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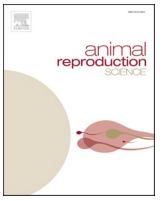

Analysis of stallion spermatozoa metabolism using Agilent Seahorse XFp Technology

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ABSTRACT

Sperm metabolism consists of a sophisticated network of biochemical reactions and varies between species, resulting in different metabolic strategies for ATP production to maintain sperm functionality. ATP can be produced through glycolysis or in the mitochondria by oxidative phosphorylation (OXPHOS). Since OXPHOS is the predominant metabolic pathway in horses spermatozoa, various assessments of mitochondrial activity are used to evaluate fertility, utilizing techniques such as fluorescent probes analysed via microscopy or flow cytometry, and polarographic electrode assays to measure current flow in response to an applied voltage. Though, these methods are limited by low throughput, as they assess mitochondrial activity at a single time point under a specific treatment condition. This study explores, for the first time, the application of the Agilent Seahorse XFp Technology to evaluate metabolism in stallion spermatozoa. This method enables real-time measurement of cellular metabolism across multiple samples or experimental conditions simultaneously. Ejaculates from eight different stallions were collected, and pools were prepared from three of them. Sperm viability and mitochondrial activity were evaluated by fluorescence microscopy, sperm motility by a computer-assisted sperm analysis system, and sperm metabolism was analysed via the Seahorse XFp analyser. Results confirmed a preference for OXPHOS over glycolysis in ATP production in stallion sperm, with mitochondria contributing significantly to total ATP generation. The Seahorse XFp Technology proved effective in evaluating equine sperm bioenergetics, offering insights into metabolic pathways critical for sperm function. In conclusion, this technology grants a new method for high-throughput analysis of sperm metabolism and quality, which could be applied to future reproductive studies in male equine fertility.

1. Introduction

Equine spermatozoa are highly specialized haploid cells, whose sole purpose is to fertilise the oocyte, thus transferring paternal DNA and resulting in the production of offspring. Semen is deposited in the female reproductive tract after ejaculation, and spermatozoa travel through it until they reach the utero-tubal junction and remain attached to the oviductal epithelium in a stage of relative quiescence. At the same time as ovulation, spermatozoa undergo capacitation and hyperactivation, allowing them to ascend to

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the ampulla and acquire the capacity to fertilise the oocyte (Suarez, 2008; Tulsiani and Abou-Haila, 2012; White, 1982). All these functions depend on spermatic metabolism, which consists of a sophisticated network of biochemical reactions capable of converting nutrients into metabolic substrates (Storey, 2008).

Metabolism in spermatozoa varies between species (Bucci et al., 2022), resulting in different metabolic strategies for energy production, ultimately responsible for phosphorylating adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Du Plessis et al., 2015). Sperm cells may use preferably the anaerobic pathway (glycolysis) or the aerobic one (oxidative phosphorylation (OXPHOS)) to obtain energy: in mouse and human spermatozoa, glycolysis has been described as the main pathway for obtaining energy in the form of ATP, whereas in boar and horse it is OXPHOS (Calvert et al., 2018; Hutson et al., 1977; Marin et al., 2003; Storey, 2008; Blanco Prieto et al., 2023). Moreover, different studies have associated various sperm functions with a specific metabolic pathway. Glycolysis has been considered responsible for maintaining sperm motility, hyperactivation, and capacitation in humans and pigs. However, while sperm motility in mice also depends on glycolysis, their hyperactivation and capacitation are linked to ATP produced by OXPHOS (Breininger et al., 2017; Du Plessis et al., 2015; Miki, 2007; Nesci et al., 2020; Prieto et al., 2023; Stendardi et al., 2011).In horses, OXPHOS has been described as the main mechanism of energy production, closely related to sperm motility and membrane integrity, but glycolytic pathway is considered responsible for sperm velocity (Darr, Varner, et al., 2016; Davila et al., 2016; Giaretta et al., 2022; Gibb et al., 2014). Hence, energy production may be restricted to one or more pathways depending on the metabolic state and the sperm function for which this energy is required (Ferramosca and Zara, 2014; Ford and Rees, 2020).

The OXPHOS pathway occurs in the mitochondria, which act as a control centre. The activity of these organelles is indistinguishably linked to fertilizing capacity in the stallion (Fawcett, 1970; Meyers et al., 2019). Mitochondria, apart from being central to energy production, perform many other functions, such as redox regulation, calcium homeostasis, and apoptosis, being also involved in many signalling pathways (Boguenet et al., 2021; Peña et al., 2009). For these reasons, beyond spermatic metabolism, the study of mitochondrial activity in sperm bioenergetics is a key focus for determining the fertility potential of ejaculates. Different tools are used to study it, identifying the specific function of each respiratory complex of OXPHOS both by testing selective inhibitors of each complex or by fuelling the respiratory chain by substrates that enter specific steps (Davila et al., 2015, 2016; Ramió-Lluch et al., 2014). Diverse fluorescent dyes as MitoTracker, JC-1 or DiOC6(3) among others, can be used to assess mitochondrial mass and activity by flow cytometry and fluorescent microscopy (Llavanera et al., 2022). As well, reactive oxygen species (ROS) production may be interesting to delineate mitochondrial function/dysfunction and it can be detected by Dihydroethidium, MitoSOX or CellROX (Dikalov and Harrison, 2014). Regarding ATP production, it has been analysed by an ATP determination kit based on the luciferase's requirement for ATP in producing light (Balao da Silva et al., 2013; Davila et al., 2015). Moreover, mass spectrometry is another tool that has been used in spermatology, providing detailed insights into the molecular composition and dynamics of spermatozoa, helping to better understand sperm function and fertility (Blaurock et al., 2022; Ortiz-Rodriguez et al., 2020; Ortiz-Rodríguez et al., 2021).

Despite the interest in these techniques for evaluating sperm fertility, they have certain limitations, as they assess sperm parameters at a single time point under specific treatment conditions. In contrast, the Seahorse XFp analyzer (Angilent) technology, an innovative tool used in bull, boar, mice, and human spermatozoa (Algieri et al., 2023; Freitas-Martins et al., 2024; Prieto et al., 2023; Tourmente et al., 2015), enables real-time measurement of cellular metabolism across multiple samples or experimental conditions simultaneously. This method provides a detailed characterization of the balance between glycolytic and oxidative metabolism by simultaneously assessing the oxygen consumption rate (OCR), an index of oxidative metabolism, and the extracellular acidification rate (ECAR), an index of glycolysis. In addition, the Seahorse XFp is contributing to a deeper understanding of sperm physiology. Contrary to previous assumptions, recent studies in humans have shown that higher-quality spermatozoa are more closely associated with oxidative metabolism than with glycolysis (Freitas-Martins et al., 2024). In boar spermatozoa, our laboratory has demonstrated that ATP production primarily depends on mitochondrial oxidative metabolism in freshly ejaculated spermatozoa. However, under liquid storage conditions, oxidative metabolism decreases, while the glycolysis activity remains constant (Prieto et al., 2023). Hence, in the present study, we aimed to use the innovative Seahorse XFp analyzer (Agilent) technology to assess, for the first time, the metabolism of freshly ejaculated stallion spermatozoa. Our goal was to delineate the main metabolic strategies used by equine sperm for energy production and to establish this technology as a new tool for studying stallion sperm physiology, which could be useful for optimizing artificial reproductive techniques.

2. Materials and methods

2.1. Semen collection and processing

Ejaculates were collected using a pre-warmed, lubricated Colorado model artificial vagina from eight stallions of different breeds. Semen was extended 1:2 and transported at 22 ºC within 1 h to the Laboratory of the Department of Veterinary Medical Sciences at the University of Bologna, where it was evaluated and pooled. Different samples were created by pooling semen from three stallions, washed, and resuspended in Tyrodes medium pH 7.4 (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM NaHCO3, 1 mM Na-Pyruvate, 21.6 mM Na-Lactate, 2 mM $CaCl_2$*$2H_2O$, 3.1 mM KCl, 0.4 mM $MgSO_4$*$7H_2O$, 0.3 mM NaH_2PO_4*H_2O, 0.3 % BSA) at the required concentration for the next experimental step.

2.2. Sperm function analysis

2.2.1. Sperm viability and mitochondrial activity

Twenty-five microliters from pooled samples at 2 $\times 10^6$ spermatozoa/ml were incubated with 2 μL of a 300 mM propidium iodide

(PI) stock solution (final concentration 20 mM), 2 μL of a 10 mM SYBR-14 stock solution (final concentration 0.7 mM), both obtained from the LIVE/DEAD sperm viability kit (Molecular Probes, Invitrogen, Milan, Italy) and 1 μL of a 300 mM JC-1 solution (final concentration 10 mM) for 20 min at 37 °C in the dark. Ten μL of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were counted using Nikon Eclipse E600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands) at 1000x magnification and separated in 4 different subpopulations: L^+, live spermatozoa (SYBR-14 positive and PI negative) with active mitochondria (orange JC1 emission); L^-, live spermatozoa with inactive mitochondria (green JC1 emission); D^+, dead spermatozoa (SYBR-14 negative and PI positive) with active mitochondria and D^-, dead spermatozoa with inactive mitochondria.

2.2.2. Sperm motility assessment

Sperm motility was measured from pooled samples at 30 $\times 10^6$ spermatozoa/ml by a computer-assisted sperm analysis system, using the open-source Image J BGM plugin as described by Giaretta et al. (2017). Briefly, a Nikon Eclipse 400 microscope (Nikon Europe BV, Badhoeverdop, The Netherlands) with a 20x plan objective with positive phase-contrast was used. The microscope was equipped with a warmed stage and a Basler ACE camera 2048 ×1536, 120 fps, mono 2/3" IMX252, CMOS, Global shutter, C-Mount, USB3 Vision (Basler, distributed by ADVANCED TECHNOLOGIES, Trezzano SN - Milan – Italy).

Videos were recorded for 3 seconds at a resolution of 1600 ×1200 pixels and 60 frames/sec (fps). Videos were saved on a hard drive in *.avi* format using the Pylon Viewer 64-bit software (Pylon Release 6.3.0.23157, Copyright C 2006–2012 Basler) for analysis using the BGM Image J plugin.

Prior to any observation, sperm were loaded on a fixed height Leja Chamber SC 20–01–04-B (Leja, The Netherlands). At least five videos of separate fields, were recorded per sperm sample and the central second of each video was analyzed. Sperm motility endpoints assessed were percent of total motile sperm (TM), percent of progressive sperm (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF) and lateral head displacement (ALH). The setting parameters used by the program were the following: 60 frames per second, number of frames 45, threshold path minimum; minimum VAP for motile 20 μm/s; minimum VCL for motile 30 μm/s; VAP cut off for progressive cells 15 μm/s; STR cut off for progressive cells 45 %.

2.3. Spermatozoa metabolism analysis

The ATP Rate Assay and Cell Mito Stress Test programs on the Agilent Seahorse XFp analyzer were used to assess spermatozoa bioenergetics. This is a specialized instrument used to measure the metabolic activity of live cells in real time, assessing key cellular functions, including mitochondrial respiration and glycolysis, by detecting changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). OCR is a measure of cellular respiration expressed in pmol/min, whereas ECAR is an index of glycolysis expressed in mpH/min. Characterization of cell number was performed seeding 1 $\times 10^6$, 1.5 $\times 10^6$ and 3 $\times 10^6$ cells in 150 μL per well in XFp cell culture miniplates (Agilent) coated with 10 μL/well fibronectin 1 mg/ml for 1 h and 30 min at 37°C and the ATP Rate Assay program was performed. Cells were spun down at 1200 g for 1 min, the supernatant was removed and switched to fresh Tyrode's medium 7.4 enriched with a mixture of 10 mM glucose, 2 mM glutamine and 1 mM pyruvate prewarmed at 37°C. XFp sensor cartridges were hydrated using XFp calibrant for a minimum of 4 h at 37°C. Prior to experiment, cartridges' injecting ports were loaded as per the guidelines of each program in order to obtain the following final concentrations. For the ATP rate assay, port A has 1.5 μM oligomycin (Oligo) and port B has 0.5 μM rotenone (Rot) plus 0.5 μM antimycin A (AA). These compounds are chosen in the ATP rate assay kit for their modulating activity in the mitochondrial respiration electron transport chain (ETC) and complex V or ATP synthase. As a matter of fact, Oligo is a known inhibitor of complex V or ATP synthase and its addition causes a drop in the OCR value since ATP is not synthetized anymore (Nesci et al., 2021). At this time point, the OCR value recorded indicates only the ETC oxygen consumption. Rot and AA are inhibitors of complex I and complex III respectively, definitely stopping the mitochondrial respiration. Thus, Oligo lets us obtain the mitoATP production, while Rot/AA injection allows the instrument to rely on the glycoATP production rate. For the Cell Mito Stress Test a titration of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone FCCP was performed using 1.5 μM Oligo in port A, 1, 2 and 4 μM FCCP in port B, and 0.5 μM Rot plus 0.5 μM AA in port C. Both programs were run at 37°C in three independent experiments. Data were analyzed using the WAVE software (Agilent) and normalized on the cell viability obtained through microscopic epifluorescence analysis.

2.4. Statistical analysis

Data were analyzed using the R statistical environment v. 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria). Statistical analyses on cell metabolism were performed by SIGMASTAT software. The analysis of variance followed by Students–Newman–Keuls' test when F values indicated significance ($p<0.05$) was applied. Unless otherwise stated, results are presented as the mean ± standard deviation.

3. Results

3.1. Sperm viability and mitochondrial activity

At least 200 spermatozoa were counted in each sample and classified as L^+, L^-, D^+, or D^- to determine sperm functionality. Sperm pools presented 69.11±6.66 % of live spermatozoa with high mitochondrial membrane potential (L^+).3.1.2. Sperm motility

Cinematic sperm parameters were measured in each sample by a computer-assisted sperm analysis system, and the results are presented in Table 1.

3.2. Spermatozoa metabolism

The first part of the study was conducted to define the best condition for stallion sperm metabolism assessment.

As far as sperm concentration concerns, ATP production of 1.5 $\times 10^6$ cells/well resulted significantly higher compared with that of 1 $\times 10^6$ cells/well while was not significantly different form that of 3 $\times 10^6$ cells/well (Fig. 1A).

The concentration of 2 μM FCCP showed to be adequate to stimulate maximal respiration in stallion spermatozoa. Conversely, 1 μM was not suitable to stimulate mitochondrial respiration, whereas 4 μM was an overdose responsible for the inhibitory effect on respiratory complexes (Fig. 1B).

Therefore, the 1.5 $\times 10^6$ cells/well concentration and the concentration of 2 μM FCCP were chosen for the subsequent experiments.

3.2.1. Evaluation of spermatozoa metabolism

The SeaHorse Analyzer measured a total ATP production rate of 481.7 ± 98.7 pmol/min. However, not only did the Real-time ATP Rate assay measure the overall rate of ATP generation in living cells, but it also discriminated between ATP produced by glycolysis (glycoATP production rate) and mitochondria (mitoATP production rate). Indeed, in our experiment horse spermatozoa mitochondria produced the majority of ATP (447.8 ± 102.2 pmol/min), while glycoATP produced only 33.9 ± 14.2 pmol/min (Fig. 2A, B). Furthermore, the ATP Rate Index, which indicated the ratio between mitoATP production rate and glycoATP production rate, was equal to 7.6 ± 4.5 (Fig. 2C). The value higher than 1 shows that horse spermatozoa presents a preference for the OXPHOS pathway as its metabolic phenotype.

To evaluate the bioenergetics parameters of mitochondria, the cellular respiratory profile of spermatozoa was determined by the subsequent injections of mitochondrial modulators, *i.e.*, oligomycin, FCCP and rotenone mixed with antimycin A (Fig. 3). Prior to employing modulators, the basal respiration was measured and found to be 46.5 ± 5.0 pmol/min. The addition of oligomycin led to minimal respiration, allowing us to measure proton leak (8.7 ± 4.0 pmol/min, difference between basal respiration and respiration in the presence of oligomycin) and ATP-linked respiration or ATP production (39.5 ± 5.1 pmol/min). Following FCCP injection, spermatozoa faced a disruption of the H^+ gradient across the inner mitochondrial membrane, collapsing the electrochemical potential that mitochondrial use to generate ATP. This results in maximum respiration, as the electron transport chain works harder in an attempt to restore the H^+ gradient. Under our experimental conditions, however, we only found an increase in respiration that did not exceed the baseline (39.1 ± 8.9 pmol/min). Furthermore, the difference between maximal and basal respiration provided information on the spare respiratory capacity; on the other hand, in this case, it cannot be determined because no greater respiration was observed in the presence of FCCP. All data were subtracted from non-mitochondrial respiration, registered in the presence of rotenone and antimycin A (23.5 ± 4.0 pmol/min). Moreover, the coupling efficiency was about 0.85 a.u. (the maximal value of 1.0 a.u. is obtained when all the basal respiration is sensitive to oligomycin), whereas the cell respiratory control ratio (cRCR), the ratio of the uncoupled rate (FCCP) to the rate with oligomycin present, was 5.2 a.u.

4. Discussion

The results presented in this paper delineate, for the first time, the metabolic profile of energy obtainment strategy in freshly ejaculated stallion spermatozoa using the Seahorse XFp analyzer (Agilent) technology. These findings underscore the critical role of intact, functional mitochondria in maintaining horse sperm function. Semen samples were pooled from three stallions, with eight different horses used in total. This approach was chosen to minimize the high inter-individual fertility variability common in equine, with the aim of applying this technology in future research on equine fertility and assisted reproductive techniques. Additionally, the samples used in this study exhibited average quality, with a viability of at least 65 % and good motility parameters.

The first step of our study was to establish the optimal settings for analysing equine sperm metabolism using the Seahorse XFp. It was shown that, as in boar sperm analysis (Barranco et al., 2024; Prieto et al., 2023), 1.5 $\times 10^6$ spermatozoa per well is the appropriate concentration for determining sperm metabolism. Using 1 $\times 10^6$ spermatozoa per well resulted in lower ATP concentrations, while 3 $\times 10^6$ did not show significant differences, possibly due to well saturation, as it is likely that no more sperm could adhere to the well. In contrast, the adequate cells amount for humans has been established as 4 $\times$ 10^6 spermatozoa per wells (Freitas-Martins et al., 2024), and 2 $\times 10^6$ for mice (Tourmente et al., 2015). Regarding the different inhibitors of the ETC and ATP synthase, we used the same concentrations as in previous experiments with boar spermatozoa: 1.5 μM oligomycin (ATP synthase inhibitor), 0.5 μM rotenone (complex I inhibitor) and 0.5 μM antimycin A (complex III inhibitor) (Barranco et al., 2024; Prieto et al., 2023). On the other hand, the optimal concentration of FCCP to stimulate maximal respiration in stallion spermatozoa is 2 μM. A concentration of 1 μM did not

Table 1
Motility features of sperm samples.

TM (%)	PM (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
50.1±1.9	31.2±1.6	187.1±7.8	75.9±3.7	48.9±2.7	6.8 ±0.3	26.6±0.3	62.3±0.9	26.3±1

Percent of total motile sperm (TM), percent of progressive sperm (PM), curvilinear velocity (VCL), mean velocity (VAP), straight-line velocity (VSL), lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), and linearity (LIN). Data are reported as mean ± SEM.

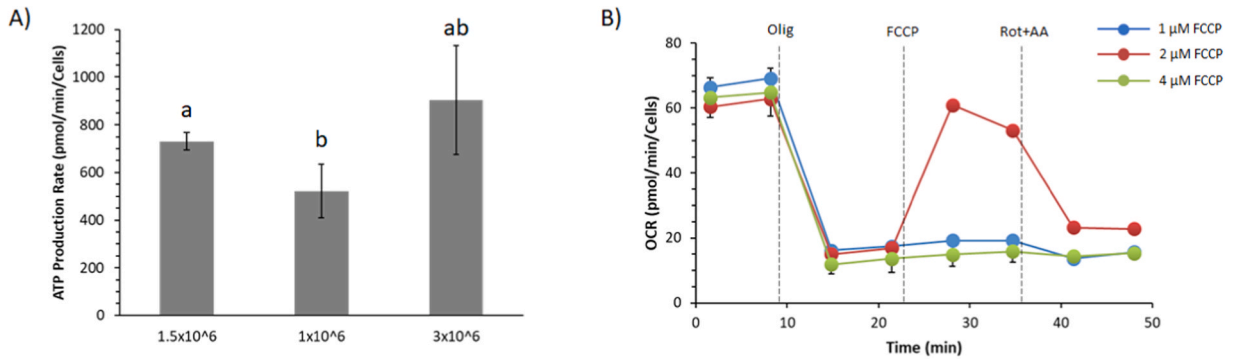

Fig. 1. A) Characterization of cell concentration via ATP production rate. B) Titration at 1 µM (blue), 2 µM (red), and 4 µM (green) of FCCP. Different letters indicate different values among treatments in ATP production rates. $p<0.05$ were obtained using one-way ANOVA followed by Student–Newman–Keuls test. Data are reported as mean ± SEM.

stimulate mitochondrial respiration, while 4 µM, unlike in boar spermatozoa (Barranco et al., 2024; Prieto et al., 2023), resulted in an overdose, leading to an inhibitory effect on the respiratory complexes.

Our study clearly shows that horse spermatozoa produce energy for cell activities and use the oxidative metabolism of mitochondria to synthesize ATP. This feature characterizes a phenogam profile typical of the aerobic metabolic state of cells. We observe that mitochondrial respiration responds to specific inhibitors of oxidative phosphorylation, such as rotenone, antimycin A and oligomycin; however, maximal respiration is not stimulated by the uncoupler FCCP beyond basal respiration levels. Evidently, ATP synthase produces ATP by exploiting the maximal respiration of mitochondria in accordance with its 85 % coupling efficiency. As a consequence, the ability of the spare respiratory capacity, to define the tendency of the cell to modify its bioenergetics in order to meet the increasing demand for energy, was not detected in sperm. The result of this investigation suggests that the metabolism of freshly ejaculated horse spermatozoa might have a limited capacity to adapt to an increase in energy requirement (Brand and Nicholls, 2011). However, we can speculate that equine sperm mitochondria work at maximum respiration rate to meet cellular energy needs under our experimental conditions (10 mM Glucose, 2 mM glutamine, 1 mM Na-Pyruvate, and 21.6 mM Na-Lactate). Indeed, the cell respiratory control ratio, which is an excellent marker of mitochondrial dysfunction in cells, obtained a value higher of 1 and the OXPHOS efficiency is almost 100 %. Thus, we can exclude an impaired mitochondrial activity in freshly ejaculated spermatozoa from stallions with average quality parameters. In addition, equine spermatozoa are reported to rely on mitochondrial activity to sustain cellular function, in particular motility (Davila et al., 2015; Giaretta et al., 2017; Gibb et al., 2014; Mislei et al., 2020; Peña et al., 2015); therefore, mitochondrial functional integrity seem to be crucial to maintain sperm functionality. Our results showed that 93 % of ATP is produced through OXPHOS, while only 7 % is generated from glycolysis. It should also be noted that previous studies have indirectly demonstrated that oxidative phosphorylation in mitochondria and glycolysis are coupled (Davila et al., 2016) and that this feature is fundamental in particular to maintain motility. In our labs we have also studied these features using the same approach, namely, inhibiting specific mitochondrial complexes to verify their role in horse mitochondrial sperm function and, more generally to verify whether impairment of mitochondrial function may also affect any other physiological functions. And, indeed, we confirmed that impaired mitochondrial function has a great impact on sperm motility, while ROS production does not seem to be deeply impacted (Giaretta et al., 2022).

Oxygen consumption rate has previously been studied using polarographic technique by Darr et al. (2016), (2017), but it has the limitation of low throughput, as they assess mitochondrial activity at a single time point under a specific treatment condition (Schober et al., 2007). Darr et al. (2016), (2017) underlined the importance of this parameter in determining stallion sperm quality, because it correlates with some other important parameters, such as motility and viability (Darr et al., 2016, 2017). Specifically, they investigated sperm oxygen consumption in freshly ejaculated semen, as we did for our experiments and the results agree in terms of high basal oxygen consumption, not statistically different from maximal respiration capacity.

Although recent research from different labs have highlighted the specific importance of mitochondrial ATP production in stallion spermatozoa (Aitken et al., 2018; Gibb et al., 2014, 2015; Gibb and Aitken, 2016; Swegen et al., 2016; Varner et al., 2015), it is well known that sperm metabolism is a complex mechanism, not just dependent on one unique pathway. The presence and function of hexose transporters (GLUTs) into the plasma membrane lipidic bilayer is important for maintaining sperm functionality, and, consequently, the different diluents used for semen preservation are composed of different sugars such as glucose or fructose (Bucci et al., 2010, 2011). Nevertheless, glucose toxicity has been described in stallion spermatozoa during conservation, as well as the importance of pyruvate metabolism and the existence of a non-canonical metabolism of glutamate (Ortiz-Rodríguez et al., 2021), which underscores the complexity of equine sperm metabolism. These findings have led to the formulation of new diluents to preserve semen, and a new protocol for the liquid storage of equine spermatozoa for up to seven days has been developed (Gibb et al., 2018). The use of the Seahorse XFp analyzer in this type of research could enhance our understanding and contribute to further advancements in ejaculates preservation, as it offers several advantages, including a high-throughput format, automated injection of mitochondrial effector drugs, and the ability to measure OXPHOS and glycolysis in the same assay (Tourmente et al., 2015). Therefore, future studies will be aimed at evaluating the dependence, flexibility, and capacity of sperm metabolism for the respective cellular fuels, i.e., glucose,

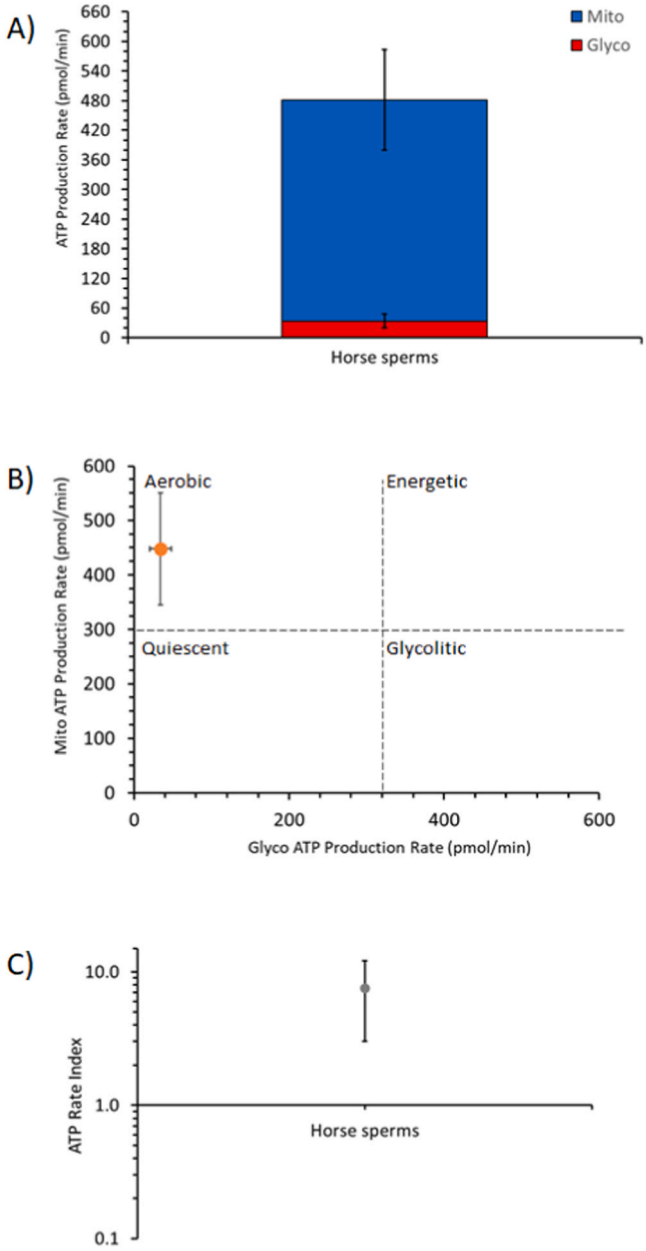

Fig. 2. Bioenergetic metabolism in sperm cells. A) Evaluation of the real-time ATP production by mitochondrial OXPHOS or by glycolysis. B) Energy map relative to the metabolic state of sperm cells by evaluating the two main metabolic pathways, i.e. mitochondrial respiration and glycolysis, to identify one of the four states of cell energy metabolism. C) The ATP rate index, calculated as the ratio between the mitochondrial ATP production rate and the glycolytic ATP production rate represent a valuable metric for detecting the metabolic phenotype (>1 cellular ATP was derived from mitochondria, <1 ATP was produced by the glycolytic pathway), is shown on the y-axis (logarithmic scale). Data expressed as column charts (A plots) and points (B, C plots), represent the mean ± SD (vertical or horizontal bars) from three experiments carried out on different cell preparations.

pyruvate, glutamine, and fatty acids, among others, in horse spermatozoa.

In conclusion, the Agilent Seahorse XFp Technology cell system (Agilent, Santa Clara, CA) can be used to add information on sperm quality analysis in horses, as well as to study and enhance various artificial reproductive techniques, including sperm conservation, capacitation, and *in vitro* fertilization. This system provides a powerful tool for measuring cellular respiration and metabolic activity, allowing for a deeper understanding of sperm physiology. Additionally, its application can lead to improvements in the efficiency and success rates of artificial reproductive technologies by identifying optimal conditions for sperm preservation and function, contributing to the development of new protocols for the treatment of male subfertility in equines, potentially benefiting the broader field of reproductive medicine.

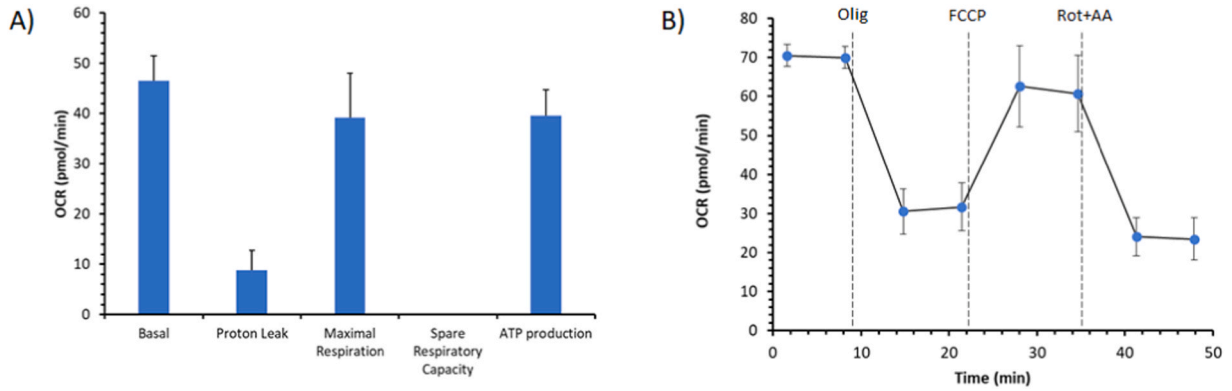

Fig. 3. The mitochondrial respiration profile in sperm lines is evaluated as OCR. A) Mitochondrial parameters (basal respiration, proton leak, maximal respiration, spare respiratory capacity, ATP production) of sperm cell oxidative metabolism. B) Mitochondrial respiration profile in spermatozoa under basal respiration conditions and after the addition of 1.5 μM olig, 2.0 μM FCCP, and a mixture of 0.5 μM Rot + 0.5 μM AA. Compound injections are shown as dotted lines. Data expressed as column charts (A plots) and points (B plots) represent the mean ± SD (vertical bars) from three experiments carried out on different sperm cell preparations.

CRediT authorship contribution statement

Diego Bucci: Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Jose Manuel Ortiz-Rodriguez:** Writing – original draft, Investigation, Formal analysis. **Silvia Granata:** Investigation, Formal analysis. **Laura Tovar-Pascual:** Investigation, Formal analysis. **Marcella Spinaci:** Writing – review & editing, Conceptualization. **Salvatore Nesci:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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