocity along the true trajectory in μm/s, VAP defined as the average velocity in μm/s, and the VSL defined as the straight-line velocity in μm/s. This information, after re-analysis, may be used to improve our understanding of the regulation of stallion sperm motility, and to develop on farm tests to check sperm quality. The data set has been deposited to the ProteomeXchange Con-

sortium [2] *via* the PRIDE partner repository with the dataset identifier PRIDE PXD025807, https://www.ebi.ac.uk/pride/archive/projects/PXD025807

2. Experimental Design, Materials and Methods

2.1. Sample Preparation

The same protocol as described in [3–9] was used. Stallion ejaculates were washed in PBS (600gx 10'), then the pellet formed by the spermatozoa was frozen immediately in liquid nitrogen and kept frozen at -80 °C. Once the pellets were thawed the absence of debris and potential contaminating cells was assessed using phase contrast microscopy. For the analysis, proteins were extracted from 200 $\times$ 10^6 spermatozoa. The protein extraction was initiated with the solubilization of the sperm proteins in the lysis buffer which composition follows, (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7M urea, 2M thiourea and 40 mM Tris (pH 10.4) as described in [1]. For the quantification of proteins we used the 2-D Quant Kit (GE Healthcare, Sevilla Spain) as indicated by the manufacturer, to normalize samples to 100 μg of protein. In the next step 200 µL of the solution obtained containing 100 μg of protein was mixed with 100 μl of 25 mM ammonium bicarbonate buffer pH 8.5 (100 μg of protein in 300 μL of solution). Then, proteins were reduced by adding 30 µL of 10 mM DTT and incubated at 56 °C for 20 min. In the next step, proteins were alkylated by adding 30 µL of 20 mM IAA solution, and incubated at room temperature in the dark for 30 min. The last step was digestion of the proteins, adding 2 μL of Trypsin Proteomics Grade (Sigma) (Trypsin solution: 1 g/L in 1 mM HCl) and incubating for at least 3 h at 37 °C. After incubation, the reaction was stopped with 10 μL of 0.1% formic acid, and the peptide solution was filtered through 0.2 μm (hydrophilic PTFE) into 2 mL dark glass vials. Finally, the protein samples were dried using a nitrogen current with the vial in a heating block at 35 °C. The dried samples were diluted in 20 μl of buffer A, (water/acetonitrile/formic acid (94.9:5:0.1)).

2.2. UHPLC-MS/MS Analysis

The separation and analysis were conducted in an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High Speed Binary Pump, coupled to a Mass Spectrometer (Agilent 6550 Q-TOF, Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface, controlled by the MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01), following previously published protocols [2,4,5,9]. The digested proteins were injected onto an Agilent Advance Bio Peptide Mapping HPLC column (2.7 μm, 150 $\times$ 2.1 mm, Agilent technologies), at 55 °C, at a flow rate of 0.4 ml/min. The gradient program was initiated with 2% of buffer B (water/acetonitrile/formic acid, 10:89.9:0.1) remaining in isocratic mode for 5 min, after this time, linearly was increased up to 45 % B for 40 min, and up to 95 % B over additional 15 min and finally remained constant for 5 min. After this initial 70 min run, 5 min followed, for the conditioning of the column for the next run using the initial condition. The mass spectrometer operated in positive mode. The nebulizer gas pressure was set to 35 psi, the drying gas flow was set to 10 l/min at a temperature of 250 °C, and the sheath gas flow was set to 12 l/min at 300 °C. The voltages of the capillary spray, fragmentor and octopole RF Vpp were, respectively, 3500 V, 340 V and 750 V. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass ranges were 50–1700 m/z and scan rates were 8 spectra/sec for MS and 3 spectra/sec for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of -4.8. The same ion was rejected after two consecutive scans.

2.3. Data Processing

Data were analyzed using the Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) as described in previous publications from our laboratory [5,6]. The raw data were extracted under default conditions: selection of non fixed or variable modifications; [MH]+ 50–10,000 m/z; maximum precursor charge +5; retention time and m/z tolerance ± 60 s; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against the updated protein database (Uniprot/Horse) was done as follows: non fixed modifications were selected and as variable modifications carbamidomethylated cysteines, tryptic digestion with 5 maximum missed cleavages, ESI-Q-TOF instrument, minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm and calculation of reversed database scores. The auto-validation strategy used was auto-threshold, in which the peptide score is automatically optimized for a target % FDR (1.2%), and immediately protein polishing validation is then performed to increase the sequence coverage of validated results with the restriction of a new maximum target protein FDR (0 %).

3. Ethics Statement

Stallions were maintained following specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). The ethical committee of the University approved this study REF IB-20008.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

Data Availability

Stallion sperm proteins in 10 stallions with different motility (Original data) (ProteomeXchange).

CRediT Author Statement

Gemma Gaitskell-Phillips: Methodology, Writing – review & editing; **Francisco E. Martín-Cano:** Methodology; **José M Ortiz-Rodríguez:** Methodology; **Antonio Silva-Rodríguez:** Methodology; **Eva da Silva-Álvarez:** Methodology; **Maria C. Gil:** Supervision, Formal analysis; **Cristina Ortega-Ferrusola:** Supervision, Formal analysis; **Fernando J. Peña:** Formal analysis, Funding acquisition, Writing – review & editing, Supervision, Validation.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dib.2022.108578.

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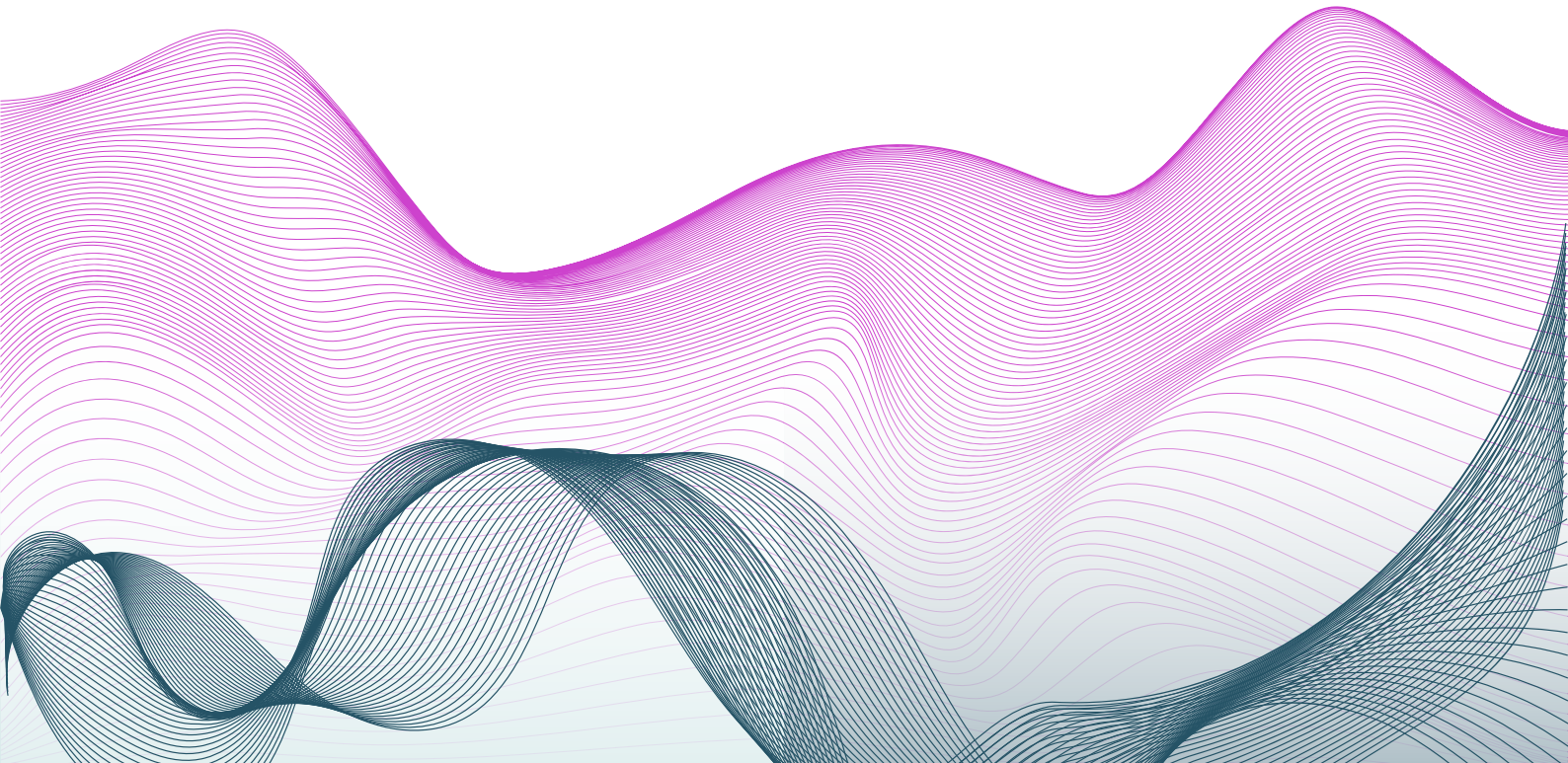
D I S C U S S I O N



In this thesis fresh and frozen thawed samples of equine spermatozoa and seminal plasma were analysed using mass spectrometry proteomic analysis, specifically shot-gun proteomics. In fresh samples of spermatozoa, a total of 903 proteins were identified and in frozen thawed samples a total of 3116 proteins were found to be present. Meanwhile through seminal plasma analysis, 3544 proteins were identified. Quality of spermatozoa samples was classified for identification of discriminant variables using established methods such as sperm motility analysis and flow cytometry, to create classifications and identify direct associations between the presence or absence of specific proteins and sperm characteristics. In addition, how the seminal plasma proteome influences the need for centrifugation of samples to maintain quality during cooling for short-term storage was studied. A bottom-up approach was used, with bioinformatics, gene ontology and network analysis, categorizing proteins depending on their biological and molecular functions.

All analyses were performed using semen samples obtained from 10 stallions routinely used at stud during the breeding season, with initial motility parameters considered to be within normal reference ranges according to current clinical guidelines (203,204). Ejaculates from the stallions included in the study were analysed immediately and baseline quality parameters for motility recorded. Seminal plasma was separated from spermatozoa by centrifugation immediately after semen extraction and seminal plasma samples were processed appropriately for proteomic analysis and stored at -80°C until further analysis was carried out. The same ejaculates were then used to prepare spermatozoa samples for the other experimental conditions analysed, including cooled extended samples, cooled centrifuged extended samples and cryopreserved samples of spermatozoa for analysis post thaw.

Seminal plasma proteins are predictive of the outcome of cooling spermatozoa

Proteomic analysis of seminal plasma led to identification of several proteins with potential for use as biomarkers. Specific proteins present in seminal plasma were linked to the tolerance of ejaculates to withstand cooling at 5°C for 48 hours, as well as predictors for sperm motility and velocities in both fresh and thawed semen. Findings support a significant role of seminal plasma in sperm functionality.

In the case of tolerance to refrigeration ejaculates were classified into three groups, according to motility after 48 hours of storage at 5°C. These included samples where quality remained good, after dilution with a commercial extender only (good group), those where quality remained good, but only after centrifugation was performed to remove seminal plasma (good RSP) and samples where quality was poor (poor), independent of the treatment of the sample.

Samples which were classified as poor and did not tolerate cooling were found to have numerous proteins which were less abundant than good and good RSP groups. Proteins found to be less abundant in poor samples were linked to redox regulation and antioxidant activity, including **catalase, peroxiredoxin 6, superoxide dismutase (SOD)** and **heat shock protein-90** (HSP-90) (205). Some of which have been reported in previous studies, such as superoxide dismutase and glutathione peroxidases (206,207). More recently SOD-3 found in seminal plasma has also been linked to a possible mechanism for sperm selection and clearance in the mare reproductive tract (208). The relative lack of abundance of **HSP-90** is of interest because it is activated by changes in redox regulation when reactive electrophilic aldehydes are generated due to the loss of redox homeostasis (209). All the above point to ineffective redox regulation being the cause of lack of tolerance of these ejaculates to cooling. In addition, other proteins linked to cell metabolism such as **oxoglutarate dehydrogenase, glyceraldehyde 3 phosphate**

dehydrogenase and proteins like phosphoinositide 3-kinase (PI3K) involved in cell viability were also reduced in stallions from the poor group.

The poor group was also found to lack glucagon receptors, which is of significance and has also recently been described in human sperm cells (210). This study found that the protein kinase inhibitor H89 reversed glucagon fuelled phosphatidylinositol-3-kinase (PI3K)/AKT signalling, indicating that glucagon signalling is dependent on protein kinase A, whilst a stimulatory action of glucagon on lactate dehydrogenase and glucose-6-phosphate dehydrogenase was also observed. Another study found that glucagon-like peptide-1 receptor agonists (GLP1-RAs) improved sperm metabolism and motility in vitro in diabetic and/or obese patients (211).

In the good group which tolerated cooling over 48 hours at 5°C without significant loss in sperm quality the protein peroxiredoxin 6 was most abundant, in addition to catalase, however the later was not found to be significantly different from the good RSP group. ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1), a protein known to play a role in post-transcriptional modification of proteins was also found to be highly enriched in the good group of stallions. This gene has previously been linked to duration of sperm storage in chicken oviducts (212) and has also been identified as being expressed significantly differently in pigs and downregulated in the endometrium when insemination was performed using a full ejaculate compared to a control group with only Beltsville Thawing Solution, a protein free extender (213). This result is unsurprising given that correct functioning of spermatozoa is heavily dependent on post transcriptional modifications.

Finally, comparison of results from stallions from the good group of chillers and the good RSP group led to the identification of proteins which are worthy candidates for biomarkers. Annexin A2 in particular was highly enriched in stallions which required the removal of seminal plasma (good RSP), and barely detectable in good chillers. Annexins, a family of membrane phospholipid-binding proteins have multiple functions in cells. These include cell proliferation, differentiation, apoptosis, migration, membrane repair and inflammatory response amongst others (214). In humans, expression of certain types of annexins seem to be linked to DNA fragmentation (215) as well as to the fertilisation process in normozoospermic men with unexplained infertility (216).

Even though annexin A2 appears to be a promising marker for differentiation of samples which require seminal plasma removal to meet sperm quality parameters after cooling for 48 hours the molecular basis behind this is yet to be fully understood. Possible mechanisms providing an explanation for the poor quality in these samples when seminal plasma is not removed (good RSP group) include increased inflammatory responses (217) and accelerated apoptosis (218,219). Network analysis supported the possible theory that annexins in the stallion stimulate ROS production, a mechanism involved in these responses (217) with interactions shown with other proteins related to cell survival and immune regulation. In addition, annexin A2 has been identified as being present in the oviduct in several species, including the human, pig, cow, cat, dog and rabbit and has been linked to sperm oviduct interaction and binding (220–222). It is therefore plausible that higher levels of this protein in seminal plasma could therefore lead to acceleration of processes normally taking place in the oviduct, leading to increased senescence in these samples (221,223).

Enrichment analysis identified proteins in seminal plasma with essential roles in hexose metabolism, extracellular vesicles, and responses to hydrogen peroxide, with enrichment of all of these terms. Both hexose metabolism and extracellular vesicles are linked, and act as a support system for spermatozoa.

Prostasomes, a particular type of extracellular vesicle which is the most prevalent in equine seminal plasma (85), are responsible for expression of glycolytic enzymes which possess ATP production capacity (85,224,225). The abundance of seminal plasma proteins in carbohydrate metabolism has also been linked to stallion fertility (108) and the proteins needed for execution of these metabolic pathways are present in equine prostasomes (85), which corresponds with enrichment findings forming part of this thesis.

Another term which was found to be enriched was the response to stress and hydrogen peroxide. The key antioxidant catalase has previously been reported as being present in stallion seminal plasma and is principally derived from prostatic secretions. Catalase is able to scavenge H_2O_2 and modulates how susceptible spermatozoa are to oxidative stress (226). Other terms which were significantly enriched in relation to redox regulation included nicotinamide adenine dinucleotide regeneration. The final group of enriched terms were related to post-transcriptional modifications in proteins, in particular SUMOylation. This is significant as spermatozoa are translationally and transcriptionally silent cells and rely heavily on post-translational modifications for their regulation (100,227), suggesting an important role in terms of seminal plasma involvement in sperm functionality. This was backed up further by functional network analysis, which revealed a highly interconnected seminal plasma protein network, including pertinent functions such as regulation of ovulation, transforming growth factor beta (TGF-β) and mitochondrial membrane permeability. It has previously been suggested that ovulation induction factors are present in equine seminal plasma (228), an aspect which in needs additional study.

Seminal plasma proteins can be used as biomarkers for motility in fresh spermatozoa

Seminal plasma proteins were also identified as possible biomarkers for total motility percentages and velocities. Samples containing spermatozoa with lower percentages of total motility, curvilinear velocity (VCL) and average path velocity (VAP) were associated with the presence of specific proteins. Those with higher parameters for straight line velocity (VSL) were also linked to the presence of two specific proteins.

One of the proteins identified as a discriminatory protein in seminal plasma in poor quality samples was fructose bisphosphate aldolase. This protein acts as a catalyst in the reversible reaction splitting fructose 1,6 bisphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GP3); intermediate metabolites in glycolysis, where phosphate groups are subsequently eliminated in a further irreversible reaction. A by-product of this elimination of phosphate groups is methylglyoxal, a strongly electrophilic 2-oxoaldehyde with carbonyl groups that spontaneously react with nucleophiles, in lipids and DNA to form advanced glycation end (AGE) products (136). AGE products may be cytotoxic and mutagenic but are also involved in regulatory functions. Methylglyoxal can form adducts with superoxide dismutase 1 (SOD1), which has recently been identified in publications forming part of this thesis (21,229) as one of the most significant antioxidant systems in spermatozoa. Consequently, these adducts limit the antioxidant capacity of the enzyme and therefore promote oxidative stress (230). In addition, the extremely toxic nature of methylglyoxal in equine spermatozoa has also recently been described (3). High glucose extenders, such as those predominantly used in a commercial setting can therefore cause production of large amounts of methylglyoxal, leading to sperm malfunction, such as reductions in sperm motility and velocities (3), explaining findings linking high levels of this enzyme with poor total motility, VCL and VAP.

Good VSL in equine spermatozoa was linked to the presence of glutathione peroxidase and triosephosphatase isomerase in seminal plasma. Although these proteins have not previously been identified in equine seminal plasma, BLAST analysis for glutathione peroxidase revealed that the proteins were 89% homologous to epididymal secretory glutathione peroxidase in *Sus scrofa*, 85.5% homologous to the same protein in *Canis lupus familiaris* and 79.3% in *Homo sapiens*. Glutathione peroxidase is responsible for catalysing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide and constitutes a glutathione peroxidase-like protective system to combat peroxide damage in membrane lipids in the spermatozoa (231,232). An unsurprising finding, that a protein with a role in lipid peroxide detoxification is linked to improved sperm kinematics. It is well recognised that spermatozoa require tight redox regulation, and that a loss of this equilibrium can promptly lead to malfunction (100). The relationship of this finding may suggest different mechanisms for regulation of specific aspects of sperm kinematics, with motility, VCL and VAP having a similar mechanism for regulation, and with different mechanisms involved in the regulation of VSL. These findings are of particular relevance in a clinical setting as they can be rapidly applied as biomarkers of sperm functionality.

Seminal plasma proteins can be used to predict motility of spermatozoa post thaw

The next logical step in the analysis of seminal plasma proteins was the analysis of the seminal plasma proteome in relation to sperm quality post thaw. Motility parameters such as total and linear motility and VCL were used in addition to percentages of viable sperm populations and those showing high mitochondrial membrane potential defined using flow cytometry to provide baselines for classification of sperm quality. Findings revealed significant enrichment of three proteins in seminal plasma of stallions with better motility parameters post thaw. These were peptidyl arginine deiminase 2 (PAD2), rRNA adenine N (6)-methyltransferase and KIAA0825. In addition, other proteins were seen in this same group of stallions, including Rho guanine nucleotide exchange factor 28, endoplasmatic reticulum protein 44 and two uncharacterised proteins (F6SCY and A0A3Q2HPE3) which corresponded to the RAPGEF6 gene, with guanyl nucleotide exchange factor activity as molecular function which is necessary for small GTPase mediated signal transduction. The second protein corresponded with the ARHGEF18 gene, which also has guanyl-nucleotide exchange factor activity and participates in small GTPase mediated signal transduction processes.

PAD2 is a calcium dependent enzyme responsible for the catalysation of the deimination of arginine residues from proteins, forming citrulline as a result of citrullination, a little understood post translational modification, which has been linked to epigenetic regulation, immunity, inflammation and transcription regulation as well as cancer in human and mouse models (233–236). It is likely that PAD2 plays a role in fertility, as studies have found that the human female knockout phenotype for the gene coding for a similar protein (PADI6) is sterile due to a cleavage failure in fertilised eggs (237,238). There is a possibility that this protein is vehiculated to the oocyte by the spermatozoa, and this concept would be an interesting area of further research given there are a set of embryo proteins which are exclusively of paternal origin (97). Rho guanine nucleotide exchange factor 28 belongs to the family of guanine nucleotide exchange factors, a family of proteins which regulate differentiation, proliferation and morphogenesis, playing essential regulatory roles in embryo development (239).

Although the protein KIAA0825 is linked to better total and linear motility post thaw, and is of unknown function in the equine, the murine ortholog is known to be expressed during limb development, in addition to variants of the gene connected to recessive non-syndromic post axial polydactyly type A in humans (240–242). Other proteins which were linked to better post thaw motility included rRNA adenine N(6)-methyltransferase, a protein involved in rRNA methylation, coded for by the transcription factor B1 mitochondrial (TFB1M) gene. Knockouts for this gene show changes in mitochondrial function, reduced ATP production and increased ROS levels in response to cellular stress (243), as well as reduced expression of mitochondrial OXPHOS components (244), a possible explanation for the detection of higher levels of this protein and improved post thaw motilities.

The uncharacterised protein A0A3Q2IAZ9 which corresponds to the supercomplex assembly factor 1 (SCAF1) gene was also more abundant in seminal plasma in stallions with higher percentages of linear motility post thaw. The SCAF1 gene codes a protein with RNA polymerase II C-terminal domain binding, to which potential early embryonic development functions could possibly be linked, given the recent discoveries regarding the importance of vehiculation of paternal proteins in sperm and seminal plasma (97). In addition, other studies have shown that it may be linked to efficient OXPHOS modulation and optimisation of metabolic resources in zebrafish (245) as well as being essential for interaction between complex II and complex IV in its long isoform (246).

Proteins indicating better overall performance post thaw were also identified in an aim to define discriminatory proteins for classification of stallions, according to post thaw sperm quality. Four seminal plasma proteins were identified as being good discriminants for overall motility post thaw and these included peptidyl arginine deiminase 2, rRNA adenine N(6)-methyltransferase, KIAAA0825 and an additional uncharacterised protein corresponding with the SCAR-1 gene, in addition to two other proteins from the same group consisting of an ATR serine/threonine kinase and the solute carrier family 25 member 37 (SLC25A37).

The presence of single stranded DNA activates ATR serine/threonine kinase (247), which takes part in DNA repair and plays an important role in meiosis in the male germinal epithelium (248), it is probable that this is the link between the presence of higher levels of this protein in seminal plasma and better sperm quality post thaw, due to an increased resistance to the stress caused by cryopreservation. The SLC25A37 gene encodes the mitochondrial metal transporter mitoferrin 1, which plays an essential role in mitochondrial iron homeostasis, in addition to the functionality of proteins involved in OXPHOS (249), most likely linking this protein with better mitochondrial functionality, and as a result cryoresistance.

Despite the limited time that seminal plasma proteins are in contact with spermatozoa during processing of equine semen, it is becoming increasingly clear that they play an essential role in sperm functionality. It is likely that many of these proteins are vehiculated through extracellular vesicles that bind to sperm membranes (51,250) and may subsequently be incorporated, participating in sperm physiology. With further research many of the discriminant biomarkers identified in seminal plasma can be applied in clinical practice to improve results and quality when storing spermatozoa, as well as predicting the outcomes of ARTs.

Sperm proteins involved in mitochondrial metabolic functions and fertilisation are predominant in stallions with better motility in fresh spermatozoa

Unsurprisingly, given the proteins which have been identified linking sperm quality with proteins present in seminal plasma, the proteome of the stallion spermatozoa in fresh samples from normal ejaculates was also shown to clearly influence sperm motility. Proteins which are discriminant variables of good and poor sperm motility and velocity were identified. The relationship linking motility and the sperm proteome has previously been reported, but the majority of studies have been performed in humans comparing asthenospermic and normospermic samples (95,251–255) or good versus low quality spermatozoa after colloidal centrifugation for recovery of high-quality spermatozoa (15,16,95).

Discriminant markers identified for motility and velocity parameters showed a high degree of specificity. These potentially represent interesting findings in relation to movement regulation of spermatozoa and the measurements obtained using computer assisted sperm analysis (CASA) and molecular biology. In total 903 proteins were identified in fresh stallion spermatozoa. Of these 24 were found to be connected to the percentage of total motility in a given sample. These proteins were analysed using gene ontology (GO) and this revealed enrichment of terms including single fertilisation and fertilisation, linking motility and fertility in the stallion at a molecular level as well as correlating with findings from studies in a clinical setting (68,256). Other GO and reactome pathways showed prevalence of mitochondrial proteins and mitochondrial metabolic pathways, the TCA cycle and OXPHOS, with significant roles in pyruvate metabolism. These results support current thinking regarding OXPHOS as the predominant metabolic pathway responsible for ATP production for motility in the stallion spermatozoa (120,125,127,138,257).

Proteins identified as discriminant for good motility included hexokinase-1, one of the multiple isoforms of the protein responsible for phosphorylating glucose and other hexoses in the first step of glycolysis, which, in the past has been identified as a marker of good motility in stallion spermatozoa (15). It is located in the outer mitochondrial membrane, and it is likely that increased glycolytic flux accumulation of glucose-6-phosphate may inhibit its activity, leading to an increase in production of mitochondrial ROS, loss of redox homeostasis regulation and activation of the mitochondrial apoptotic pathway (258). It is possible that this mechanism also exists in spermatozoa, as increased ROS production has been linked to high glucose concentrations in storage extenders (3).

Aconitase hydratase mitochondrial was also more abundant in stallion spermatozoa with better motility. This protein is responsible for catalysis of the conversion of citrate to isocitrate via cis-aconitate and its identification underpins the importance of mitochondrial ATP production for sperm motility and reinforces previous research (120,121,127,259). Other proteins identified as being more abundant in stallions with better motility included f actin-capping protein subunit alpha, phosphoinositide phospholipase C and elongation factor Thermo unstable (Ef-Tu). To the contrary, mannosidase alpha 2 class 2C member 1 and angiotensin 1 converting enzyme were discriminant proteins in stallions with lower percentages, although still within normal ranges, of total motility in fresh spermatozoa.

Discriminant proteins were also identified for poor linear motility in fresh spermatozoa. In these samples cytochrome C and heat shock 70 kd protein 9B (fragment) were much more abundant. The higher levels of cytochrome C present in these samples reinforce the role of apoptotic mechanisms (5,206,218,260–265) in sperm damage, which in addition to ferroptosis (266) have recently come to be considered key causes of sperm malfunction.

In addition, protein composition of fresh spermatozoa majorly influenced sperm velocities. VCL and VAP appeared to have a similar profile, and VSL emerged as having a very specific profile of differentially predominant proteins. Enrichment analysis of proteins differentially expressed in relation to VCL and VAP showed enriched annotations of both GO terms and KEGG pathways which were related to mitochondria and mitochondrial metabolism, OXPHOS and the TCA cycle. These findings are of interest as they imply that mitochondrial metabolism is responsible for production of energy responsible for sperm velocities. This goes against the idea that glycolysis plays a key role in supporting sperm velocities, a concept which arose from research which observed a drop in sperm velocities due to inhibition of glycolysis with 2-deoxyglucose (2-DG), the non-metabolizable analogue. A plausible explanation for this finding is a result of ATP depletion due to futile phosphorylation of 2-DG by hexokinase, as incubation of stallion spermatozoa in a glucose free media does not affect sperm velocities if other necessary substrates for the TCA cycle are provided (127).

ATP synthase subunit alpha, aconitase hydratase mitochondrial and phosphoinositide phospholipase C were identified as discriminant variables for fresh spermatozoa with good VCL and VAP. In addition to these proteins ADT/ATP translocase 2-like protein was enriched. Regarding VSL, differential proteins present were connected to GO terms unfolded protein binding and sperm capacitation. Poor VSL in fresh spermatozoa was linked to heat shock protein HSP-90 alpha, chaperonin containing TCP1 subunit 6A and seminal plasma protein 1. A number of proteins identified were also validated using western blotting, including ATP synthase, HSP90, HSP70 and cytochrome C, all of which reinforce the participation of mitochondria in sperm energetics (3) and apoptotic processes in sperm malfunction (5).

As anticipated due to the type of breeding selection that takes place in equines (17), the differences between individuals were found to be much more significant than those between breeds, despite the fact that the stallions used in the study were of genetic proximity, including, predominantly Iberian breeds (Andalusian, Lusitano and Spanish Sport Horse) and Arabs, in addition to crosses between these breeds, which reflect the type of horse most in demand in the geographical area around the Veterinary Teaching Hospital at the University of Extremadura in Cáceres, Spain.

Overall, findings regarding the proteome of fresh spermatozoa in relation with motility and velocities are supportive of the theory that stallion spermatozoa predominantly depend on mitochondrial metabolism to sustain these parameters, but also provide interesting new insights behind the utility of different CASA parameters and their connections with molecular mechanisms. Findings suggest that lower VSL values are linked to capacitation like changes, with HSP90 (267) found to be more abundant in fresh spermatozoa from stallions with lower VSL. Further backing up this finding was the enrichment of the GO term sperm capacitation in these stallions.

Proteins in spermatozoa most significantly reduced by cryopreservation

Proteomic analysis was also performed on spermatozoa post thaw in an aim to better understand the mechanisms responsible for decreased sperm quality and death due to cryopreservation and for identification of proteins and their potential for use as additional discriminate biomarkers. It is well recognised that the process of cryopreservation exposes spermatozoa to significant insult, including osmotic stress during both freezing and thawing, in addition to exposure to toxic cryoprotectants used in cryopreservation media. These stresses mean that even with optimised cryopreservation protocols

it is common for half or more of spermatozoa processed in this way to be lost to osmotic induced necrosis, especially during thawing (268,269). Spermatozoa which survive also undergo changes as a result of osmotic stress in the mitochondria, with increased production of ROS, depletion of antioxidant reserves and ultimately oxidative stress triggering accelerated senescence and eventually premature death (154).

As also seen in previous research published in other species significant changes in the stallion sperm proteome were identified as a result of cryopreservation (14,270–273). The gene ontology terms which were enriched in proteins present in the greatest amounts in fresh spermatozoa echoed the significance of metabolism and redox reactions, as well as the already well recognised importance of mitochondria in spermatozoa. However, in frozen thawed spermatozoa only the KEGG pathway for RNA degradation was enriched. Other proteins which experienced highly significant changes due to cryopreservation were evaluated and are valuable discriminant markers for the major consequences caused in spermatozoa by this commonly used technique.

A highly significant decrease in the amount of superoxide dismutase (Cu-Zn) (SOD1) was detected in spermatozoa after cryopreservation. This enzyme constitutes a front-line defence against oxidative stress in the majority of organisms (274–276), and although previous studies have reported that it is affected by cryopreservation (14,277), this was the first time it was identified using bioinformatic analysis as a discriminant variable, constituting one of the most highly significantly different proteins between fresh and frozen-thawed spermatozoa. A finding strongly supporting the theory that changes in redox regulation and oxidative stress are key components in cryodamage. The increased levels of 4-hydroxynonenal (4-HNE) detected in cryopreserved spermatozoa corroborate this finding.

In addition to SOD1, other proteins with oxidoreductase activity were also identified as discriminant variables for frozen thawed spermatozoa. These included aldoketoreductase family 1 member b (AKR1B1), which has functions of special relevance in the context of sperm biotechnologies, including catalysis of NADPH-dependent reduction of carbonyls, detoxification of lipid derived unsaturated carbonyls, such as crotonaldehyde, 4-HNE, trans-2-hexenal, trans-2,4-hexadienal and glutathione conjugates of carbonyls (GS-carbonyls) as well as catalysing the reduction of various phospholipid aldehydes such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphoethanolamin (POVPC) and related phospholipid aldehydes generated from the oxidation of phosphatidylcholine and phosphatidylethanolamines (278).

In the context of the spermatozoa, the role of this enzyme in detoxification of 4-HNE, which has been found to be extremely toxic to spermatozoa is of interest (279) with other studies demonstrating that cryopreservation causes significant increases in the 4-HNE levels found in spermatozoa (280), which was also evident in the results of the present thesis. This provides a plausible molecular explanation for much of the molecular damage which occurs as a consequence of cryopreservation, with reductions in the amounts of key antioxidant proteins. With this in mind, it seems a logical assumption that spermatozoa containing greater amounts of AKR1B1 may have a better survival rate after cryopreservation.

In fresh spermatozoa samples several proteins were detected as being more abundant. Many of these provide feasible explanations for the drop in fertility experienced after cryopreservation. Higher levels of lysozyme B (LYZLB) were detected in spermatozoa in fresh samples. Geneontology.org links this protein to the following three biological processes, including defence response to Gram negative and

positive bacterium and fusion of sperm to egg plasma membrane involved in single fertilization (281), providing another explanation for the reduced fertility commonly seen with cryopreserved spermatozoa (282). A serine/threonine-protein phosphatase was also more abundant in fresh sperm. BLAST analysis disclosed that this equine protein was 100% homologous with the human serine/threonine-protein phosphatase PP1 – alpha catalytic subunit, an essential protein for spermatogenesis, motility, and normal morphology in spermatozoa (283–285).

The reduced amounts of numerous proteins in spermatozoa post-cryopreservation can be explained by the well-recognised and studied effects that are caused by freezing and thawing, with reductions in antioxidant proteins explained by exhaustion due to the oxidative stress which the technique causes (100), and reductions in other proteins as a result of protein degradation due to the osmotic stress to which spermatozoa are exposed (194,271,273,286).

To the contrary a highly significant increase was found in another 12 proteins in thawed samples of spermatozoa, two of which were significantly enriched. One of these proteins was transmembrane protein like 5 with increased levels likely attributable to the intense stress that the plasma membrane of the spermatozoa is exposed to during cryopreservation (269,287). Increases in the amounts of the olfactory receptor were also noteworthy. The olfactory receptor is a membrane protein, which is part of the class A rhodopsin-like family of G protein-coupled receptors (288,289), with staining patterns used for detection suggesting it mediates chemoreceptive mechanisms to help control motility and facilitate the acrosomal reaction (290). Increases in this membrane receptor are likely caused by the significant disruption of the sperm membrane during the process of cryopreservation.

The fact that spermatozoa are both translationally and transcriptionally silent cells makes increases in amounts of certain proteins difficult to explain. It is possible that the stress caused by cryopreservation may have caused some proteins to be excluded from the membrane or proteins that have undergone post translational modifications to be excluded. Increases in the amounts of specific proteins after cryopreservation may possibly be caused by changes in secondary and tertiary structures of specific proteins (271), however this is an area which requires additional research.

Differences in the sperm proteome of fresh spermatozoa can explain variability in ejaculates post thaw

In addition to identification of significantly enriched or reduced protein levels in spermatozoa as a result of cryopreservation, differences in the sperm proteome can be used to explain variability post thaw. Good and poor outcomes after cryopreservation and their relationship with the sperm proteome were used to identify discriminatory variables. Factors used to differentiate post thaw outcomes included motility, viability, and mitochondrial membrane potential.

A particularly interesting finding was that sperm motility and velocities from fresh samples were not necessarily good predictors of post thaw motility, and unexpectedly linear motility in fresh samples was higher in stallions where outcomes of cryopreservation were poor. Remarkably, age did not seem to have a major impact on the outcome of cryopreservation either. However, there were obvious differences in the levels of proteins present in fresh samples of spermatozoa when comparing good and poor freezers and the impact of cryopreservation was different on the proteome of stallion spermatozoa in both groups.

Although there were similarities in some of the aspects that the impact that cryopreservation had on both groups, with reductions in levels of some proteins and increases in others, there were also specific proteins which were associated with ejaculates from stallions which have better motility post thaw. Six such proteins were present in fresh samples and three of these were **mitochondrial proteins** (K9K273, A0A3Q2I7V9 and F7CE45), reinforcing how imperative these organelles are for sperm function (120,291), in particular for the production of ATP for sperm motility via the OXPHOS pathway.

F7CE45, also known as **acetyl-CoA acetyltransferase 1**, is a catalyst in the last step of the mitochondrial beta oxidation pathway (292) as well as playing an important role in the metabolism of ketone bodies (293) with subsequent effects on motility (294). As discussed previously spermatozoa are able to produce energy for motility from the beta oxidation pathway (6,16), providing an explanation linking the presence in greater amounts of this protein in fresh samples and improved motility post thaw.

Another of the proteins present at higher levels in fresh spermatozoa was **F6YTG8 (alpha mannosidase)**, a protein which plays a part in catabolism of oligosaccharides (295), preventing their accumulation. In more recent research, it has been proposed that this protein may play a role in preventing mitochondrial dependent apoptosis (296). As a significant proportion of the damage incurred during cryopreservation involves mitochondrial apoptotic pathways (5,265) this function provides an explanation for this finding. In addition, a study in ovine semen, found a positive correlation between alpha mannosidase and a positive outcome after cryopreservation (297).

The protein **latherin** (F6YU15) was also detected for the first time in equine spermatozoa. This protein is present in equine saliva and sweat and is a powerful surfactant (298,299) and is the cause of the foam formed on horse's skin during intense exercise. To date it is unclear what the function of this protein is in spermatozoa, although inhibition of the growth of biofilms due to its antibacterial properties have been attributed to it (300), with a possible contribution to endometrial health; a possibility which warrants further research.

Four proteins were found to be discriminatory for predicting good mitochondrial membrane potential post thaw. These included **peroxiredoxin-like 6 protein**, a major antioxidant defence in spermatozoa (149,151) which was also detected in seminal plasma and linked to the ability of spermatozoa to tolerate cooling for short term storage, with higher levels linked to better quality after 48 hours storage at 5°C (301). Considering that many aspects of cryodamage are a result of oxidative stress (302,303), it is logical that samples richer in this antioxidant protein are more resistant to cryopreservation.

Stallions in the good group of freezers were also found to have a greater abundance of a glycolytic enzyme called **phosphoglycerate mutase** (PGAM), which is upregulated in many types of cancer cell (304). It catalyses conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) during glycolysis. When there is overexpression of this protein in cancer cells there is an increase in 2-PG and a decrease in 3-PG, as well as expression of higher levels of lactate and increased flux through the pentose phosphate pathway (304), resulting in production of more reducing power in the form of NADPH. This mechanism may provide an explanation for the improved survival of ejaculates with higher levels of PGAM after cryopreservation, although additional research is needed.

The protein **GRAM domain containing 1A**, a cholesterol transfer protein which plays a role in the early stages of autophagosome formation (305) was also more abundant in stallion spermatozoa with good mitochondrial membrane potential post thaw. Mitophagy has recently been connected with sperm quality (306), so the abundance of this protein in good freezers may be one of the explanations. The

final protein which was more abundant in good freezers was an uncharacterised protein similar to actin-1. There is a possibility that this protein is also connected to mitophagy as actin structures enclose damaged mitochondria during mitophagy (307), however additional research is required to fully characterise this protein and identify its role in spermatozoa.

Differences in abundances in proteins were also seen related to good and poor membrane integrity post thaw. There were 92 proteins common to both good and poor groups, and 64 related specifically to the good group with high viability and good membrane integrity post thaw, and 19 specific to the poor group showing low viability and poor membrane integrity post thaw. Again, bioinformatic analysis was successful in disclosing discriminant variables in fresh semen, which were predictors of viability post thaw. These proteins included the chaperonin containing TCP1 subunit 8 and testis expressed 101 which were more abundant in spermatozoa with higher membrane integrity post thaw. The chaperonin containing TCP1 complex plays a major part in the initial stages of fertilisation as a mediator of sperm-oocyte interaction (308–310). The testis expressed 101 protein is also involved in fertilisation, facilitating sperm binding to the zona pellucida, as well as being involved in migration of spermatozoa within the oviduct (311,312). These findings reinforce the need for thorough assessment of sperm membranes in the reproductive examination of stallions, as proteins with key, direct roles in fertilisation are present in spermatozoa in stallions with better viability post thaw.

Phosphoproteomics is a valuable technique, elucidating new mechanisms of cryodamage

Phosphorylation is one of the most important post translational modifications (183), causing modifications that play a vital role in regulation of processes vital for spermatozoa function, including fertilisation, motility, capacitation and the acrosome reaction (185). This thesis looked at the stallion spermatozoa phosphoproteome for the first time, revealing links between the presence of specific phosphoproteins to sperm functionality and the effects of cryopreservation on the phosphoproteome. Some of the changes which are evident offer a molecular explanation for the reduction in functionality of spermatozoa as a result of cryopreservation.

Findings from recent research using multiparametric flow cytometry and bioinformatics contradict a previous theory based on changes in intracellular Ca^{2+} which claimed that spermatozoa experience accelerated capacitation after thawing and die rapidly in the female reproductive tract (313,314). Increases in tyrosine phosphorylation after thawing are not evident and the number of spermatozoa which show a capacitation compatible phenotype is very low (5). As cryopreservation is known to cause major negative effects on sperm functionality changes in the stallion phosphoproteome were linked to changes in sperm quality.

SPATA18, also known as Mieap, a protein previously described in rat, mouse, boar, ram and human spermatozoa (315–319), was found to be significantly reduced in spermatozoa after cryopreservation. This protein is involved in mitochondrial maintenance and had not previously been described in the stallion. The protein detected showed a 79.9% homology with the human orthologue (Q8TC71), a product of the p53 gene which plays an essential role in mitochondria quality control and has previously been described (320,321). The detection of this protein further supports current thoughts on the pivotal role of the mitochondria in the stallion spermatozoa. As mentioned previously in relation to other findings in this thesis spermatozoa mitophagy has been described as a possible system for quality control (306) and the presence of the SPATA18 protein is yet another finding that supports this theory.

In addition, SPATA18 was detected using image flow cytometry and western blotting. It appears that phosphorylation of SPATA18 is necessary for its activation in the stallion spermatozoa. Once activated it induces accumulation of lysosomal proteins within the mitochondria as a response to mitochondrial damage and eliminates oxidised proteins to repair unhealthy mitochondria (320–323).

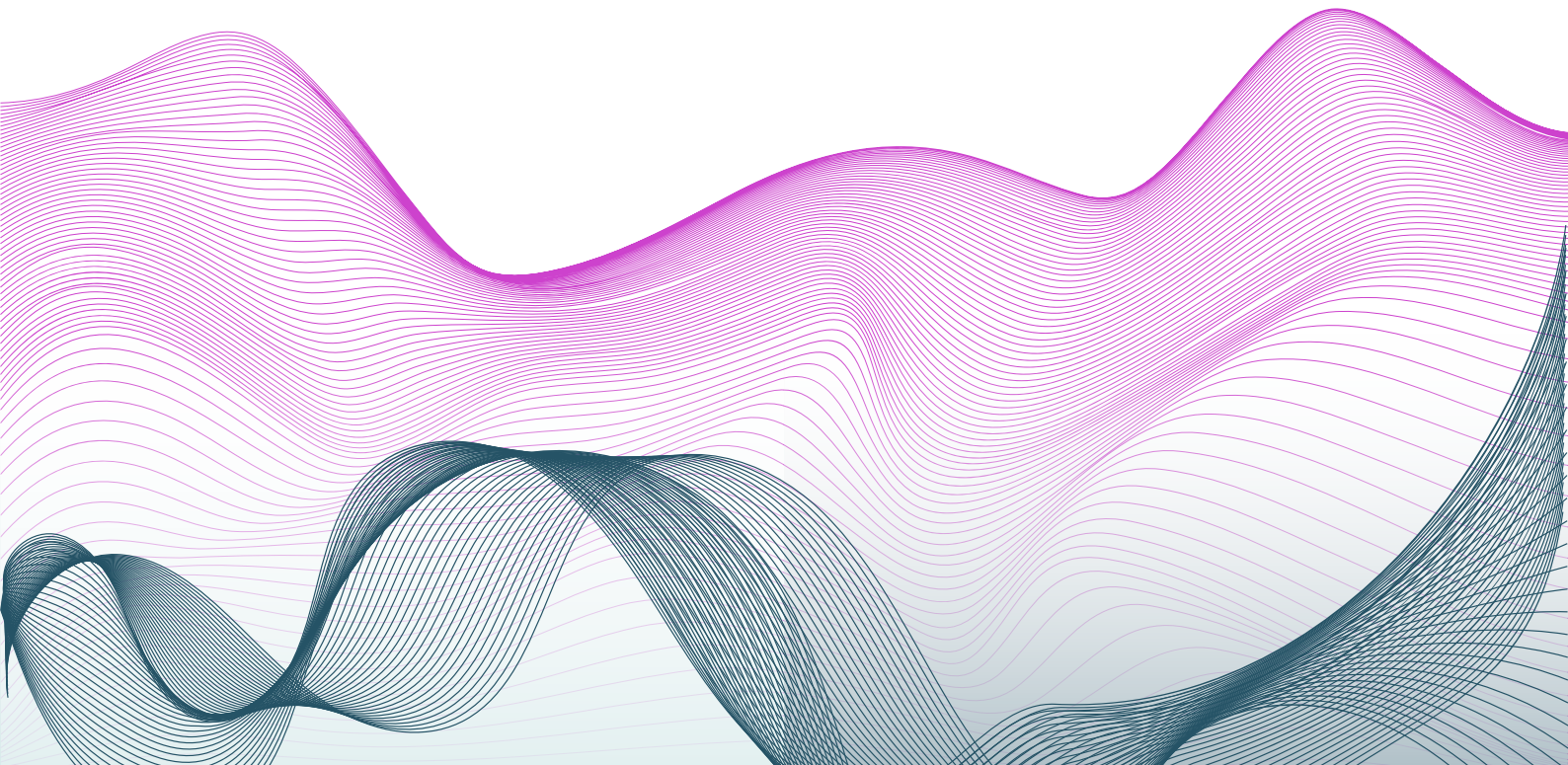
In the context of the spermatozoa, it is well recognised that cryopreservation causes excessive oxidative stress and that mitochondrial proteins are a preferred target for electrophilic aldehydes, such as 4-HNE originating from lipid peroxidation (209,324). It is highly likely that that this phosphoprotein becomes dephosphorylated as a result of the stress caused by cryopreservation, further adding to mitochondrial damage. *In silico* analysis for investigation of potential kinases regulating the phosphorylation of SPATA18 was used, and PKCß was identified as being responsible for its phosphorylation. When functional kinase assays were performed using PKCß inhibition these showed that the inhibition of the phosphorylation sites of this protein led to apoptotic changes in stallion spermatozoa, which are already well described (123,259,325,326). Results from PKCß inhibition further contributed to supporting the link between apoptotic changes and the desphosphorylation of SPATA18 during cryopreservation.

Two other phosphoproteins, the A-kinases anchoring proteins (AKAP) 3 and 4, with significant roles in sperm functions such as capacitation, the acrosome reaction and motility were significantly reduced after cryopreservation. These proteins, in particular AKAP4 have previously been proposed as potential biomarkers of sperm quality in stallions, with AKAP4 being identified as being highly susceptible to 4-HNE adduction (15). The changes observed further support the findings of this previous report, in addition to revealing a role as a biomarker for sperm quality post cryopreservation.

A decrease in the overall phosphorylation of Ca^{2+} binding tyrosine phosphorylation regulated (CABYR) was also observed because of cryopreservation. CABYR plays a key role in regulating capacitation and the acrosome reaction (327) and upon capacitation it is phosphorylated by tyrosine. This finding also goes against the above mentioned cryocapacitation theory. CABYR has also been linked to fibrous sheath integrity in mice and humans, as well as to progressive motility (328,329). The predicted kinases revealed that CABYR is regulated by GSK3ß, with the kinase inhibition assay supporting the role of this protein in stallion spermatozoa, with incubation in the presence of GSK3ß leading to increases in the percentages of apoptotic spermatozoa. Although the main role of this protein which has been discovered so far by researchers is capacitation, these findings suggest other significant roles in sperm functionality, similar to those which have recently been proposed in mouse spermatozoa (330). A recent report in human spermatozoa has shown that cryopreservation dephosphorylates the GSK3α isoform of this protein, a discovery which aligns with results of this thesis.

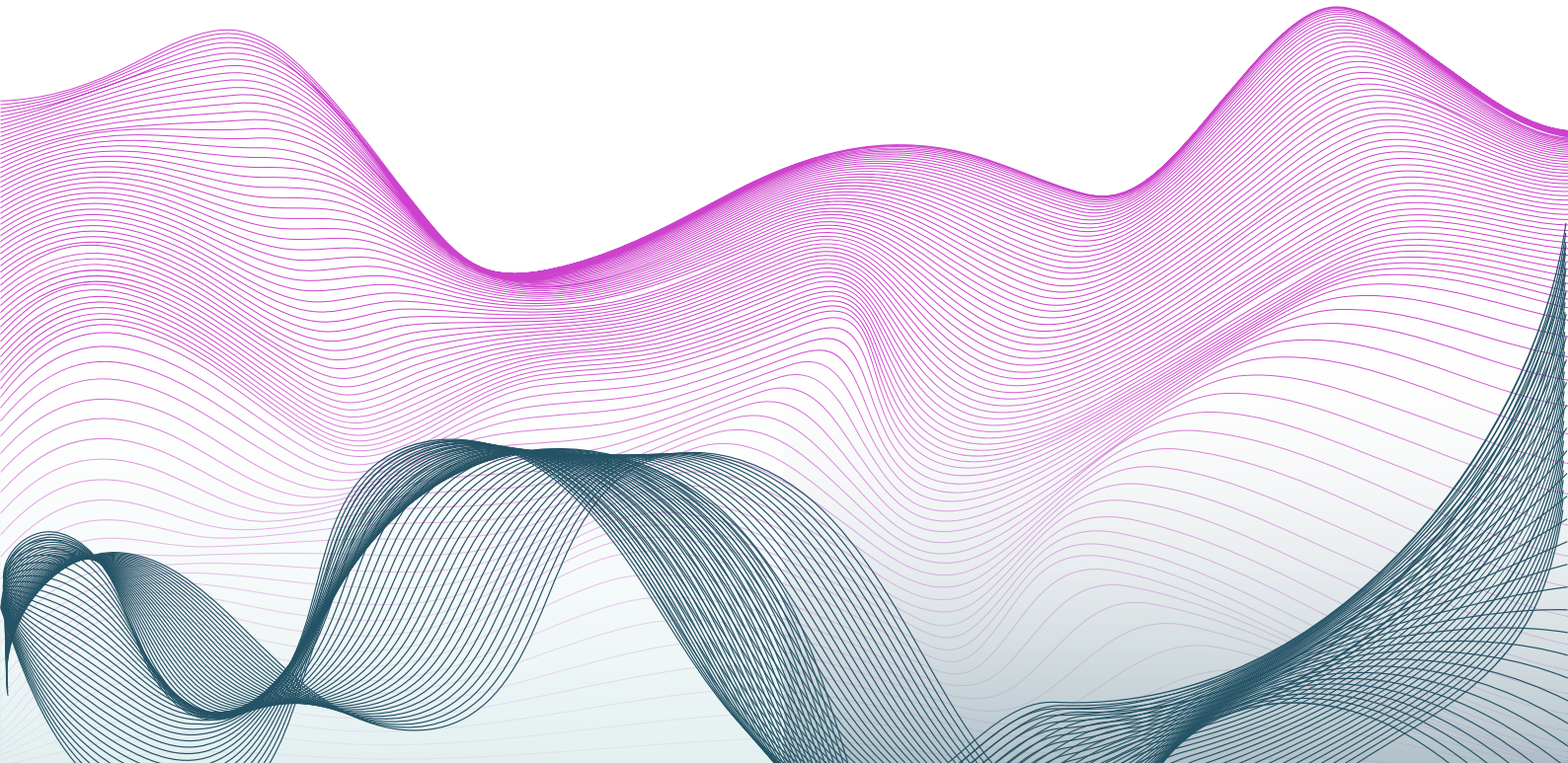
It is clear from findings presented in this thesis that there are many factors which play a part in the proteome of both seminal plasma and spermatozoa. Biotechnologies commonly used in equine reproduction have an undeniable effect on sperm quality, and the extent of this effect is largely influenced by individual variability and can be predicted by identification of the presence or absence or certain biomarker proteins. Identifying the differences in protein levels before and after application of biotechnologies and as predictors of sperm quality in fresh semen is an essential step forwards to further our understanding at a molecular level of how these technologies affect spermatozoa and the possible long-term consequences they may have. It is clear from the proteins identified that an optimal metabolism is key to maintaining good sperm function, with changes in levels of numerous proteins linked to processes such as redox regulation, antioxidant activity, ROS production, mitochondrial function, apoptosis, mitophagy and fertilisation and sperm binding to name a few, clearly correlating

C O N C L U S I O N S



1) Bioinformatic analysis of the equine seminal plasma proteome revealed major roles related to hexose metabolism, which may be mediated by extracellular vesicles. Seminal plasma also plays a major role in functionality, redox regulation and immune response regulation in spermatozoa.
2) Specific seminal plasma proteins can be used as discriminant markers for motility and velocity in addition to tolerance of cooling.
3) Seminal plasma proteins have potential to predict the outcome of cryopreservation in the stallion.
4) Mitochondrial proteins involved in the tricarboxylic acid cycle and oxidative phosphorylation are linked to higher percentages of total motility, VCL and VAP in stallion spermatozoa.
5) Cryopreservation causes major changes in the stallion sperm proteome. These changes provide a molecular explanation for the low fertility outcomes associated with the use of frozen thawed spermatozoa in equines.
6) Specific proteins in fresh samples of stallion spermatozoa can be used as discriminant variables to predict the response of ejaculates to cryopreservation.
7) Reduced motility in stallion spermatozoa after cryopreservation can be explained by changes to the phosphoproteome and damage to key proteins responsible for ensuring mitochondrial health.

A C K N O W L E D G E M E N T S



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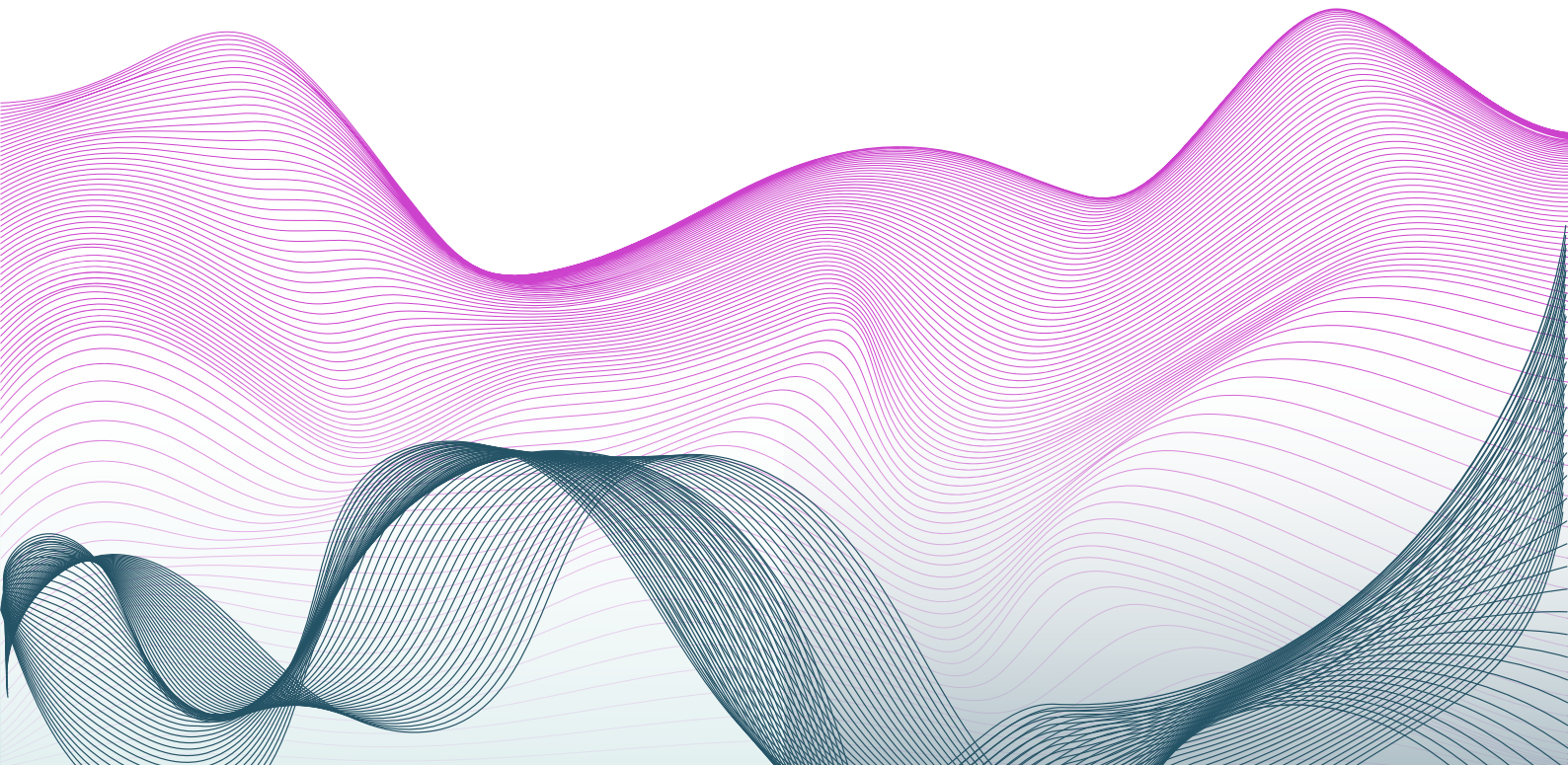
A N N E X E S



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196

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

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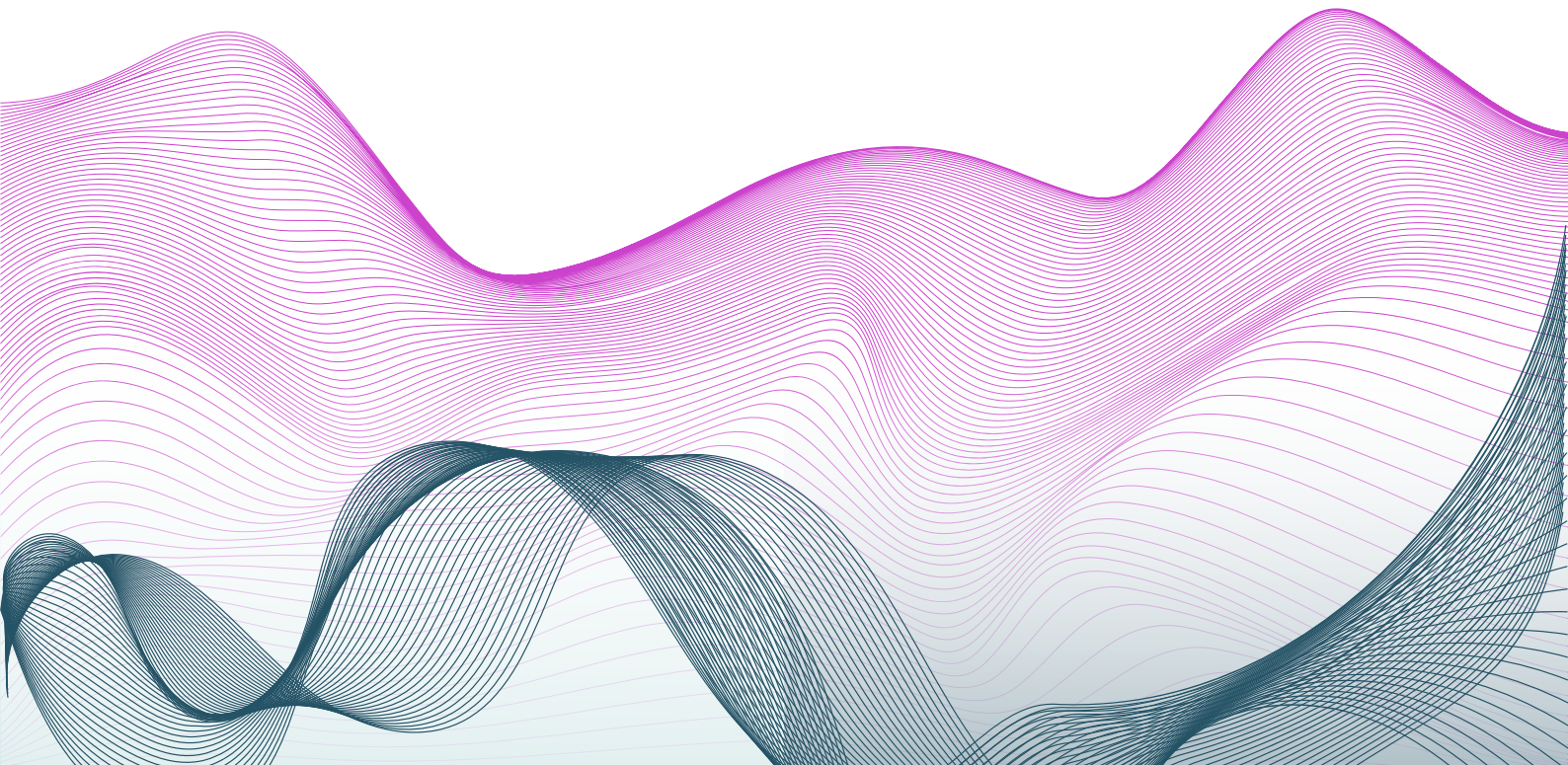

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