REPRODUCTION
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Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation

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Abstract

To investigate the hypothesis that oxidative phosphorylation is a major source of ATP to fuel stallion sperm motility, oxidative phosphorylation was suppressed using the mitochondrial uncouplers CCCP and 2,4,-dinitrophenol (DNP) and by inhibiting mitochondrial respiration at complex IV using sodium cyanide or at the level of ATP synthase using oligomycin-A. As mitochondrial dysfunction may also lead to oxidative stress, production of reactive oxygen species was monitored simultaneously. All inhibitors reduced ATP content, but oligomycin-A did so most profoundly. Oligomycin-A and CCCP also significantly reduced mitochondrial membrane potential. Sperm motility almost completely ceased after the inhibition of mitochondrial respiration and both percentage of motile sperm and sperm velocity were reduced in the presence of mitochondrial uncouplers. Inhibition of ATP synthesis resulted in the loss of sperm membrane integrity and increased the production of reactive oxygen species by degenerating sperm. Inhibition of glycolysis by deoxyglucose led to reduced sperm velocities and reduced ATP content, but not to loss of membrane integrity. These results suggest that, in contrast to many other mammalian species, stallion spermatozoa rely primarily on oxidative phosphorylation to generate the energy required for instance to maintain a functional Na^+/K^+ gradient, which is dependent on an Na^+-K^+ antiporter ATPase, which relates directly to the noted membrane integrity loss. Under aerobic conditions, however, glycolysis also provides the energy required for sperm motility.

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Introduction

The breeding season of domestic horses is constrained by both physiological and regulatory factors, such that adequate fertility of stallions and mares is critical to reproductive success. However, in contrast to other domestic species, stallions are selected primarily on the basis of pedigree, performance and conformation, with little consideration given to reproductive soundness or fertility (Varner *et al.* 2015). This has contributed to the marked variability in fertility and semen quality seen between individual stallions and breeds, and male factor sub-fertility is a common problem in some horse breeds (Peña *et al.* 2011). Spermatozoa are highly specialized cells with the primary task of delivering a haploid set of male chromosomes to the oocyte during fertilization. During their generation in the testes, spermatozoa lose most of their cell organelles, with the notable exceptions of the acrosome (derived from the Golgi apparatus) and the mitochondria, where the latter become concentrated in the mid-piece. In recent years, the mitochondria of stallion spermatozoa have become the focus of research interest. Alongside their role as generators of ATP, sperm mitochondria play important roles during fertilization, and in the regulation of sperm lifespan, via the activation of an apoptosis-like mechanism (Ortega-Ferrusola *et al.* 2009*a*, Amaral *et al.* 2014) thought to be linked to their role as a source of reactive oxygen species (Koppers *et al.* 2008). Moreover, the sensitivity of spermatozoa to osmotic stress (Macías-García *et al.* 2012) is linked to mitochondrial malfunction (low ATP levels distort the functional Na^+-K^+ gradient allowing water influx into the cell

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(Woo *et al.* 2000). Mitochondria dysfunction is also a potential source of reduced sperm quality in the stallion (Garcia *et al.* 2012, Pena *et al.* 2015, Plaza-Davila *et al.* 2015), and it has been proposed that evaluation of mitochondrial activity is a robust tool for assessing sperm function and the ability to withstand biotechnological procedures (Ortega-Ferrusola *et al.* 2009*a*, 2010, Martin-Munoz *et al.* 2015). Despite all these potential contributions to sperm function, the role of mitochondria in the regulation of sperm motility remains a point of discussion. It has been proposed that equine spermatozoa are highly dependent on oxidative phosphorylation for motility, whereas sperm from other species rely mostly on glycolysis to provide ATP for flagellar propulsion (Mukai & Okuno 2004, Nascimento *et al.* 2008, Mannowetz *et al.* 2012, Odet *et al.* 2013, Ferramosca & Zara 2014). Previously, we studied the effect of inhibiting the adenine nucleotide translocator (ANT), a specific ATP/ADP transporter, on sperm function (Ortega-Ferrusola *et al.* 2010). The ATN catalyses the transmembrane exchange of ATP, generated in the mitochondria by oxidative phosphorylation, for cytosolic ADP (Klingenberg 2008). Our findings suggested that equine spermatozoa depend primarily on oxidative phosphorylation for the maintenance of active motility. It is generally agreed that functional mitochondria are essential for normal stallion sperm function. To improve our understanding of the importance of oxidative phosphorylation as a source of ATP for stallion sperm motility, oxidative phosphorylation was suppressed using mitochondrial uncouplers (carbonyl cyanide m-chlorophenyl hydrazine; CCCP and 2,4, dinitrophenol; DNP), and by inhibiting complex IV and ATP synthase. In addition, we studied the role of glycolysis using 2-deoxyglucose (2-DG). As mitochondrial dysfunction can also lead to oxidative stress, we simultaneously monitored the production of reactive oxygen species. Our results suggest that, in contrast to many other mammalian species, stallion spermatozoa rely primarily on oxidative phosphorylation to generate the energy required for motility and the maintenance of membrane integrity, and that mitochondrial dysfunction may lead to a loss of motility via a decrease in ATP production in addition to an oxidative mechanism.

Material and methods

Reagents and media

Ethidium homodimer; 5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethyl benzimidazolyl carbocyanine iodine (JC-1); YoPro-1; CellRox Deep Red Reagent; Hoechst 33342 and the ATP detection Kit were all obtained from Molecular Probes. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), oligomycin-A, 2,4 dinitrophenol (DNP), sodium cyanide (NaCN), 2-deoxyglucose (2-DG) and all other chemicals were purchased from Sigma.

Semen collection and processing

Semen was collected from 7 Purebred Spanish horses (PRE) (three ejaculates each) individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. Stallions were maintained according to Institutional and European Animal Care Regulations (Law 6/2913 June 11th and European Directive 2010/63/EU), and semen was collected on a regular basis (two collections/week) throughout the 2013 and 2014 breeding seasons. All procedures used in this study were approved by the ethical committee of the University of Extremadura (Ref AGL2013-43211-R). Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina with an inline filter to eliminate the gel fraction. After collection, the semen was immediately transported to the laboratory for evaluation and processing. The ejaculate was extended 1:2 in INRA-96 diluent (IMV L'Aigle, France), centrifuged at 600 ***g*** for 10 min to remove the bulk of the seminal plasma and re-suspended at 40×10^6 spermatozoa/ml in BWW medium (Aitken *et al.* 2015) supplemented with 1% polyvinyl alcohol. All of the experiments followed a split sample design, with each ejaculate divided to yield control and treatment groups. After centrifugation, aliquots of stallion semen extended in BWW were supplemented with the different mitochondrial uncouplers or ATP inhibitors and incubated in a water bath at 37°C up to 3 h. Treatments included CCCP (0 (vehicle), 100 nM, 500 nM, 5 μM and 10 μM), 2,4-DNP (0 (vehicle), 10, 50, 200 and 400 μM), NaCN (0 (vehicle), 2, 5 and 10 mM), oligomycin-A (0 (vehicle), 30 and 60 μM). In an additional set of experiments, glucose was replaced by 2-DG. After 1 and 3 h of incubation, aliquots were removed for flow cytometric, computerized motility and ATP content analysis.

Sperm motility

Sperm motility and kinematics were assessed using a computer-assisted sperm motility analysis (CASA) system (ISAS: Proiser, Valencia, Spain) with samples loaded into 20 μm deep Leja chambers (Leja Products B.V. Nieuw Vennep, The Netherlands) placed on a warmed (37°C) stage. The analysis was based on the examination of 60 consecutive digitalized images in a lapsed time of 1 s (60 Hz) using a negative phase-contrast objective at a 100 times magnification. The number of objects incorrectly identified as spermatozoa was minimized retrospectively using the playback function. With respect to the motility parameters used, spermatozoa with a VAP <15 μm/s were considered to be immotile, whereas spermatozoa with a velocity >35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were considered to show linear motility and spermatozoa with a curvilinear velocity (VCL) >45 μm/s were designated as rapid. Absolute sperm motility parameters assessed by CASA included: curvilinear velocity (VCL; μm/s), linear velocity (VSL; μm/s) and mean path velocity (VAP; μm/s).

Flow cytometry

Flow cytometric analyses were conducted using a MACSQuant Analyser 10 (Miltenyi Biotech, Pozuelo de Alarcón, Spain) flow cytometer equipped with three lasers, emitting at 405, 488 and 635 nm and 10 photomultiplier tubes (PMTs); (excitation

405 nm, emission 450–450 nm band pass), (excitation 405 nm, emission 525–550 nm band pass), (excitation 488 nm, emission 525–550 nm band pass), (excitation 488 nm, emission 585–640 nm band pass), (excitation 488 nm, emission 655–730 nm; 655 nm long pass and split at 730 nm), (excitation 499 nm, emission 750 nm long pass), (excitation 635 nm, emission 655–730 nm band pass; 655 nm long pass and split at 730 nm) and (excitation 635 nm, emission filter 750 nm long pass). The system was controlled using MACSQuantify software (Miltenyi Biotech). Sperm subpopulations were made on the basis of two fluorescent properties and visualized as two-dimensional dot plots (using logarithmic increase of detected emission signals at a fixed PMT sensitivity setting). On the two-dimensional intensity dot-plots, fixed four quadrants were used to quantify the number of sperm in each subpopulation.

Confocal laser microscopy

Confocal microscopy was used to image sperm using a FV1000 spectral confocal microscope (Olympus). Sperm were stained with JC-1 and excited using a 488 nm Argon laser. PMTs were used for fluorescence detection using a 510–535 nm spectral interval for green fluorescence (low mitochondrial membrane potential) and a 580–620 nm spectral interval for orange–red fluorescence (high mitochondrial membrane potential). A water immersion 60× objective was used. Plate temperature was set to 30°C to decrease sperm motility. Acquisition time was set at 3.5 s and resolution at 1600 × 1600 pixels.

Simultaneous flow cytometric assessment of early membrane changes, viability and oxidative stress (reactive oxygen species, ROS)

The following stock solutions were prepared in DMSO: YoPro-1 (25 µM), ethidium homodimer-1 (1.17 mM), and CellROX (5 mM). Hoechst 33342 (1.62 mM in water) was used to identify spermatozoa and allow debris to be gated out of the analysis. A sperm suspension (1 mL) containing 5×10^6 spermatozoa/mL was stained with 1 µL YoPro-1, 1 µL CellROX and 0.3 µL Hoechst 33342. After thorough mixing, the sperm suspension was incubated at RT in the darkness for 25 min. The spermatozoa were then washed in PBS, and then incubated for 5 min with 0.3 µL ethidium homodimer before analysis in the flow cytometer. This staining protocol was a modification (Gallardo-Bolanos *et al.* 2014) of previously published protocols (Nunez-Martinez *et al.* 2007, Ortega-Ferrusola *et al.* 2009*b*,*c*) and distinguishes four sperm subpopulations while simultaneously measuring oxidative stress. The first subpopulation, positive for only Hoechst 33342, was considered to be alive and without any membrane alterations. The YoPro-1-positive cells, emitting green fluorescence, were considered to show signs of early damage indicating a shift to a different physiological state, because sperm membranes become slightly permeable during the first steps of damage, which enables YoPro-1 but not ethidium homodimer to cross the plasma membrane. Neither probe enters intact cells. Finally, two subpopulations of dead spermatozoa were also detected. These were either apoptotic (spermatozoa stained both with YoPro-1 and ethidium homodimer, emitting both green and red fluorescence) or necrotic (cells stained with ethidium homodimer only and emitting only red fluorescence). Spermatozoa exhibiting oxidative stress emit fluorescence in the far-red spectrum. The positive controls for oxidative stress were samples that were supplemented with 800 µM $FeSO_4$ and 200 µL of H_2O_2 (Sigma) to stimulate the Fenton reaction.

Evaluation of mitochondrial membrane potential (ΔΨm)

JC-1 is a amphipathic component has the unique ability to differentially label mitochondria with low versus those with high membrane potential and was used to monitor mitochondrial membrane potential, as described previously (Ortega-Ferrusola *et al.* 2009*a*). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength (590 nm) when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers that emit in the green wavelength (525–530 nm) when excited at 488 nm. Mitochondrial staining was performed by adding 0.5 µL of a 3 mM stock solution of JC-1 (in dimethyl sulfoxide (DMSO)) to 1 mL of a sperm suspension in PBS (5×10^6/mL). The samples were incubated at 37°C in the darkness for 40 min before flow cytometric analysis, and the staining patterns were also monitored using confocal laser microscopy.

Determination of intracellular ATP

Intracellular ATP content in sperm lysates was measured using the ATP determination Kit (A22066) following the manufacturer's instructions, as described previously (Balao-da Silva *et al.* 2013). The assay is based on the requirement of luciferase for ATP to produce light (emission maxima ~560 nm at pH 7.8). The ATP content was normalized to pM/100 µg of protein. Measurements were made in a Tecan Infinite M200 Microplate Reader (Tecan, Männedorf, Switzerland).

Statistical analysis

Three ejaculates were collected from each of the 7 individual stallions. All experiments were repeated at least three times with independent samples (three separate ejaculates from each of the seven stallions). The normality of the data was assessed using the Kolmogorov–Smirnoff test. As the data showed equivalence of variance, the results were analyzed by ANOVA followed by a Tukey post hoc test to perform pairwise comparisons (SPSS 19.0 for Mac). Differences were considered significant when $P < 0.05$, and are indicated as: $^*P < 0.05$ and $^{**}P < 0.01$. Results are displayed as mean ± S.D.

Results

Effect of CCCP and DNP, uncouplers of the mitochondrial proton gradient, on mitochondrial membrane potential of stallion spermatozoa

To establish the effect of CCCP and DNP on sperm mitochondria, a dose and time effect experiment

was conducted. Stallion sperm were incubated in increasing concentrations of CCCP and DNP for up to 3 h, and mitochondrial membrane potential was evaluated flow cytometrically after JC-1 staining. This staining allowed the differentiation of sperm with high inner mitochondrial membrane potential (IMMP; Fig. 1A) and low membrane potential (Fig. 1B). CCCP concentrations in excess of 500 nM significantly ($P<0.05$) decreased mitochondrial membrane potential in a dose-dependent manner after both 1 and 3 h of incubation (Fig. 2A and B, respectively). At a concentration of 100 nM, CCCP had no effect on mitochondrial membrane potential. By contrast, 1- and 3-h incubation with DNP had no effect on IMMP (Fig. 2C and D, respectively).

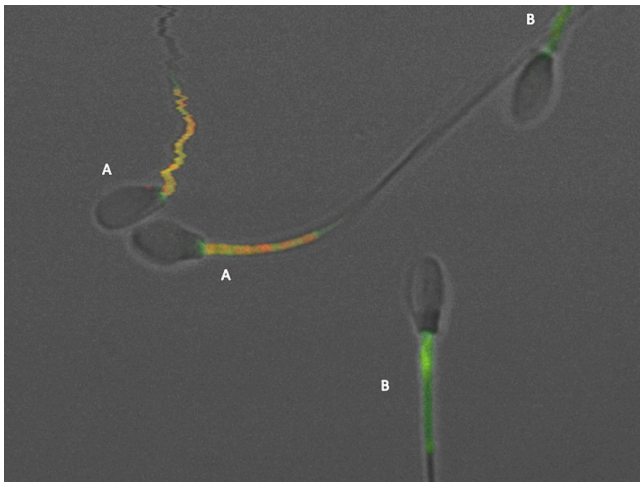

Figure 1 Confocal laser microscopic images of the staining patterns of stallion spermatozoa after loading with JC-1. A indicates spermatozoa with active mitochondria and high Δψm. B indicates spermatozoa with inactive mitochondria and low Δψm. 100× objective.

Inhibition of mitochondrial respiration reduces mitochondrial membrane potential

When complex IV was inhibited using sodium cyanide, there was a significant drop ($P<0.05$) after 3 h of incubation only, which was only evident at a sodium cyanide final concentration of 10 mM (Fig. 2F), with no effect after 1 h (Fig. 2E). Incubation of stallion spermatozoa in the presence of the F_0F_1-ATP synthase inhibitor oligomycin-A, resulted in a marked and rapid drop in IMMP (Fig. 2G (1 h of incubation) and H (3 h of incubation)). The percentage of spermatozoa showing low IMMP increased from 28% in control to 68 and 63% ($P<0.01$) in samples treated with 30 and 60 µM oligomycin-A, respectively.

Uncoupling the mitochondrial proton gradient reduces the ATP content of stallion spermatozoa

To determine the effect of mitochondrial uncoupling on ATP production, stallion sperm were incubated in the presence of CCCP and DNP before the assessment of ATP content. CCCP concentrations 500 nM and above reduced the ATP content of stallion spermatozoa (Fig. 3A). Concentrations of DNP above 400 µM also reduced ATP content of stallion spermatozoa (Fig. 3B).

Inhibition of mitochondrial respiration reduces intracellular ATP in stallion spermatozoa

Inhibition of complex IV using NaCN (2–10 nM) also resulted in a reduced ATP content (Fig. 3C). Inhibition of the F_0F_1-ATP synthase of stallion spermatozoa by incubation with oligomycin-A, resulted in a dramatic reduction of the ATP content of stallion spermatozoa (Fig. 3D).

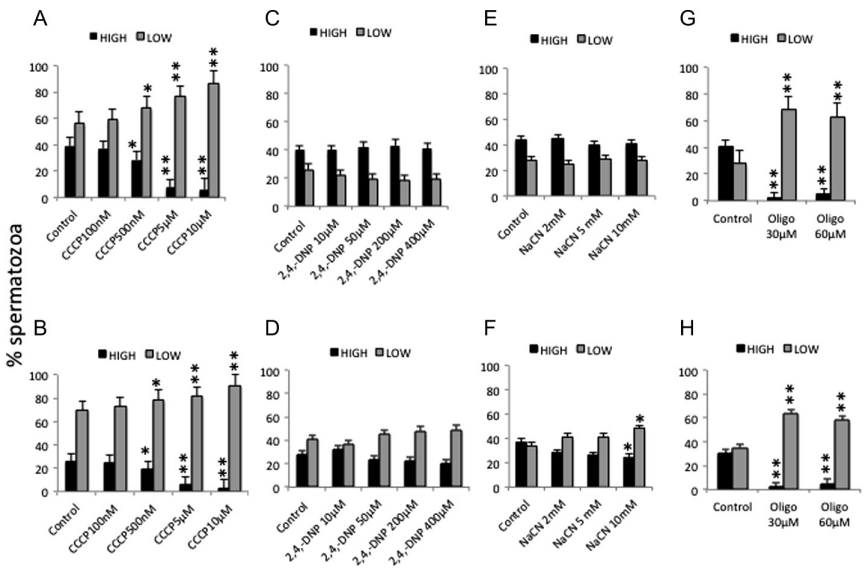

Figure 2 Effect of uncoupling agents; CCCP; (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration; NaCN (panels E and F) and oligomycin-A (panels G and H) on mitochondrial membrane potential, assessed using JC-1 staining. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to 3 h: panels A, C, E and G depict results after 1 h of incubation; panels B, D, F and H are results after 3 h of incubation at 37°C. Results are depicted as means ± S.D. Comparisons were made between each sample and its control. $n=7$ stallions. *$P<0.05$, **$P<0.01$.

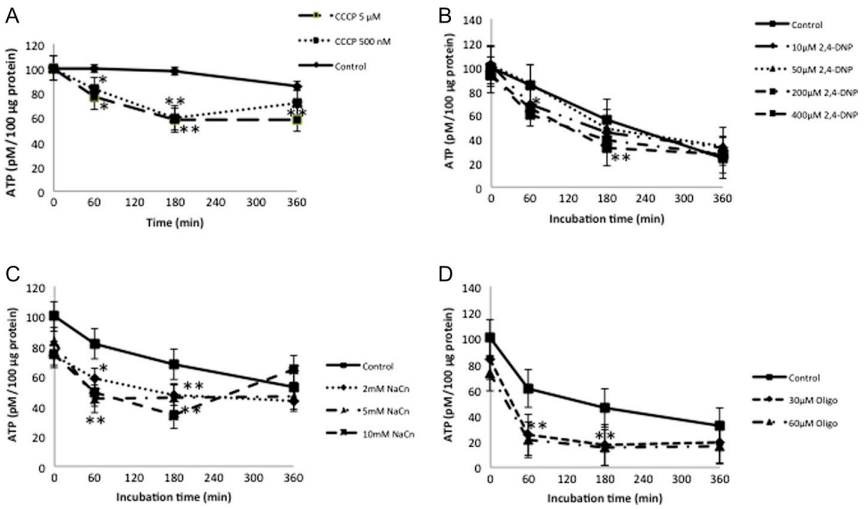

Figure 3 Effect of uncoupling agents CCCP (panel A) and DNP (panel B) and inhibitors of mitochondrial respiration NaCN (panel C) and oligomycin-A (panel D) on ATP content of stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors. Results are portrayed as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{**}P < 0.01$.

Effect of uncoupling the mitochondrial proton gradient on sperm motility and kinematics

When stallion spermatozoa were incubated in the presence of increasing doses of CCCP to uncouple mitochondrial activity, a marked reduction in overall sperm motility was observed in the presence of concentrations of 500 nM CCCP after 1 (Fig. 4A) and 3 h (Fig. 4B) of incubation ($P < 0.01$); this was accompanied by a concomitant reduction in the percentage of progressively motile sperm ($P < 0.05$) after 3 h of incubation, a reduction that was even bigger after only 1 h of incubation ($P < 0.01$). At 100 nM, CCCP only caused a decrease in the percentage of progressively motile sperm after 3 h of incubation ($P < 0.05$; Fig. 4). Mitochondria uncoupling also had a profound effect on sperm kinematics, with significant ($P < 0.05$) reductions in sperm velocity at CCCP concentrations >500 nM after both 1- and 3-h incubations (Fig. 5). DNP had a less pronounced effect; incubation of stallion sperm in the presence of 400 µM DNP reduced the percentage of total motile sperm after 1 and 3 h of incubation, but had no effect on the percentage of progressively motile sperm (Fig. 4). However, DNP did reduce the sperm velocity when present in the media at a final concentration of 400 µM (Fig. 5).

Inhibition of mitochondrial respiration dramatically reduces sperm motility and kinematics

Stallion sperm was incubated in the presence of the inhibitor of complex IV NaCN and the F_0F_1-ATP synthase inhibitor oligomycin-A. Both inhibitors had a dramatic effect on the percentage of motile and progressively

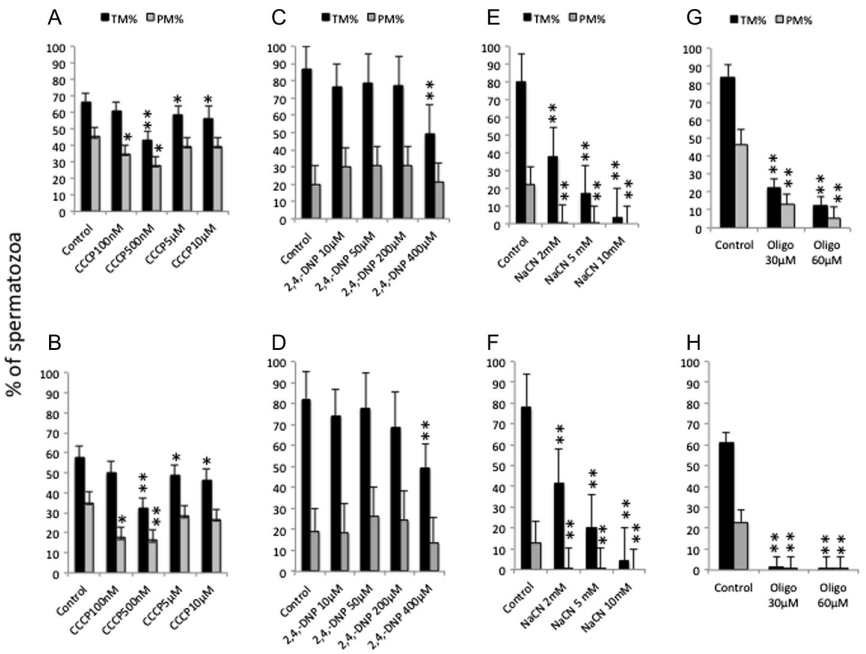

Figure 4 Effect of uncoupling agents CCCP (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration NaCN (panels E and F) and oligomycin-A (panels G and H) on the percentages of total (TM%) and progressively motile (PM%) spermatozoa measured by computer-assisted sperm analysis (CASA). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors, for up to 3 h. Results are depicted as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{**}P < 0.01$.

motile sperm. In the absence of the sodium cyanide, 80% of the sperm were motile. By contrast, in the presence of 2 mM NaCN, only 40% or less of the sperm were motile (Fig. 4E and F; for 1- and 3-h incubation, respectively; $P<0.01$). Progressive sperm motility was nearly abolished in the presence of this inhibitor (Fig. 4E and F). The ATP synthase inhibitor oligomycin-A also induced a dramatic drop in the percentages of total and progressively motile spermatozoa (Fig. 4G and H for 1- and 3-h incubation, respectively). In negative controls, 80% total and 46% progressive motilities were noted, whereas in the presence of 60 µM oligomycin-A, values fell to 12 and 4%, respectively, after 1 h of incubation at 37°C (Fig. 4G). Sperm motility was almost completely abolished after 3 h of incubation with 60 µM oligomycin-A (Fig. 4H).

The inhibitory effect on sperm velocity was more prominent when mitochondrial respiration was inhibited than when the mitochondrial proton gradient was uncoupled (Fig. 5). All tested doses of both inhibitors (oligomycin-A and NaCN) caused a significant reduction in sperm velocity ($P<0.01$).

Uncoupling the mitochondrial proton gradient has no effect on the percentage of intact sperm

When the mitochondrial proton gradient was inhibited by incubating stallion spermatozoa with CCCP or DNP, no effect on the percentage of spermatozoa with intact membranes was observed at any time of incubation examined (Fig. 6A, B, C and D).

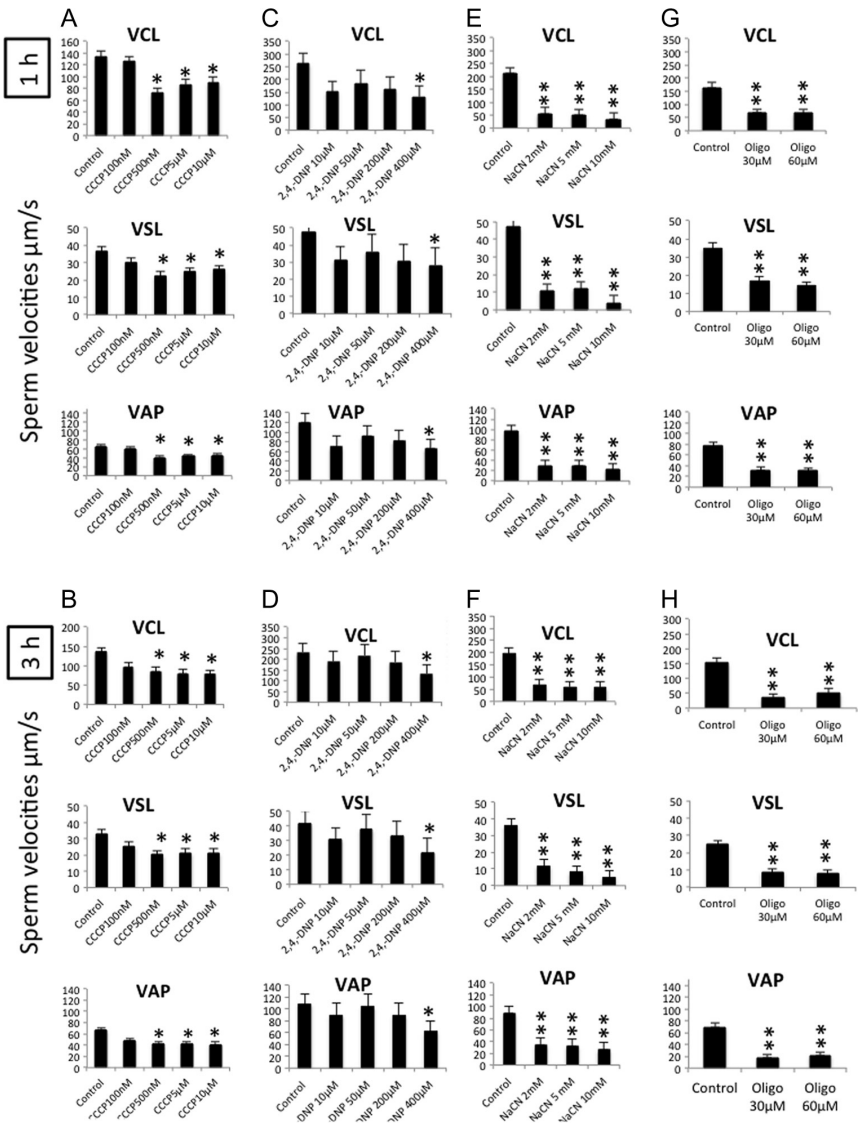

Figure 5 Effect of uncoupling agents CCCP (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration NaCN (panels E and F) and oligomycin-A (panels G and H)) on stallion sperm velocities after CASA analysis; VAP, average path velocity (µm/s); VCL, curvilinear velocity (µm/s); VSL, straight line velocity (µm/s). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to 3 h. Results are depicted as means ± S.D. Comparisons were made between each sample and its control. $n=7$ stallions. $^{*}P<0.05$, $^{**}P<0.01$.

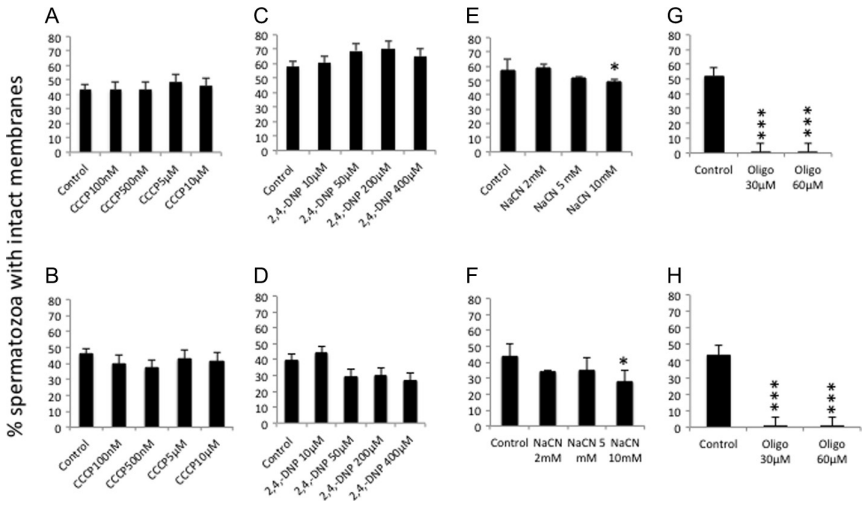

Figure 6 Effect of uncoupling agents CCCP (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration NaCN (panels E and F) and oligomycin-A (panels G and H) on the percentages of intact spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to 3 h. Results are depicted as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{***}P < 0.001$.

Uncoupling the mitochondrial proton gradient in stallion sperm allows trans-membrane influx of YoPro-1

To examine the sensitivity of sperm membrane integrity to mitochondrial uncoupling, stallion spermatozoa were incubated in the presence of CCCP and DNP. Neither uncouplers had affected membrane integrity during the incubation (Fig. 6). However, YoPro-1 fluorescence increased when CCCP was present at concentrations greater than 5 µM (Fig. 7A and B) and when DNP was present at a concentration of 400 µM, after 3 h of incubation (Fig. 7D), and DNP after 1 h of incubation had no effect (Fig. 7C).

Inhibition of mitochondrial respiration affects membrane integrity and permeability, with inhibition of ATP synthase inducing loss of sperm membrane integrity

When complex IV was inhibited (inducing the previously noted marked decrease in ATP content; Fig. 3), there was a significant drop in the percentage of spermatozoa with intact membranes after both 1 and 3 h of incubation (Fig. 6E and F); this drop was accompanied by an increase in the percentage of spermatozoa with increased membrane permeability (Fig. 7E and F). When ATP synthase was inhibited using oligomycin-A (causing the largest depletion of cellular ATP content; Fig. 3), membrane integrity was lost in nearly all sperm after 1- and 3-h incubations (Fig. 6G and H; ($P < 0.001$), this drop was due to both an increase in the permeability of the membrane (increased YoPro-1 influx), and complete loss of plasma membrane integrity (Fig. 7G and H)).

Uncoupling the mitochondrial proton gradient in stallion sperm increases or decreases reactive oxygen species (ROS) generation in a dose-specific fashion

As some effects of mitochondrial dysfunction may be due to increased ROS production, the effect of CCCP and DNP on ROS production was also assessed. CCCP increased ROS production after 1 h of incubation

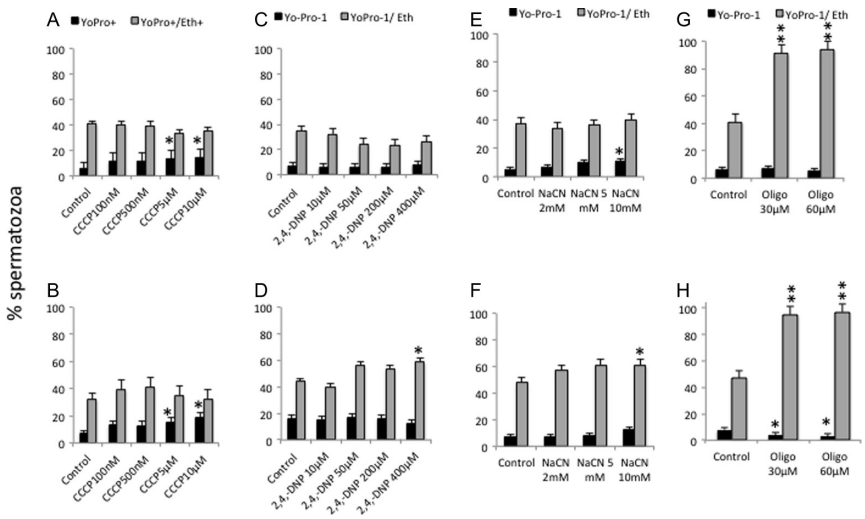

Figure 7 Effect of uncoupling agents CCCP (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration NaCN (panels E and F) and oligomycin-A (panels G and H) on the percentage of spermatozoa with signs of increased membrane permeability (YoPro+) or loss of membrane integrity (YoPro+ Eth+). Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to 3 h. Results are shown as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{**}P < 0.01$

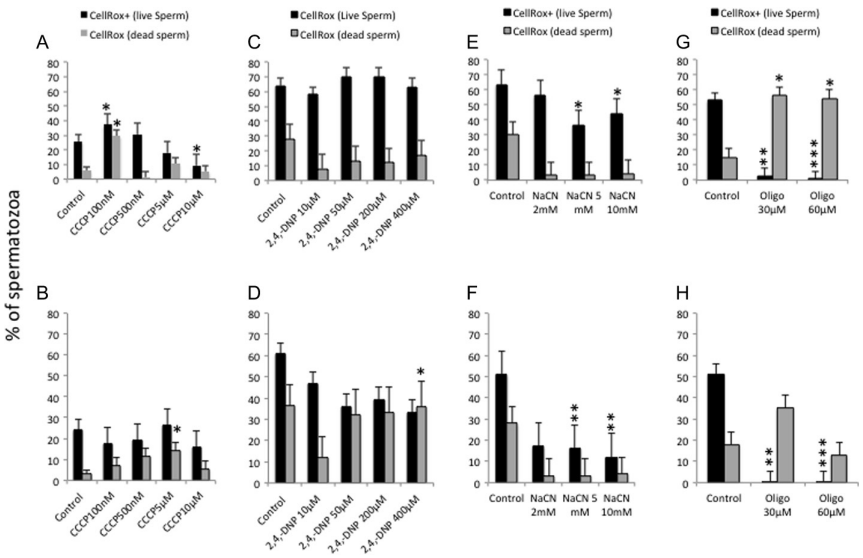

Figure 8 Effect of uncoupling agents CCCP (panels A and B) and DNP (panels C and D) and inhibitors of mitochondrial respiration NaCN (panels E and F) and oligomycin-A (panels G and H) on the production of reactive oxygen species (ROS) by stallion spermatozoa. ROS production was assessed flow cytometrically. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of the mitochondrial inhibitors for up to 3 h. Results are portrayed as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

at 100 nM but decreased ROS production at 10 μM (Fig. 8A). After 3 h of incubation, no effect of CCCP on ROS production was apparent when compared with control sperm, with the exception of an increase in ROS in dead cells in the presence of 5 μM CCCP (Fig. 8B). DNP only induced an increase in ROS production in membrane-deteriorated spermatozoa after 3 h of incubation (Fig. 8D).

Effect of inhibiting mitochondrial respiration on the production of ROS by stallion spermatozoa

Inhibition of complex IV resulted in a reduction of the percentage of live stallion spermatozoa showing elevated ROS production after both 1 ($P < 0.05$) and 3 h ($P < 0.01$) of incubation at 5 and 10 mM (Fig. 8D and E). When ATP synthase was inhibited using oligomycin-A, there was a dramatic decrease in ROS production in the live sperm subpopulation ($P < 0.001$), whereas in the population of spermatozoa with permeable membranes ROS production increased only after 1 h of incubation ($P < 0.01$) (Fig. 8F and G).

Inhibition of glycolysis decreases stallion sperm ATP content and reduces sperm motility and velocity, but has no effect on membrane integrity

Although ATP production is inhibited when mitochondrial aerobic function (oxidative phosphorylation) is inhibited, alternative glycolytic production of ATP may rescue sperm motility and preserve membrane integrity. To test the importance of glycolytic ATP production, stallion spermatozoa were incubated in the presence of 2-DG instead of glucose, to inhibit glycolysis. Incubating with 2-DG resulted in a reduction of ATP content in stallion spermatozoa (Fig. 9A). In addition, incubation with 2-DG resulted in a reduction in the percentage of total motile sperm, but not in the percentage of progressively motile spermatozoa (Fig. 9B and C). Sperm velocities were

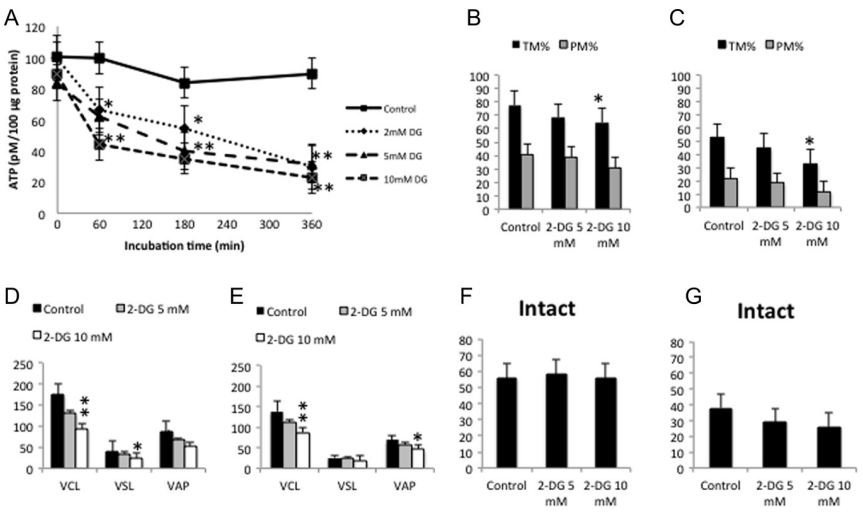

Figure 9 Effect of inhibition of glycolysis ATP content (panel A) on sperm motility (panel B) after 1 h of incubation, (panel C after 3 h) and velocities (panel D 1 h; panel E 3 h), and membrane intactness (panel F 1 h; panel G 3 h) in stallion spermatozoa. Samples were washed and resuspended in BWW supplemented with 1% PVA in the presence of 5 or 10 mM 2-DG for up to 3 h. Results are portrayed as means ± S.D. Comparisons were made between each sample and its control. $n = 7$ stallions. $^{*}P < 0.05$, $^{**}P < 0.01$.

also reduced in the presence of 2-DG (Fig. 9D and E), although membrane intactness was not affected (Fig. 9F and G).

Discussion

Mitochondrial oxidative phosphorylation takes place around the inner mitochondrial membrane and comprises the electron transport chain and ATP synthase. The respiratory chain generates a proton concentration gradient (change in pH) and a trans-membrane potential ($\Delta\Psi m$) across the inner membrane, which together allow the production of ATP by ATP synthase (Erkkila *et al.* 2006). We evaluated the effect of uncoupling the mitochondrial electron chain and inhibiting mitochondrial respiration on sperm quality parameters. Exposing stallion spermatozoa to CCCP reduced sperm motility and increased sperm labeling with YoPro-1, without a complete loss of membrane integrity. Incubation with DNP also resulted in reduced motility, but had no effect on membrane integrity. In contrast to CCCP, DNP was not able to decrease mitochondrial membrane potential. Inhibition of complex IV of the electron transport chain (ETC) resulted in a marked drop in sperm motility and, in particular, sperm velocity; however, effects on membrane integrity were only evident when sodium cyanide was used at a final concentration of 10 mM. Inhibition of the F_0 part of H^+-ATP synthase using oligomycin-A significantly reduced sperm motility and velocity. Moreover, inhibition of ATP synthase led to an almost complete loss of sperm membrane integrity. These results indicate that mitochondrial ATP is essential for stallion sperm function, confirming previous reports (Ortega-Ferrusola *et al.* 2009*a*, Gibb *et al.* 2014). In addition, we demonstrate for the first time that mitochondrial ATP synthase activity is crucial for the maintenance of an intact sperm membrane.

At present, it is assumed that mammalian sperm rely predominantly on glycolytic ATP production for motility (Mukai & Okuno 2004). This assumption was based on the effect of CCCP on mouse sperm; CCCP did not affect sperm motility in glucose-containing incubation media. By contrast, we showed that uncoupling the mitochondrial electron chain using CCCP or DNP decreased both the percentage of motile stallion sperm and the velocity of motion, despite the presence of glucose in the media. We showed a similar effect on stallion sperm of inhibiting complex IV or ATP synthase. Uncoupling mitochondria or inhibiting mitochondrial respiration, with the consequent reduction in sperm ATP content, showed that motility of stallion spermatozoa is highly dependent on aerobic ATP production (Gibb *et al.* 2014) in clear contrast to other mammalian species, which are reported to rely more on non-aerobic glucose consumption via glycolysis (Storey 2008).

Although reduced ATP levels were observed, ATP was not completely depleted by inhibiting mitochondrial function, and likewise, sperm motility was not completely abolished. Although oligomycin-A was able to disrupt sperm membrane permeability and dramatically reduce sperm motility after 3 h of incubation, sperm velocities were not so dramatically affected. One possible explanation is that other sources of ATP are present in stallion sperm. To test this hypothesis, glycolysis was inhibited using 2-DG. Inhibition of glycolysis lead to reduced ATP content without any effect on membrane integrity. In addition, 10 mM 2-DG reduced the percentage of motile sperm and sperm velocity without affecting the percentage of progressively motile sperm. These findings suggest that ATP generated by glycolysis has a role in providing energy for motility.

Although disruption of mitochondrial function in stallion sperm leads to reduced motility, this could be a function of either reduced ATP production (Gibb *et al.* 2014) or increased reactive oxygen species production (Koppers *et al.* 2008). To determine whether the effect was most likely a function of decreased ATP or increased ROS production, the two parameters were measured simultaneously. Uncoupling mitochondria and inhibiting mitochondrial respiration reduced ATP content. The most prominent reduction in ATP was induced by oligomycin-A and coincided with the highest incidence of collapse of MMP, motility and membrane integrity of the spermatozoa. At the same time, ROS increased in dead sperm, but significantly decreased in the live sperm subpopulation. Interestingly, sperm velocities, although reduced, were not affected as markedly as motility or membrane permeability. Inhibition of complex IV markedly reduced motility and velocities, but had no effect on mitochondrial membrane potential, and only compromised membrane integrity at a final concentration of 10 mM NaCN. Moreover, production of ROS was reduced. These findings indicate that the changes observed after reduced ATP production are related to oxidative stress and, in the case of oligomycin-A incubations, also affect the membrane integrity.

We further investigated whether these effects were related to ATP depletion and/or increased leakage of reactive oxygen species. Oligomycin-A treatment induced a massive increase in ROS production in dead sperm, but reduced ROS in live sperm; sodium cyanide increased ROS production in dead spermatozoa. This contradictory effect may be explained by the fact that in live cells oligomycin-A will inhibit ATP synthase and thus inhibit the speed of oxidative phosphorylation and the flux of electrons through the electron transport chain (ETC). Dead spermatozoa sperm are known to give nurse in radical production (Aitken *et al.* 2015). CCCP increased ROS in live and dead sperm, which can be explained by the decoupling effect of CCCP

causing a back flow of protons from the inter-membrane space back to the matrix of the mitochondria without ATP production and O_2 consumption. Under such circumstances in live cells, the flow of electrons through the ETC will increase and increased ROS production is expected as was observed. DNP only increased ROS production in dead sperm after 3 h of incubation, possibly the effect decoupling drug (working similarly as CCCP) is weaker and not affecting ROS production in live sperm and only after prolonged incubation in deteriorated sperm. The relationship between ROS production and sperm function is poorly understood, with conflicting reports on their role in, or impact on, sperm function (reviewed by Varner *et al.* 2015), although the importance of degenerating sperm in the generation of reactive oxygen species has been emphasized recently (Aitken *et al.* 2015). Our results support this last publication, and suggest the need to adequately interpret ROS production in equine semen. High ROS is a by-product of intense aerobic activity in stallion sperm (Gibb *et al.* 2014). ROS does not appear to contribute to reduced sperm motility under uncoupling conditions, but ROS production becomes more intense when ATP synthase is inhibited. Mitochondrial inhibitors can both increase or decrease ROS production depending on the dose used (Xi *et al.* 2005). In somatic cells, a small mitochondrial depolarization can apparently lead to an increase in ROS generation, whereas a more profound mitochondrial depolarization reduces ROS. This is consistent with the concept that, under resting conditions, 1–2% of the O_2 used in the electron transport chain (ETC) is not completely reduced, leading to the generation of $O_2^{\bullet-}$ (Turrens 2003). In accordance with this observation, in our study, 100 nM CCCP increased ROS production after 1 h of incubation, and reduced the percentage of progressively motile sperm after 3 h. This latter effect could be attributed to ROS oxidizing sperm proteins involved in the regulation of motility. Many types of amino acids can be oxidatively modified, although their susceptibilities vary (Bourdon & Blache 2001). Direct oxidation is mostly mediated by $HO^{\bullet}$ and $NO^{\bullet}$. Among the amino acids, those containing sulfur such as methionine and cysteine are preferred targets.

We tested a wide range of mitochondrial inhibitors (CCCP, DNP, NaCN and oligomycin-A), and stallion sperm behaved largely as described for somatic cells. At lower concentrations and after shorter (e.g. 1 h) incubations, CCCP resulted in increased ROS production; however, this was not accompanied by decreased motility or by mitochondrial membrane depolarization. Higher concentrations tended to reduce ROS production, but the effect was only significant after 1 h of incubation. These effects are somewhat difficult to explain if one assumes that ROS are universally deleterious to stallion sperm function. The fact that increased ROS production was not initially associated with impaired sperm function may indicate, as has been suggested previously, that increased ROS production is not necessarily detrimental, and may instead reflect an active sperm metabolism (Gibb *et al.* 2014). Only when ROS exposure is continued over a longer period of time, may the deleterious effects become apparent as a reduction in the percentage of progressively motile sperm, such as that seen 2 h after the increase in ROS observed in our study. Recently, Macías-García *et al.* (2012) described increased ROS production in sperm selected by single-layer centrifugation, a technique that should select 'better' spermatozoa, further suggesting that increased ROS production may simply reflect active oxidative phosphorylation in metabolically active spermatozoa. Alternatively, the effect reported by Macías-García *et al.* (2012) may relate to oxidative stress induced by colloidal centrifugation because it has recently been reported that colloidal sperm preparation media may be a source of oxidative damage due to the presence of transition metals (Aitken *et al.* 2014). Inhibition of ATP synthase resulted in a significant increase in ROS production, but only in dead spermatozoa. Recently, it has been demonstrated that an L-amino acid oxidase is present in equine spermatozoa and that this enzyme is located in the acrosome, responsive to aromatic amino acids and particularly active in non-viable cells (Aitken *et al.* 2015). Our finding confirmed this previous report, and others that indicated that dead sperm in a sample increase oxidative stress (Roca *et al.* 2013).

Interestingly, we found that uncoupling mitochondria with CCCP led to increased sperm staining with YoPro-1, while inhibiting ATP synthesis with oligomycin-A virtually reduced to 0 the percentage of spermatozoa with membranes not permeable to YoPro-1. As the plasma membrane of the sperm remained intact, the drop in ATP induced by uncoupling oxidative phosphorylation may result in insufficient outward pumping of incoming YoPro-1 by multidrug resistant (MDR) receptors, which operate in an ATP-dependent fashion. Indeed YoPro-1 seems to be more sensitive to ATP-sensitive MDR transporter deficiency than other probes, and ATP leakage through specific channels may contribute to ATP depletion (Chekeni *et al.* 2010, Gallardo-Bolanos *et al.* 2014). The significant increase in ROS production may also have contributed to reduce membrane integrity. All these findings stress the importance of mitochondrial function to stallion spermatozoa, opening new areas of research and challenging the paradigm of ROS negatively influencing sperm function.

In summary, this study provides new evidence that indicates that stallion sperm rely more on mitochondrial ATP production for their function than other mammalian sperm. The alternative pathway of glycolysis for ATP production is also present in stallion spermatozoa but is of less importance for maintaining sperm function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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