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Capacitation promotes a shift in energy metabolism in murine sperm

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In mammals, sperm acquire fertilization ability after a series of physiological and biochemical changes, collectively known as capacitation, that occur inside the female reproductive tract. In addition to other requirements, sperm bioenergetic metabolism has been identified as a fundamental component in the acquisition of capacitation. Mammalian sperm produce ATP through two main metabolic processes, oxidative phosphorylation (OXPHOS) and aerobic glycolysis that are localized to two different flagellar compartments, the midpiece, and the principal piece, respectively. In mouse sperm, the occurrence of many events associated with capacitation relies on the activity of these two energy-producing pathways, leading to the hypothesis that some of these events may impose changes in sperm energetic demands. In the present study, we used extracellular flux analysis to evaluate changes in glycolytic and respiratory parameters of murine sperm that occur as a consequence of capacitation. Furthermore, we examined whether these variations affect sperm ATP sustainability. Our results show that capacitation promotes a shift in the usage ratio of the two main metabolic pathways, from oxidative to glycolytic. However, this metabolic rewiring does not seem to affect the rate at which the sperm consume ATP. We conclude that the probable function of the metabolic switch is to increase the ATP supply in the distal flagellar regions, thus sustaining the energetic demands that arise from capacitation.

KEYWORDS

sperm metabolism, capacitation, mouse, glycolysis, OXPHOS

Introduction

After ejaculation, mammalian sperm must spend a species-specific amount of time in the female reproductive tract in order to acquire the ability to fertilize the ova. During this period, sperm undergo a series of structural, biochemical, physiological, and behavioral changes that have been collectively termed “capacitation” (Austin, 1951; Chang, 1951; De

Jonge, 2017). As a consequence of capacitation, sperm become able to orient their movement in response to chemical cues originating in the cumulus-oocyte complex (chemotaxis) (Giojalas et al., 2015; Guidobaldi et al., 2017), and undergo the acrosome reaction, which allows them to penetrate the oocyte vestments and fuse with the oolema (Buffone, 2016; Stival et al., 2016; Nixon and Bromfield, 2018). In addition to these modifications, capacitation is associated with a change in sperm movement pattern, termed “hyperactivation” (Yanagimachi, 1970). Hyperactivated movement is characterized by highly vigorous, asymmetric flagellar beating with increased amplitude (Yanagimachi, 1970; Suarez, 2008; Chang and Suarez, 2011; Goodson et al., 2011), which results in the loss of linear movement in aqueous media but increases progression efficiency in viscous media (Perez-Cerezales et al., 2016).

At the molecular level, sperm capacitation constitutes a highly complex process that includes modifications in the majority of cell components (Gervasi and Visconti, 2016). Capacitation involves hyperpolarization (Zeng et al., 1996; Fraser, 2010) and alterations in the architecture (Harrison, 1996; Visconti et al., 1999; Gadella and Harrison, 2000; Cuasnicú et al., 2016) and composition of the plasma membrane (Zarintash and Cross, 1996; Gadella and Harrison, 2000; Escoffier et al., 2012), increase in the intracellular Ca^{2+} concentration (Bailey and Storey, 1994; Arnoult et al., 1999), and elevation of intracellular pH (Zeng et al., 1996). This process also involves the activation of lipid signalling pathways (Roldan and Vazquez, 1996), and a soluble adenylyl cyclase with the subsequent increase in the levels of cAMP (Okamura et al., 1985; Garty et al., 1988; Visconti et al., 1990; Fraser, 2010), which in turn activates the cAMP-dependent protein kinase A (PKA) pathway (Harrison and Miller, 2000; Turner, 2006; Fraser, 2010), leading to a substantial increase in the phosphorylation of tyrosine and serine-threonine residues throughout the cell (Visconti et al., 1995a; Harrison, 2004; Visconti, 2009; Fraser, 2010; Krapf et al., 2010).

Previous studies on *in vitro* sperm capacitation have identified a series of medium components that are key for this process (see (Visconti, 2009; Fraser, 2010; Gervasi and Visconti, 2016) for reviews on the subject). Ions such as HCO_3^- (Lee and Storey, 1986; Shi and Roldan, 1995; Gadella and Harrison, 2000; Demarco et al., 2003; Harrison and Gadella, 2005) and Ca^{2+} (Fraser, 1977; Fraser, 1982; Navarrete et al., 2015) are necessary for the activation of the phosphorylation pathways, and a cholesterol acceptor (usually serum albumin) to facilitate the efflux of cholesterol from the membrane (Demarco et al., 2003). More recently, sperm bioenergetic metabolism has been identified as an additional central component in the acquisition of the capacitated status since a number of studies have identified the activity of particular metabolic pathways as necessary for sperm capacitation (see review in (Ferramosca and Zara, 2014)).

In order to sustain flagellar motility, mammalian sperm produce ATP by means of two main metabolic pathways, oxidative phosphorylation (OXPHOS) and aerobic glycolysis (Ford, 2006; Ruiz-Pesini et al., 2007; Storey, 2008), which occur in different cellular compartments. While OXPHOS takes place in the mitochondria located in the midpiece (i.e., the section of the flagellum most proximate to the head), glycolysis takes place in the principal piece, where glycolytic enzymes are associated with the fibrous sheath that surrounds the axoneme (Ford, 2006; Ruiz-Pesini et al., 2007; Storey, 2008; Cummins, 2009). In the laboratory mouse, the activity of both metabolic routes is necessary to maintain vigorous motility (Travis et al., 2001; Narisawa et al., 2002; Goodson et al., 2012; Odet et al., 2013; Takei et al., 2014) since sperm motility decreases to its eventual cessation under inhibition of glycolytic enzymes or absence of glycolytic substrates (Ferramosca et al., 2012; Odet et al., 2013), and alterations of mitochondrial functions are associated to male infertility as a consequence of impaired sperm motility (Narisawa et al., 2002).

Metabolic requirements for sperm capacitation in mammals seem to be species-specific (Cummins, 2009; Ferramosca and Zara, 2014). Numerous studies have revealed that an active glycolytic pathway is necessary for the normal occurrence of mouse sperm capacitation and its associated processes, including fertilization (Hoppe, 1976; Fraser and Quinn, 1981; Cooper, 1984; Urner and Sakkas, 1996; Travis et al., 2001; Urner et al., 2001; Mukai and Okuno, 2004; Travis et al., 2004; Goodson et al., 2012), and mutations that impair the catalytic function of key glycolytic enzymes (Miki et al., 2004; Odet et al., 2008; Danshina et al., 2010; Odet et al., 2011; Odet et al., 2013; Tang et al., 2013) have been found to promote disruptions in sperm capacitation, hyperactivation, and fertilizing ability (Miki et al., 2004; Danshina et al., 2010). These findings support the idea that the physiological events associated with capacitation, such as active ion transport, activation of multiple intracellular signaling pathways, extensive protein phosphorylation, and the vigorous flagellar beating typical of hyperactivation, may impose changes in sperm energetic demand (Travis et al., 2004; Ishijima, 2011).

A previous study from our laboratory (Tourmente et al., 2015a) examined the metabolism of sperm cells in the house mouse and other closely related species in survival (i.e., non-capacitating) conditions using extracellular flux analysis (Agilent-Seahorse XF24). This study revealed that these cells engaged in glycolysis and OXPHOS when glucose was the only substrate in the medium, and that the ratio of usage of each metabolic pathway was species-specific. However, until recently, there was little to no evidence regarding the changes in metabolic rate experienced by mouse sperm during the capacitation process. Two recent studies revealed that mouse sperm increase their glucose uptake rate when exposed to capacitating medium (Hidalgo et al., 2020), or during chemically induced capacitation (Balbach et al., 2020), in

response to elevated intracellular cAMP and Ca^{2+} concentrations (Hidalgo et al., 2020). Furthermore, they showed the elevation of substrate uptake was associated with its utilization to fuel the increased activities of both glycolytic and respiratory pathways (Balbach et al., 2020). Nonetheless, these results only allowed for a limited analysis of the metabolic processes involved in the change from a non-capacitated to a capacitated status, since 1) capacitation was achieved via chemical stimulation with db-cAMP and IBMX in the absence of NaHCO_3 , and 2) basal respiration and glycolysis rates were not calculated by using combinations of pathway inhibitors and, thus, phenomena such as non-mitochondrial oxygen consumption, proton leakage rate, and non-glycolytic acidification were not contemplated. Finally, there is evidence showing that an increase in mitochondrial membrane potential, which occurs only in capacitated sperm (Yang et al., 2020; Giaccagli et al., 2021), is necessary for the acquisition of the hyperactivated movement pattern. In all, this evidence supports the notion that the bioenergetics of murine sperm capacitation involves changes in both glycolytic and respiratory pathways.

The majority of studies on the relationship between sperm bioenergetics and capacitation have focused on the variations that occur in ATP production mechanisms. It is intuitive to think that higher cellular ATP content would be the immediate consequence of higher ATP production rates. Nonetheless, in order to maintain cellular homeostasis and engage in any metabolic process (capacitation included), there is a need for an equilibrium between ATP synthesis and hydrolysis. Thus, the ratio between ATP production and consumption determines the level of metabolic intensity sperm may achieve and its ability to maintain adequate motility. Comparative studies in muroid rodents showed that the species with high sperm metabolic rates (ATP production) (Tourmente et al., 2015a) can sustain higher ATP consumption rates (Tourmente et al., 2019), and consequently, faster swimming velocities for a longer time (Tourmente et al., 2015b). On the other hand, sperm from other *Mus* species have been shown to experience a decline in ATP content when incubated under capacitating conditions (Sansegundo et al., 2022), suggesting that the events involved in capacitation may impose ATP demands that even the enhanced metabolic rate of capacitated sperm might not be able to sustain for a long time.

In the present study, we used extracellular flux analysis (hereafter EFA) to evaluate which changes in the glycolytic and respiratory parameters of murine sperm occur as a consequence of capacitation. Furthermore, we examined the variations in ATP sustainability that may be imposed by capacitation. We show that capacitation promotes a shift in the usage ratio of the main metabolic pathways, from oxidative to glycolytic, probably as a manner to maintain local ATP concentration in the distal flagellar regions.

Materials and methods

Reagents

Unless stated otherwise, reagents were purchased from Merck (Madrid, Spain).

Animals, sperm collection, and incubation

B6D2F1 adult male mice (3–4 months old) were purchased from Envigo (Barcelona, Spain). The animals were kept in the facilities of the Museo Nacional de Ciencias Naturales (Madrid) in individual cages under controlled temperature (20–24°C) on a 14/10 h light/darkness photoperiod, and supplied with water and food *ad libitum*. Care and maintenance of the mice used in this study followed the Royal Decree on Protection of Experimental Animals RD53/2013 and the European Union Regulation 2010/63, and had the approval of CSIC's ethics committee and the Comunidad de Madrid (28079-47-A). Mice were sacrificed by cervical dislocation, which is considered a humane method by Spanish and European regulations. No procedures other than sacrifice for the collection of gametes were performed.

After sacrifice, mice were dissected and their caudae epididymides excised. The blood vessels, fat, and surrounding connective tissue were removed from the caudae. Each cauda epididymis was placed in a 35 mm Petri dish containing 0.4 ml of one of two variants of culture medium pre-warmed to 37°C. One cauda epididymis was placed in non-capacitating medium under air and the other one was placed in capacitating medium under 5% CO_2 /air. The compositions of both media were based on a Hepes-buffered modified Tyrode's medium (Shi and Roldan, 1995), supplemented with albumin, lactate, and pyruvate ($\text{pH} = 7.4$, osmolality = 295 mOsm kg^{-1}). The composition of the non-capacitating medium was: 132 mM NaCl, 2.68 mM KCl, 0.49 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.36 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 5.56 mM glucose, 20 mM HEPES, 1.80 mM CaCl_2 , 0.02 mM phenol red, 0.09 mM kanamycin, 4 mg ml^{-1} fatty acid-free BSA, 20 mM Na lactate, and 0.5 mM Na pyruvate. The capacitating medium differed from the non-capacitated medium in that 15 mM NaHCO_3 was added and NaCl adjusted to maintain the osmolality.

Three to five incisions were performed in the distal region of the caudae, and sperm were allowed to swim out for 5 min (Sansegundo et al., 2022). For most experiments (see below), sperm from two mice were used in each experiment. One epididymis from each of two males was placed in a Petri dish (total of two per dish) and the sperm of both was mixed during swim out. After swim out, the epididymal tissue was discarded and the sperm suspension was transferred to a plastic tube under a suitable atmosphere. Sperm concentrations were estimated for each sperm suspension by using a modified Neubauer chamber and adjusted to 100×10^6 sperm ml^{-1} with the corresponding

medium. Concentration-adjusted sperm suspensions were then incubated for 1 h at 37°C under suitable atmospheres since, after such incubation time, the mouse sperm population tends to reach the peak of capacitated cells (Sansegundo et al., 2022). In all procedures, large-bore pipette tips were used to minimize mechanical damage to spermatozoa.

In the case of sperm ATP consumption measurements, only one male was used in each experiment. Thus, sperm from one cauda epididymis was collected in each medium. In this case, the sperm concentration of each suspension was adjusted to 20×10^6 sperm ml^{-1} prior to the 1-h incubation time.

Sperm capacitation

Sperm capacitation status was assessed at the end of the 1 h incubation period, and prior to extracellular flux analysis. Capacitation was assessed using chlortetracycline (CTC, C4881) [73] combined with a vital stain (Ward and Storey, 1984; Sansegundo et al., 2022). Briefly, after a 1 h incubation under different conditions, 100 μl of the sperm suspension were mixed with 50 μl of 6 $\mu\text{g}/\text{ml}$ Hoechst 33258 bisbenzamide (B2883) and incubated for 1 min in the dark. Subsequently, the samples were centrifuged for 2 min at 100 $\times g$, the supernatant was discarded, the pellet resuspended in 100 μl of medium and fixed with an equal volume of 2% glutaraldehyde-0.165 M sodium cacodylate solution. Immediately before evaluation, an aliquot of 20 μl of sperm suspension was added to 20 μl of 250 μM CTC solution and incubated in the dark for 3 min. Spermatozoa were observed at 1,000 \times magnification under fluorescence and phase-contrast microscopy simultaneously (E-600 microscope, Nikon). Pre-fixation viability of the spermatozoa was assessed using a Nikon UV-2A 330-nm filter and fluorescence emission via a DM 400 dichroic mirror. Only the cells that excluded the Hoechst stain were considered viable. CTC staining patterns were observed using a Nikon BV-2A 405-nm filter and fluorescence emission with a DM 455 dichroic mirror, and the following patterns were distinguished (Sansegundo et al., 2022): 1) F (non-capacitated sperm): the head of spermatozoa was uniformly stained with CTC, 2) B (capacitated sperm): the post-acrosomal region of the head was not stained with CTC, and 3) AR (without the sperm acrosome): the head of the sperm cell showed little or no CTC staining. Capacitation status was evaluated in 100 viable spermatozoa with intact acrosomes per sample and expressed as the percentage of sperm showing staining pattern “B.”

Assessment of sperm metabolic rates

An XFp extracellular flux analyzer (Agilent Seahorse, Santa Clara, CA) was used to measure oxygen consumption rate (OCR)

and extracellular acidification rate (ECAR) in real-time. In this system, OCR indicates the level of respiratory activity in a population of cells, and ECAR, an estimate of the rate of lactate release to the medium, is used as a proxy for aerobic glycolytic activity. After 1 h incubation in differential conditions (i.e., capacitation period), the cells were transferred to an 8-well XFp plastic microplate. As a substantial difference from the original method developed by Tourmente et al. (Tourmente et al., 2015a), sperm were not attached to the bottom of the well. Thus, measurements were obtained from a free-swimming sperm population. This required an increase in the number of sperm added to each well in order to ensure that the 2 μl closed micro-chamber, formed between the sensor and the bottom of the plate when the instrument is measuring, contained enough cells to detect a reliable signal. Three wells were seeded with 80 μl of sperm suspension (approximately 8×10^6 sperm) incubated under non-capacitating conditions, other three wells were seeded with sperm suspension incubated under capacitating conditions, and two wells were left without cells to perform background corrections. The plate was centrifuged for 1 min at $\times 1,300 g$ in each direction to temporarily set the cells at the bottom of the well. The supernatant was removed from each well and 200 μl of unbuffered assay medium was added to the 8 wells of the plate, thus resuspending the cells. Since the presence of a pH buffer in the medium would impede an accurate measurement of ECAR, an unbuffered assay medium was used for the measurements and dilution of the metabolic modulators. In this medium, Hepes and NaHCO_3 were replaced by NaCl to preserve osmolality, and pH was adjusted to 7.4 at 37°C. After medium replacement, the plate with the sperm was placed in the XFp, and a 12 min equilibration step was allowed before measurements.

OCR and ECAR were measured at 37°C and the measurement cycle consisted of 2 min of mixing, 1 min of waiting, and 3 min of measuring. In the first set of experiments ($n = 4$), we assessed whether stable measurements of sperm metabolism could be obtained from mouse sperm samples before the addition of any compound. Thus, OCR and ECAR were recorded for 48 min (8 cycles). Subsequently, 1 μM rotenone and 1 μM antimycin A (inhibitors of the mitochondrial respiratory chain complexes I and III respectively) were added to the wells, and both metabolic variables were monitored for an additional 12 min (10 cycles, 60 min). Oxygen levels showed a marked descent during the measurement phase that fully recovered during the mixing and waiting phases (Supplementary Figure S1), indicating that the sperm concentration used in the experiment did not lead to hypoxia.

For the second set of experiments, we measured sperm metabolism both before and after the addition of metabolically active compounds, in order to calculate a range of metabolic parameters. OCR and ECAR were measured for 36 min (6 cycles) to establish a baseline value (hereafter “basal”). Subsequently, one of three metabolic modulators was added to

the wells, and sperm metabolism was recorded for 3 additional cycles (18 min). Metabolic modulators were: 1) 5 μM oligomycin A, an inhibitor of the mitochondrial ATP synthase, 2) 1 μM carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP), an uncoupler of mitochondrial OXPHOS, and 3) 50 mM 2-deoxy-D-glucose (2DOG), a glucose analog that competitively inhibits the first step of glycolysis. Finally, 1 μM rotenone and 1 μM antimycin A were added to the wells for two final measurements (11 cycles, 66 min). Oligomycin and FCCP were used in four separate experiments, while 2DOG was used in three separate experiments (total $n = 11$ for the second set). For all experiments, the first two measurements were discarded since, according to our previous experience, these tend to show a higher degree of instability. Thus, the first OCR and ECAR values considered for this study were taken 12 min after the beginning of the measurements.

At the end of the experiment, the sperm suspension in each well was mixed by pipetting, and 10 μl was collected to estimate sperm concentration using a modified Neubauer chamber. Sperm numbers in the wells were calculated and the OCR and ECAR values for each well were normalized by the number of sperm present in the 2 μl measurement volume (reported as $\text{amol of O}_2 \text{ min}^{-1} \text{ sperm}^{-1}$, and $\text{nano-pH min}^{-1} \text{ sperm}^{-1}$, respectively).

Calculation of metabolic parameters

The addition of the metabolic modulators used in the second set of EFAs allowed us to calculate several metabolic parameters (Supplementary Table S1) that have more informative value than the raw normalized measurements. They also allowed for a more accurate comparison between the two incubation conditions (Marinangeli et al., 2018; Zhao et al., 2020). Metabolic parameters were calculated for each well as the average value of the measurements taken for that well in condition A minus the average value of the measurements taken for that well in condition B (see Supplementary Table S1 for a description of the conditions and Supplementary Figure S2 for a graphical representation). Values corresponding to the basal state (no additions) were averaged across measurement cycles 3-6, values corresponding to oligomycin and 2DOG additions were averaged across cycles 7-9, and values corresponding to A + R additions were averaged across cycles 10-11. Exceptionally, for the calculation of the stimulating effects of FCCP and Oligomycin on OCR and ECAR respectively, the highest value among cycles 7-9 was used instead of the average.

In order to facilitate comparisons with previous and future studies, we also calculated the response of OCR and ECAR to metabolic modulators as a percentage of the averaged baseline values (measures 3-6) for each well. In the case of OCR, both the baseline and the stimulated/inhibited values were first corrected by subtracting the average of the measures after the addition of A + R (cycles 10-11).

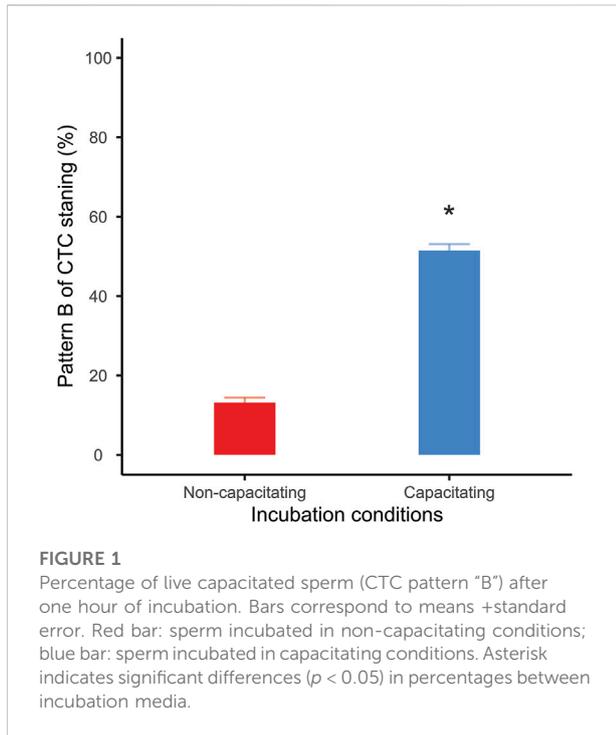
Sperm ATP content

A separate set of experiments ($n = 5$) was performed in order to compare sperm ATP consumption rates between sperm incubated in the different conditions. After 50 min of incubation under capacitating or non-capacitating conditions, each sperm suspension was divided into two aliquots. The first sperm aliquot (hereafter “control”) was used to monitor basal ATP content throughout the consumption experiment. Sperm ATP content was assessed in this aliquot at 55 and 70 min of incubation. A combination of inhibitors of OXPHOS (1 μM antimycin A, 1 μM rotenone, and 5 μM oligomycin) and glycolysis (50 mM 2DOG) were diluted in the corresponding medium and added to the second aliquot (hereafter “inhibition” treatment) after 60 min of incubation in order to fully inhibit both ATP production pathways, thus making sperm able to consume ATP without producing it (Tourmente et al., 2019). Sperm ATP content was assessed in the inhibition treatment immediately after the addition of inhibitors and after 2, 4, 6, 8, and 10 min. Although the addition of rotenone and antimycin A would suffice to stop OXPHOS-derived ATP production, oligomycin was used to prevent an artefactual ATP depletion due to the reverse action of the mitochondrial ATP synthase [55]. Since OXPHOS inhibitors were dissolved in DMSO prior to the addition to the sperm suspension, an equivalent concentration (0.7% v/v) of this solvent was added to the control treatment before the first ATP content assessment.

Sperm ATP content was measured using a luciferase-based ATP bioluminescent assay kit (Roche, ATP Bioluminescence Assay Kit HS II) based on the protocol of Tourmente et al. (Tourmente et al., 2019). Sperm suspension was diluted 1:10 in the corresponding medium, and a 100 μl aliquot of diluted sperm suspension was mixed with 100 μl of Cell Lysis Reagent and frozen in liquid N_2 . At the end of the experiment, all the aliquots were thawed at room temperature for 5 min. The resulting cell lysate was centrifuged at $12,000 \times g$ for 2 min, and the supernatant was recovered and frozen in liquid N_2 . Bioluminescence was measured in triplicate in 96-well plates using a luminometer (Biotek Synergy, Biotek Instruments Inc.). Auto injectors were used to add 50 μl of luciferase reagent to 50 μl of sample in each well, and, following a 1 s delay, light emission was measured over a 5 s integration period. Standard curves were constructed using ATP standards diluted with the corresponding media and Cell Lysis Reagent in a proportion equivalent to that of the samples. ATP content was expressed as amol sperm^{-1} .

Statistical analyses

All analyses were conducted using R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria), with $\alpha = 0.05$. To assess whether incubation conditions affected the percentage of capacitated sperm, we applied a generalized linear



mixed effect model (GLMM, *mixed* function, *afex* package) with binomial distributions and "logit" link function, including incubation conditions as a fixed factor with 2 levels (non-capacitating vs capacitating), and experiment as random factor. In the first set of EFAs the stability of the OCR and ECAR measures during the assay was assessed via linear mixed effect models (LMM, *mixed* function, *afex* package) using time as a fixed factor with 8 levels (one per measurement cycle), and experiment as random factor. In the second set of EFAs, we analyzed the effect of incubation conditions and metabolic modulators on OCR and ECAR expressed as percentages relative to the baseline using LMMs with incubation medium and treatment (basal, oligomycin, FCCP, 2DOG, and A + R) as fixed factors, and experiment as random factor. The inclusion of the experiment as a random factor allowed for the consideration of each well as an individual value in the statistical analyses, thus decreasing the impact of outliers and increasing the statistical powers of the tests by controlling for the between-experiment variability (Yepez et al., 2018). The values of the metabolic parameters calculated after EFAs were compared between incubation conditions using LMMs with incubation medium as a fixed factor and experiment as a random factor.

Non-linear regression models (*ngls* function, *nlme* package) were used to estimate sperm ATP consumption rates for sperm incubated in capacitating and non-capacitating conditions and subsequently treated with inhibitors. First, a general regression curve ($ATP = a \times e^{b \times \text{time}}$), based on the values of ATP content at the six sampling time points, was estimated for each condition

using the pooled measurements of the four experiments. The parameter "b" was defined as the ATP consumption rate, that is, the exponential rate of decline in ATP content as a function of time. In addition, a second ATP consumption rate was estimated for each individual sample and the "b" exponents of the curves were compared between conditions by means of an LMM with experiment as random factor.

All variables were \log_{10} -transformed for statistical purposes in all analyses with the exception of the values expressed as percentages, and the estimated ATP consumption rates. Significant differences between levels of fixed factors were analyzed via *post-hoc* estimated marginal means tests (*pairwise* function of the *emmeans* package).

Results

After 1 h of incubation post collection, approximately 50% of the live sperm incubated in capacitating conditions presented the pattern "B" of CTC staining. On the other hand, only 13% of the live sperm showed such pattern when incubated under non-capacitating conditions, confirming that incubation in capacitating medium promoted a significant increase in the percentage of capacitated sperm in the samples (mean \pm SE: 51.5 \pm 1.59 vs. 13.17 \pm 1.59; GLMM: $X^2 = 428.52$, $p < 0.001$; Figure 1).

The first set of EFAs showed that experimental time had no significant effects on OCR values before A + R addition in both incubation conditions (non-capacitating: LMM $X^2 = 4.13$, $p = 0.531$; capacitating: LMM $X^2 = 2.56$, $p = 0.768$); Supplementary Figures S3A,C). Similarly, experimental time did not have a significant effect on ECAR for any of the two incubation conditions (non-capacitating: LMM $X^2 = 1.59$, $p = 0.903$; capacitating: LMM $X^2 = 8.66$, $p = 0.123$); Supplementary Figures S3B,D). These results indicate that the modification applied to the EFA technique allowed us to obtain stable OCR and ECAR values for at least 54 min after introducing the samples into the XFp analyzer.

The OCR and ECAR profiles for the second set of EFAs in both incubation conditions are shown in Figure 2. In both non-capacitating and capacitating sperm, cells responded as expected to the injection of metabolically active compounds (Figure 2; Table 1). Thus, OCR significantly increased when exposed to the uncoupler FCCP, decreased when exposed to the ATP synthase inhibitor oligomycin, and further diminished when exposed to antimycin and rotenone (Figures 2A,B; Table 1). Furthermore, sperm OCR was reduced when exposed to the glycolytic inhibitor 2DOG. ECAR values decreased as expected in response to the addition of 2DOG in both incubation conditions, and increased when respiration was inhibited by the addition of oligomycin (Figures 2C,D; Table 1).

Sperm populations incubated under both conditions showed qualitatively similar responses to metabolic modulators when

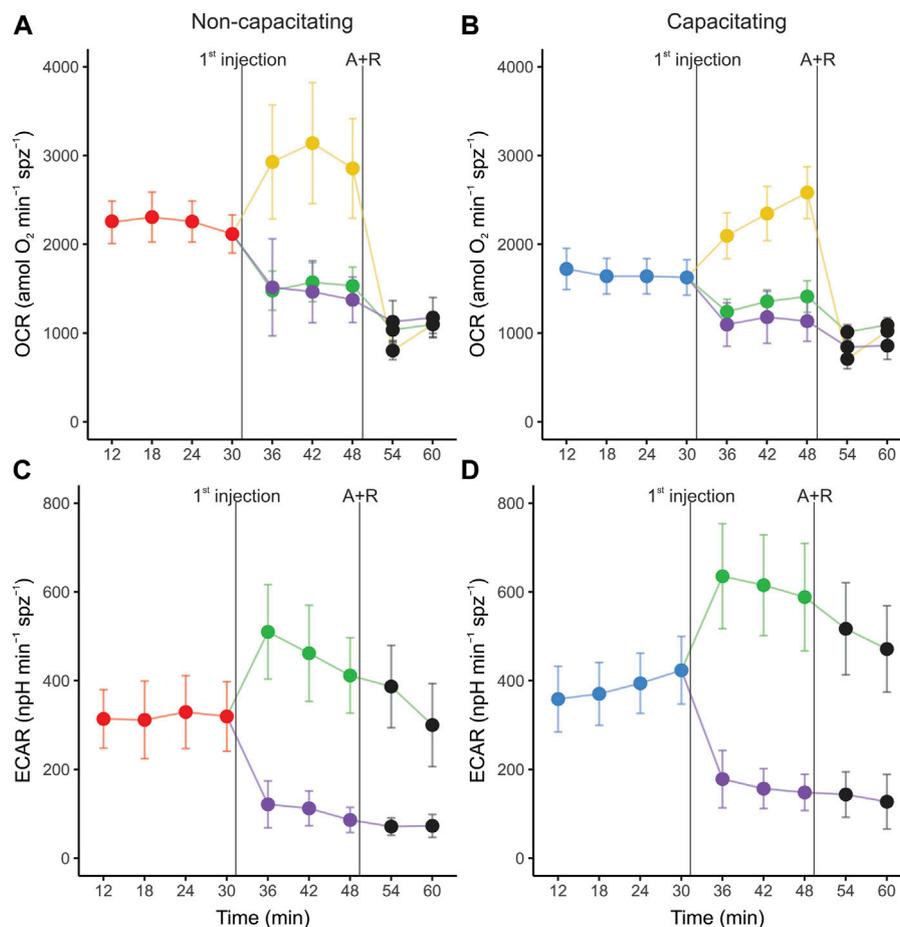


FIGURE 2

Real-time measurements of oxygen consumption rate (OCR) (A,C) and extracellular acidification rate (ECAR) (B,D) in mouse sperm before and after treatment with metabolic modulators. Sperm were incubated for 1 h in non-capacitating (A,C) and capacitating (B,D) conditions prior to extracellular flux analysis. Values have been normalized by sperm numbers inside each well. Symbols and whiskers correspond to means \pm standard error. Time = 0 was defined as the start of the first measurement cycle; measurement cycles 3 to 11 are reported. The line labeled as "1st injection" marks the addition of either 5 μ M oligomycin, 1 μ M FCCP, or 50 mM 2DOG; the line labeled "A + R" marks the addition of 1 μ M antimycin A + 1 μ M rotenone. Red and blue symbols: values before any addition (basal state) in non-capacitating and capacitating conditions respectively; green symbols: after oligomycin injection; yellow symbols: after FCCP injection; purple symbols: after 2DOG injection; black symbols: after A + R injection.

OCR and ECAR were expressed as percentages relative to the basal state. Exposure to oligomycin caused a reduction of approximately 71% in OCR (Figures 3A, 4A, Table 1). The addition of 2DOG promoted a decline in OCR that was similar to that produced by exposure to oligomycin (~70%) (Figures 3C, 4A; Table 1) and a decrease of approximately 60% in ECAR (Figures 3E, 4B; Table 1). On the other hand, some treatments elicited different responses in non-capacitating and capacitating sperm. The increase in OCR associated to the addition of FCCP was significantly higher in sperm incubated under capacitating conditions (319 % vs. 192%) (Figures 3B, 4A; Table 1). Also, the rise in ECAR as a response to oligomycin in sperm incubated under capacitating

conditions surpassed that of sperm incubated in non-capacitating conditions (90 % vs. 60%) (Figures 3D, 4B; Table 1).

Clear differences between incubation conditions emerged when the OCR and ECAR measurements were used to calculate and compare a range of metabolic parameters (Supplementary Table S1). Sperm incubated under non-capacitating conditions presented higher values in nearly all parameters associated with respiratory metabolism (basal respiration rate, proton leak, respiratory ATP production, and maximum respiration rate) (Figures 5A–D), with the exception of spare respiratory capacity, which did not show any significant differences between incubation conditions (Figure 5E). It should be noted that FCCP-stimulated OCR did not stabilize within the

TABLE 1 Effect of incubation conditions and metabolic modulators on mouse sperm OCR and ECAR. Independent variables were tested as percentages relative to the basal state. X^2 and p values were estimated using LMMs and likelihood ratio tests. Incubation medium (non-capacitating or capacitating) and treatment (basal, 5 μ M oligomycin, 1 μ M FCCP, 50 mM 2DOG, and 1 μ M antimycin +1 μ M rotenone) were used as fixed factors and experiment as random factor. Significant differences ($p < 0.05$) are shown in boldface.

Dependent variable	Independent variable	X^2	P
OCR (%)	Incubation medium	44.07	<0.001
	Treatment	673.72	<0.001
	Interaction	113.44	<0.001
ECAR (%)	Incubation medium	16.71	<0.001
	Treatment	325.89	<0.001
	Interaction	34.65	<0.001

three measurements taken in sperm incubated in capacitating conditions. Thus, there is the possibility that maximum

respiration rate and spare respiratory capacity are underestimated in these cells. Conversely, the two parameters associated with aerobic glycolysis (basal glycolysis rate and glycolytic reserve) showed higher values in sperm incubated under capacitating conditions (Figures 5G,H). The OCR/ECAR ratio supported this trend since it was significantly higher in the sperm incubated in non-capacitated conditions (Figure 5F). In all, these results confirm the occurrence of a switch in the usage ratio of the two energy supply pathways as a consequence of sperm capacitation.

Incubation conditions did not produce significant variations in the ATP content of sperm that had not been treated with metabolic inhibitors (LMM $X^2 = 0.45$, $p = 0.503$). However, these cells showed a slight (~20%) but significant reduction in ATP content throughout the experimental time (Figure 6A) (LMM $X^2 = 5.60$, $p = 0.018$). Conversely, sperm incubated in the presence of inhibitors showed a severe and sustained decrease in ATP content over time in both incubation conditions (Figure 7A). Non-linear regression analyses performed by pooling the measurements of the five individuals yielded ATP consumption curves that significantly adjusted to the “ATP = a x

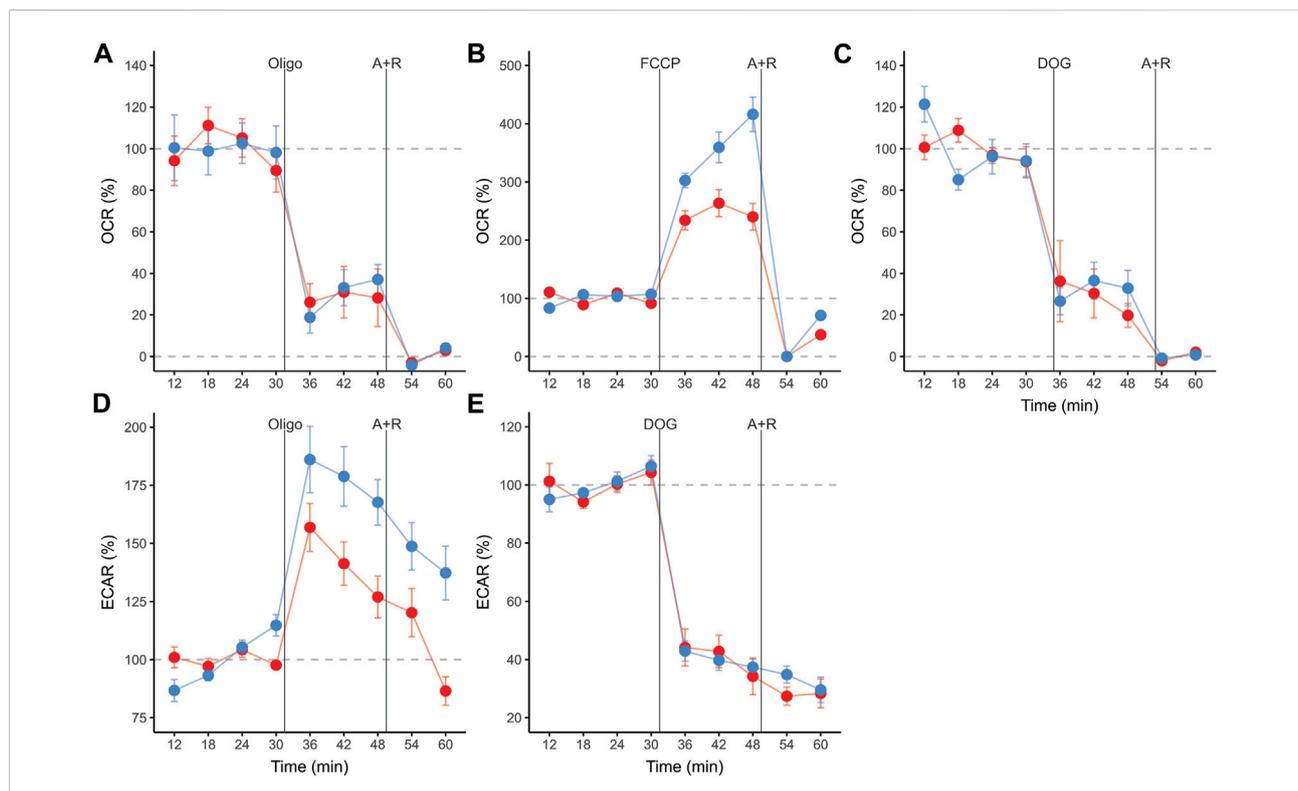
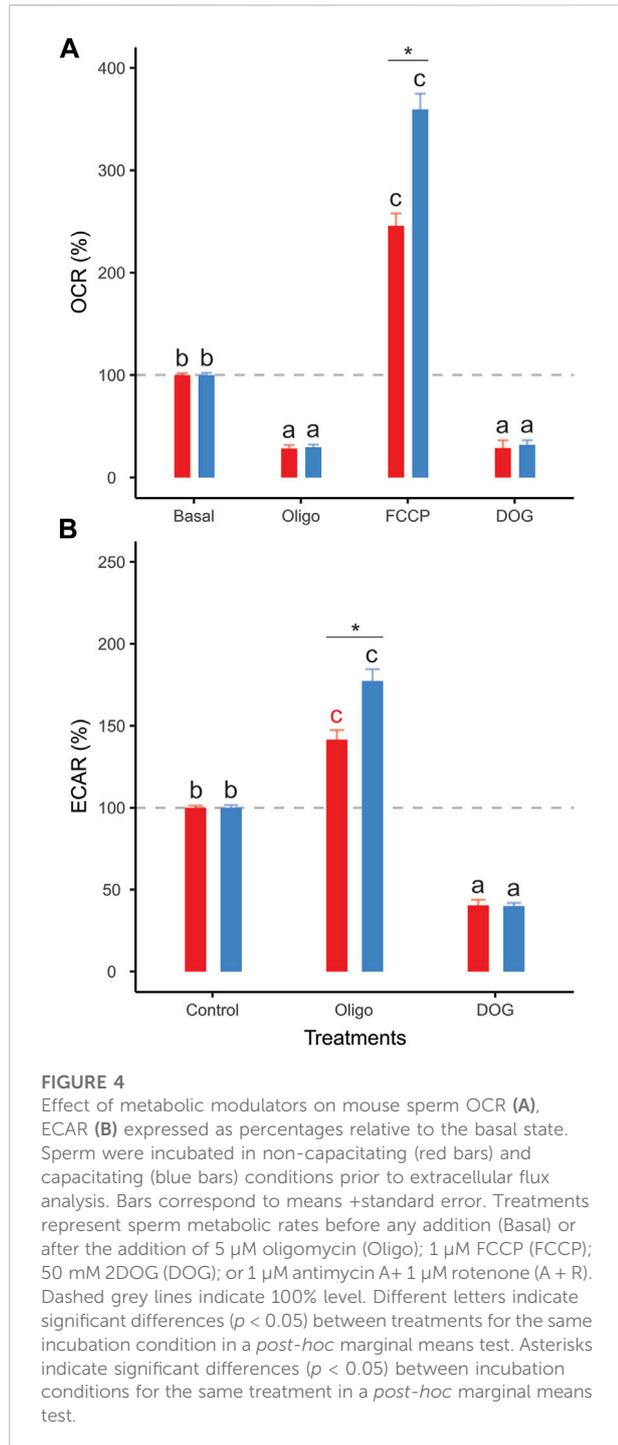


FIGURE 3

Real-time measurements of oxygen consumption rate (OCR) (A,B,C), and extracellular acidification rate (ECAR) (D,E) in mouse sperm before and after treatment with metabolic modulators. Values are expressed as percentages relative to the basal state (100%), which was defined as the mean of the four measurement cycles before any addition and is represented by the horizontal dashed line. Sperm were incubated for 1 h in non-capacitating (red symbols) and capacitating (blue symbols) conditions prior to extracellular flux analysis. Symbols and whiskers correspond to means \pm standard error. Time = 0 was defined as the start of the first measurement cycle; measurement cycles 3 to 11 are reported. Dashed grey lines indicate 100 % and 0% levels. The first vertical line marks the addition of either 5 μ M oligomycin, 1 μ M FCCP, or 50 mM 2DOG; the line labeled “A + R” marks the addition of 1 μ M antimycin A+ 1 μ M rotenone.



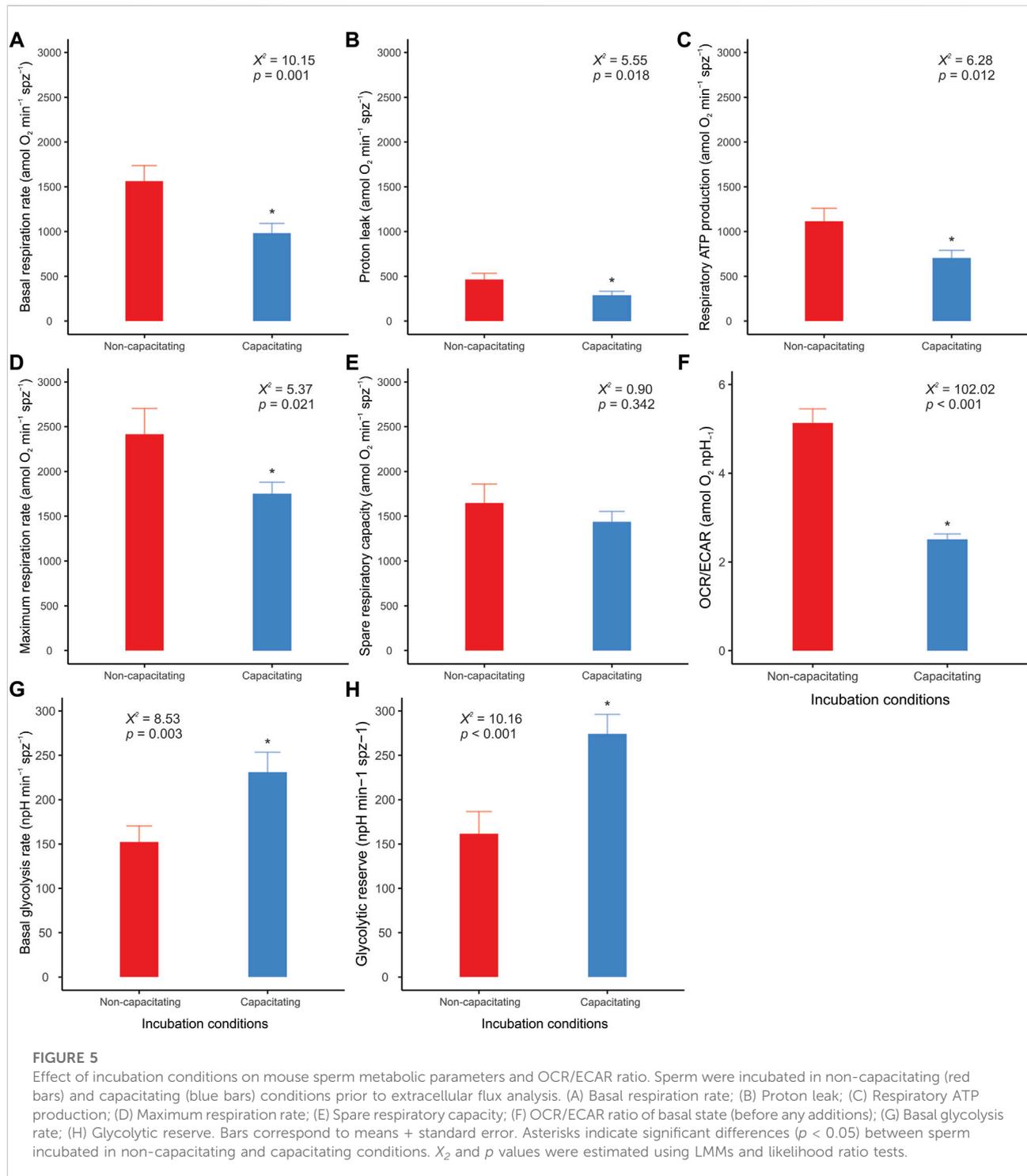
$e^{b \times \text{time}}$ model in both incubation conditions (non-capacitating: $\text{ATP} = 37.09 \times e^{-0.21 \times \text{time}}$, $F_b = 50.25$, $p_b < 0.001$; capacitating: $\text{ATP} = 30.50 \times e^{-0.19 \times \text{time}}$, $F_b = 32.33$, $p_b < 0.001$) (Figure 6B). In order to compare ATP consumption between incubation conditions, we estimated ATP consumption curves for each individual in each condition and compared the “ b ” exponents of the curve. There were no significant differences (LMM $X^2 =$

0.15, $p = 0.712$) between the values of the exponents from curves estimated for non-capacitating ($b = -0.20 \pm 0.02$) and capacitating conditions ($b = -0.19 \pm 0.03$).

Discussion

The results of our study revealed that the development of capacitation in mouse sperm is associated with changes in the energy metabolism of these cells. In the present study, sperm capacitation was achieved through the incubation of spermatozoa for 1 h in a medium containing BSA, Ca^{2+} , and NaHCO_3 . Sperm capacitation was confirmed by CTC staining in live cells. These incubation period and medium components necessary for murine sperm capacitation have been described in previous studies (Shi and Roldan, 1995; Visconti et al., 1995a; Visconti et al., 1995b; Sansegundo et al., 2022), and the changes in CTC staining patterns have been shown to strongly correlate to markers of sperm capacitation, such as progesterone-induced acrosome reaction (Gomendio et al., 2006) and hyperactivated movement (Sansegundo et al., 2022). Thus, we can confidently infer that the observed rise in CTC pattern “B” after incubation in capacitating conditions was indicative of an increased proportion of capacitated cells within the sample. Moreover, sperm populations incubated under capacitating conditions are a mix of capacitated and non-capacitated cells and, thus, the metabolic variations observed as a consequence of sperm capacitation are most likely an underestimation since they are attenuated by the presence of non-capacitated sperm.

In the present study, the increase in the proportion of capacitated cells in the sperm populations was associated with a change in their metabolic phenotype (Figure 7A). Parameters associated with respiratory metabolism (basal respiration, proton leak, respiratory ATP production, and maximal respiration) presented higher values in sperm populations with a low percentage of capacitated cells. Conversely, sperm populations enriched in capacitated sperm relied more on glycolysis, as evidenced by their higher basal glycolysis and glycolytic reserve values. This change in metabolic profile is summarized in the OCR/ECAR ratio, which decreases by 50% after incubation under capacitating conditions. It should be borne in mind that the magnitude of this metabolic switch may be underestimated by the nature of the measuring system (i.e., using ECAR as a proxy of the rate of lactate formation) since HCO_3^- formation, derived from the production of CO_2 via OXPHOS, will contribute to total ECAR in variable proportions depending on cell type and substrate (Mookerjee et al., 2015). In our study, the decrease in sperm O_2 consumption (OXPHOS) associated with capacitation leads to a stoichiometric reduction in CO_2 production. Thus, the increase in ECAR from lactate excretion (glycolysis), which results from the metabolic switch associated with capacitation, is partially attenuated because the CO_2 contribution has diminished.



Sperm incubated in both media (capacitating and non-capacitating), reacted as expected to the addition of metabolic modulators. While OCR decreased in response to oligomycin or antimycin plus rotenone and increased in response to FCCP, ECAR was reduced when sperm were exposed to 2DOG. These results allowed us to validate the robustness

of the technique, and to calculate, for the first time in mouse sperm, a range of relevant metabolic parameters that allow for a comparison between studies and species. Importantly, this is, to the best of our knowledge, the first study to achieve robust metabolic measurements using XF EFA technology in freely moving sperm, since previous work using these techniques

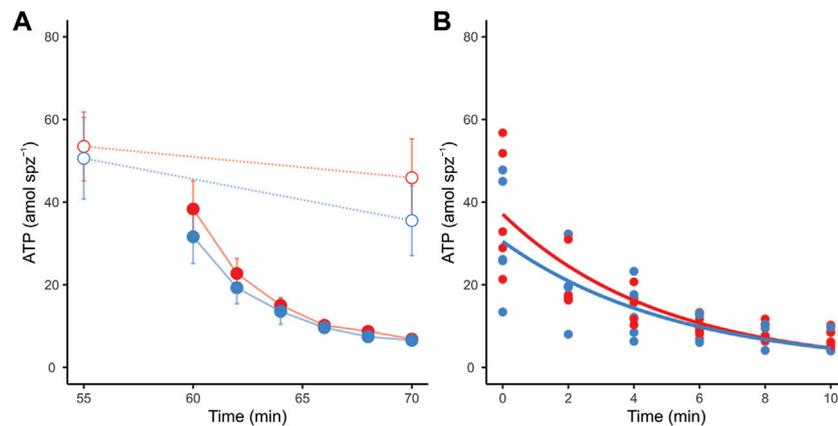


FIGURE 6

ATP consumption in mouse sperm in non-capacitating (red symbols and lines) and capacitating (blue symbols and lines) conditions. **(A)** ATP content in response to the addition of metabolic inhibitors to sperm in both incubation conditions. Dotted lines and open symbols represent control conditions (no inhibitors added); continuous lines and filled symbols represent metabolically inhibited sperm (a combination of 5 μ M oligomycin, 50 mM 2DOG, 1 μ M antimycin A, and 1 μ M rotenone was added). **(B)** Lines represent estimated sperm ATP consumption curves of metabolically inhibited sperm in non-capacitating ($ATP = 37.09 \times e^{-0.21 \times \text{time}}$) and capacitating ($ATP = 30.50 \times e^{-0.19 \times \text{time}}$) conditions. Symbols represent individual measurements.

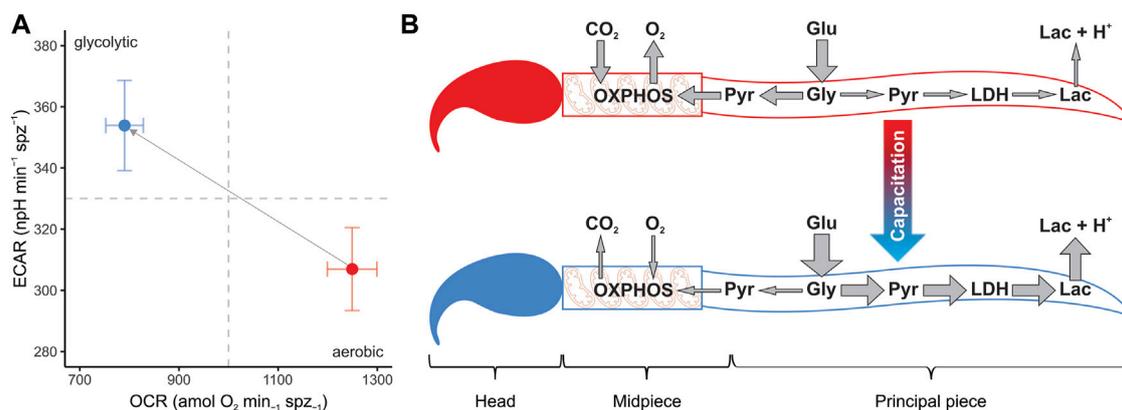


FIGURE 7

Changes in metabolic phenotype promoted by sperm capacitation in murine sperm. **(A)** Metabolic phenotype plot of mouse sperm depicting the usage of the two main metabolic pathways (OXPHOS and anaerobic glycolysis). Symbols and whiskers correspond to means \pm standard error. Red symbols: non-capacitating conditions. Blue symbols: capacitating conditions. Dashed grey lines indicate the midpoint in the measurement range for both variables. **(B)** Graphical depiction of conclusions. Capacitation promotes a metabolic shift in the mouse sperm, increasing the uptake of glucose and altering the proportion of pyruvate that enters each metabolic pathway. A greater proportion of pyruvate is reduced into lactate by the enzyme lactate dehydrogenase and transported to the extracellular environment along with protons, while a relatively minor proportion is transported to the mitochondria in the midpiece, thereby entering the OXPHOS pathway. Lac: lactate. LDH: lactate dehydrogenase. Pyr: pyruvate. Gly: glycolysis. Glu: glucose.

necessitated the use of attached cells (Tourmente et al., 2015a; Magdanz et al., 2019; Balbach et al., 2020).

Whereas the metabolism of mouse sperm responded similarly to metabolic modulation regardless of incubation conditions, there were quantitative differences in the level of these responses associated with capacitation. The increased

elevation of glycolytic rate by capacitated sperm in response to OXPHOS inhibition (glycolytic reserve) was caused by a more pronounced reaction from a higher basal glycolytic rate. On the other hand, the proportionally higher stimulation of the respiratory chain exhibited by sperm in capacitating medium was a consequence of their decreased basal respiration rate.

This evidence further supports the notion of a metabolic rewiring of capacitated spermatozoa in favor of aerobic glycolysis over OXPHOS.

A recent EFA study on mouse sperm (Balbach et al., 2020) revealed an increase in rates of OXPHOS and aerobic glycolysis that were both concomitant with the emergence of capacitation markers, and with an increase in glucose uptake. Although at first hand, the observations in this earlier study (Balbach et al., 2020) seem contradictory to our results, there are important differences between the experimental designs of both studies that make comparisons difficult. The measurements in Balbach *et al.* (Balbach et al., 2020) were obtained by subjecting sperm to capacitating conditions within the extracellular flux analyzer, with capacitation being stimulated pharmacologically via the addition of a cAMP analog (db-cAMP) and a phosphodiesterase inhibitor (IBMX) in the absence of NaHCO_3 . In our case, sperm were preincubated for 1 h under capacitating conditions (including NaHCO_3) before placing them in the analyzer, and extracellular flux measurements were performed after sperm capacitation had occurred in a high proportion of cells in the population. Although pharmacological elevation of cAMP levels is sufficient to promote the protein phosphorylation events associated with capacitation in mouse sperm (Visconti et al., 1995a; Visconti et al., 1995b; Balbach et al., 2020), the absence of HCO_3^- might affect other capacitation-related processes that depend on the concentration of this ion (Zeng et al., 1996; Demarco et al., 2003; Guerrero et al., 2011; Nishigaki et al., 2014), such as membrane hyperpolarization, intracellular pH increase, and hyperactivation, and could impact the activity of metabolic pathways (Kamp et al., 2003; Mannowetz et al., 2012; Shahrokhi et al., 2020). In addition, the effects of sperm capacitation on sperm metabolism found in the present study are smaller in magnitude than those reported by Balbach et al. (2020). The changes in metabolic activity following pharmacologically-stimulated capacitation ranged between 5 and 7-fold (for OCR) and 4-5-fold (for ECAR) in their study. In the present study, incubation under capacitating conditions resulted in a decrease of 37% in basal respiration rate and an increase of 52% in basal glycolysis rate. Finally, comparison between treatments in the previous study is problematic since phenomena such as non-mitochondrial oxygen consumption, and non-glycolytic acidification were not estimated by subtracting values after inhibition from basal measurements.

As an alternative to a general increase in sperm metabolism linked to capacitation (Balbach et al., 2020), our results suggest that sperm capacitation promotes a change in the usage ratio of the two main metabolic pathways, from a highly oxidative metabolic phenotype in non-capacitated sperm to a predominantly glycolytic one in

capacitated sperm (Figure 7B). Since oxidative phosphorylation yields more ATP per molecule of glucose consumed than aerobic glycolysis, the evidence does not support the hypothesis that sperm would be expected to reorient their metabolism to pathways that produce ATP more efficiently in response to increased ATP demands associated with capacitation (Travis et al., 2004; Ishijima, 2011). Nonetheless, some caveats could be raised.

Recent studies have found that mouse sperm capacitation is associated with increased glucose uptake (Hidalgo et al., 2020) and that active glycolysis is crucial for the maintenance of ATP concentration and bending capacity in the distal end of the flagellum (Takei et al., 2014). This suggests that 1) ATP produced in the mitochondria does not diffuse sufficiently fast in order to reach the end of the flagellum, and 2) local ATP provision is a crucial physiological variable for the motility of these cells. According to our results, capacitated mouse sperm do not appear to engage in increased ATP consumption and are able to maintain ATP levels similar to those of non-capacitated cells. Considering that aerobic glycolysis occurs along most of the principal piece, a high glycolytic rate would provide a metabolic alternative to ensure sufficient ATP supply if physiological events associated with sperm capacitation (ion transport, protein phosphorylation, or hyperactivated movement) resulted in modifications of the local ATP demands in specific flagellar regions. Local ATP generation by mechanisms other than OXPHOS is relatively common in cellular structures with high energetic demands whose dimensions or structure make them unable to accommodate mitochondria (Villar et al., 2021). In addition, it should also be borne in mind that EFA and ATP consumption analyses were performed in sperm populations with a high proportion of capacitated sperm (Sansegundo et al., 2022) and, thus, early processes that take place during the acquisition of capacitation, and that may affect energy metabolism, have been excluded from this study.

In coincidence with previous results (Balbach et al., 2020), inhibition of glycolysis resulted in a decrease of OXPHOS rates even when the culture medium was supplemented with respiratory substrates, thus indicating that the substrate used to fuel the Krebs cycle in the mitochondria of these cells is necessarily of endogenous origin. In support of this interpretation, earlier studies revealed that in a medium containing glucose, the monocarboxylate transporters expressed in the flagellar membranes of mouse sperm (MCT2) are predominantly used to export lactate instead of importing respiratory substrates (Garcia et al., 1995; Boussouar et al., 2003; Mannowetz et al., 2012). In all, the alteration of the OCR/ECAR ratio promoted by capacitation might be simply regarded as an increase in the proportion of the pyruvate that, after its generation in the principal piece, is exported as lactate instead of being transported to the mitochondria to be oxidized (see

Figure 7B for a graphical conclusion). While the diversion of potential OXPHOS substrates to an excretion pathway might appear as a waste of energetic resources for the cell, the reduction of pyruvate by lactate dehydrogenase (LDH) to produce lactate involves the regeneration of the NAD⁺ pool (from oxidized NADH), a crucial requirement for the maintenance of high glycolytic rates (Yeung et al., 2019; Luengo et al., 2021).

Recent publications have shown that mouse sperm increase their mitochondrial membrane potential (MMP) during incubation under capacitating conditions (Yang et al., 2020; Ferreira et al., 2021; Giaccagli et al., 2021) and that this event appears to be necessary to achieve hyperactivated motility (Ferreira et al., 2021; Giaccagli et al., 2021). These findings might appear to contradict our results since they were interpreted as a sign of higher OXPHOS in capacitated cells. In other words, high MMP was associated with elevated mitochondrial ATP synthesis. However, H⁺ reentry through the ATP synthase during ADP phosphorylation causes a drop in MMP with the concomitant compensatory increase in oxygen consumption, thus, higher ATP synthesis rates result in lower MMP (Brand and Nicholls, 2011). In bioenergetic terms, the transition from state 3 (active ATP synthesis) to state 4 (e.g., oligomycin inhibited ATP synthesis) would in turn lead to MMP hyperpolarization (Brand and Nicholls, 2011; Sanderson et al., 2013; Zorova et al., 2018). Thus, the increased MMP exhibited by capacitated mouse sperm when compared to non-capacitated (Yang et al., 2020; Giaccagli et al., 2021) cells would be consistent with the decrease in the oxygen consumption rates observed in our study.

Our study also revealed that inhibition of mitochondrial ATP production with oligomycin resulted in increased lactate excretion rates. This phenomenon could be due to the conversion to lactate of the excess pyruvate that remains in the cytosol (via LDH) since its usage in the Krebs cycle would be diminished as a consequence of ATPase inhibition. Alternatively, there is evidence showing that a decrease in ATP concentration produced by OXPHOS inhibition leads to the activation of the AMP protein kinase (AMPK) which, among other effects, promotes an increase in catabolic processes (i.e., glycolysis) (Martin-Hidalgo et al., 2018). Moreover, mouse sperm have been revealed to increase their glucose uptake rates under physiological conditions (Hidalgo et al., 2020). Thus, mouse sperm would be capable of shifting the burden of ATP supply to glycolysis when respiration is insufficient to meet the energetic demands of the cell. Various degrees of metabolic flexibility are observed in cell types that are frequently subjected to unpredictable environments (Mookerjee et al., 2016; Bekebrede et al., 2021). In the case of mouse sperm, this would represent a significant evolutionary advantage since it would allow them to sustain an adequate ATP supply during variations in availability and concentration of metabolic substrates that are known to exist within the female

reproductive tract (Gardner and Leese, 1990; Harris et al., 2005). On the other hand, mouse sperm seem unable to perform the reverse compensation (i.e., increasing respiratory rate when glycolytic ATP production is unavailable) since the inhibition of glycolysis by 2DOG produces a decrease in OXPHOS of a similar level to that caused by oligomycin. The inability of mouse sperm to utilize exogenous pyruvate and their dependence on glycolysis to provide endogenous respiratory substrates has been previously reported (Balbach et al., 2020), and it seems to be at odds with the significant presence of pyruvate and lactate in the mouse uterine, oviductal, and follicular fluids (Gardner and Leese, 1990; Harris et al., 2005).

In conclusion, our study has revealed that mouse sperm exhibit a shift in the usage ratio of the main metabolic pathways, from oxidative to glycolytic, as a consequence of capacitation. These changes do not seem to affect intracellular ATP concentration and ATP consumption rates. Thus, the probable function of this metabolic switch is not to increase overall ATP production, but to sustain ATP levels while ensuring the local provision of ATP to flagellar regions with specific ATP demands that arise from capacitation. The molecular mechanisms that govern and regulate these phenomena are still poorly understood, and further research is needed to address these issues.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by National Museum of Natural Sciences, Spanish Research Council and Madrid Regional Government.

Author contributions

ERRS, ER, MT, and ES conceived the study. ES and MT performed experiments. MT, ES, and ERRS analyzed data. MT, ES, ER, and ERRS wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.950979/full#supplementary-material>

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