Received: 11 July 2022 | Accepted: 13 September 2022

DOI: 10.1111/rda.14261

Reproduction in Domestic Animals WILEY

SHORT COMMUNICATION

Effects of cryopreservation on the mitochondrial bioenergetics of bovine sperm

Cristina Algieri[1] | Olga Blanco-Prieto[1] | Marc Llavanera[1,2] | Marc Yeste[2,3] | Marcella Spinaci[1] | Gaetano Mari[1,4] | Diego Bucci[1] | Salvatore Nesci[1]

[1]Department of Veterinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Bologna, Italy

[2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Unit of Cell Biology, Department of Biology, Faculty of Sciences, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain

[3]Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain

[4]National Institute for Artificial Insemination—AUB INFA, University of Bologna, Bologna, Spain

Correspondence
Diego Bucci, Department of Veterinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia, Bologna 40064, Italy.
Email: diego.bucci3@unibo.it

Funding information
Fondazione Cassa di Risparmio in Bologna

Abstract

This study evaluated the bioenergetic map of mitochondria metabolism in cryopreserved bovine sperm. The detected oligomycin-sensitive basal respiration supported ATP production; frozen–thawed spermatozoa were found to have a coupling efficiency higher than 0.80. Cell respiration, however, was not stimulated by the protonophoric action of FCCP, as its titration with 1, 2, 4 and 6 μM did not stimulate the uncoupling activity on oxidative phosphorylation as highlighted by unresponsive oxygen consumption. The unusual effect on the stimulation of maximal respiration was not related to fibronectin- or PDL-coated plates used for cellular metabolism analysis. Conversely, irradiation of frozen–thawed bovine sperm with the red light improved mitochondrial parameters. In effect, the maximal respiration of red-light-stimulated sperm in PDL-coated plates was higher than the non-irradiated. In spite of this, red-light irradiation had no impact on membrane integrity and mitochondrial activity evaluated by epifluorescence microscopy.

KEYWORDS
bioenergetics, mitochondria, red-light stimulation, spermatozoa

Cryopreservation enables the long-term storage of sperm from genetically superior, selected animals (Fickel et al., 2007; Kumar et al., 2019; Yánez-Ortiz et al., 2021). While cryopreservation is an advantageous technique from several points of view, sperm can be damaged during freeze-thawing and, as a result, their quality, function and fertilizing ability can be impaired (Adamkovicova et al., 2016; Díaz et al., 2019). This detrimental impact is the consequence of different processes that take place during freezing and thawing, such as the formation of ice microcrystals, which may induce mechanical damage to biological membranes; and the generation of excessive amounts of reactive oxygen species, which may increase DNA fragmentation and induce lipid peroxidation thus altering sperm structure and function (Upadhyay et al., 2021; Waterhouse et al., 2010). Yet, preserving mitochondrial functionality during cryopreservation is crucial for maintaining sperm fertilizing ability and guaranteeing the reproductive success (Amaral et al., 2013; Khan et al., 2021).

In recent years, different strategies aimed at minimizing mitochondrial cryodamage, such as the enrichment of freezing media with antioxidants, proteins or cryoprotective agents (Hezavehei et al., 2018). Red-light stimulation could be a potential strategy to improve sperm cryotolerance, as this procedure has been reported

Cristina Algieri and Olga Blanco-Prieto contributed equally as first authors.

to have a positive impact on sperm motility and fertilizing ability (Yeste et al., 2018). This appears to be the result of an improvement at the mitochondrial level, accelerating respiration and ATP synthesis (Gao & Xing, 2009). In particular, the mechanism of photobiomodulation from red to near-infrared light has been attributed to the activation of mitochondrial respiratory chain components, such as cytochrome c oxidase (Blanco-Prieto et al., 2020). The latter is an integral membrane protein that has strong absorbance at the far-red to near-infrared spectral range (Beauvoit et al., 1995). This could stimulate an increase in electron transfer to cytochrome *c* oxidase (Pastore et al., 2000), increasing mitochondrial activity (Eells et al., 2004; Karu, 2010).

Against this background, and because determining the energy metabolism may predict the function and survival of cryopreserved sperm (Marchetti et al., 2004), this study sought to address whether energy production in frozen–thawed bovine sperm was altered following red-light stimulation.

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

No ethics approval was needed as commercial frozen semen was used in this study.

One straw of frozen semen (0.5 ml, nine ejaculates from nine different bulls—nine replicates) was thawed for each replicate thawed for 30 s at 37°C. In a first phase, we characterized the normal metabolism of frozen–thawed bovine sperm: each well of Agilent Seahorse XF polystyrene cell culture plates was covered with 10 µl of 1 mg/ml fibronectin and incubated at 37°C for 90 min. Parallel analyses on Agilent Seahorse XF poly-D-lysine (PDL)-coated cell culture plates were carried out (Moraes et al., 2021). Semen was centrifuged at 800×*g* for 2 min, supernatants were removed, and sperm pellets were resuspended in Tris-Glucose-Citrate medium (TGC: 313.7 mM Tris, 33.3 mM Glucose, 114.5 mM citrate, pH 6.8). Subsequently, a suspension of 2×10^6 cells was placed onto each well of the plate and centrifuged at 1200×*g* for 1 min at 20°C to promote their stickiness to the adhesion-promoting agent. Finally, the supernatant was removed and replaced with 180 µl of Tyrode's medium (Llavanera et al., 2022) plus 5.56 mM Glucose, 2 mM Glutamine and 1 mM Pyruvate. Cellular respiration was determined through the Mito Stress Test by Seahorse technology, and the following parameters at 37°C were measured: real-time oxygen consumption rate (OCR), basal respiration (before the addition of 1.5 µM oligomycin); maximal respiration (after the addition of 4 µM carbonyl cyanide-p-trif luoromethoxyphenylhydrazone, FCCP), proton leak (the difference between basal respiration and respiration in the presence of oligomycin) and non-mitochondrial respiration (OCR in the presence of 0.5 µM rotenone plus 0.5 µM antimycin A, the inhibitors of the respiratory chain), which was subtracted from all the above parameters. Finally, ATP turnover was obtained from the difference between basal respiration and proton leak (OCR in the presence of oligomycin), whereas the spare respiratory capacity, which is the difference between maximal and basal respiration, was considered as the ability to respond to increased energy demand (Bernardini et al., 2021).

To test red-light stimulation effects on substrate oxidation/mitochondrial respiration, after thawing, straws were irradiated with red-light using the Maxicow instrument (IUL S.A., Barcelona, Spain), following the schedule: red light (620–630 nm) for 1 min,

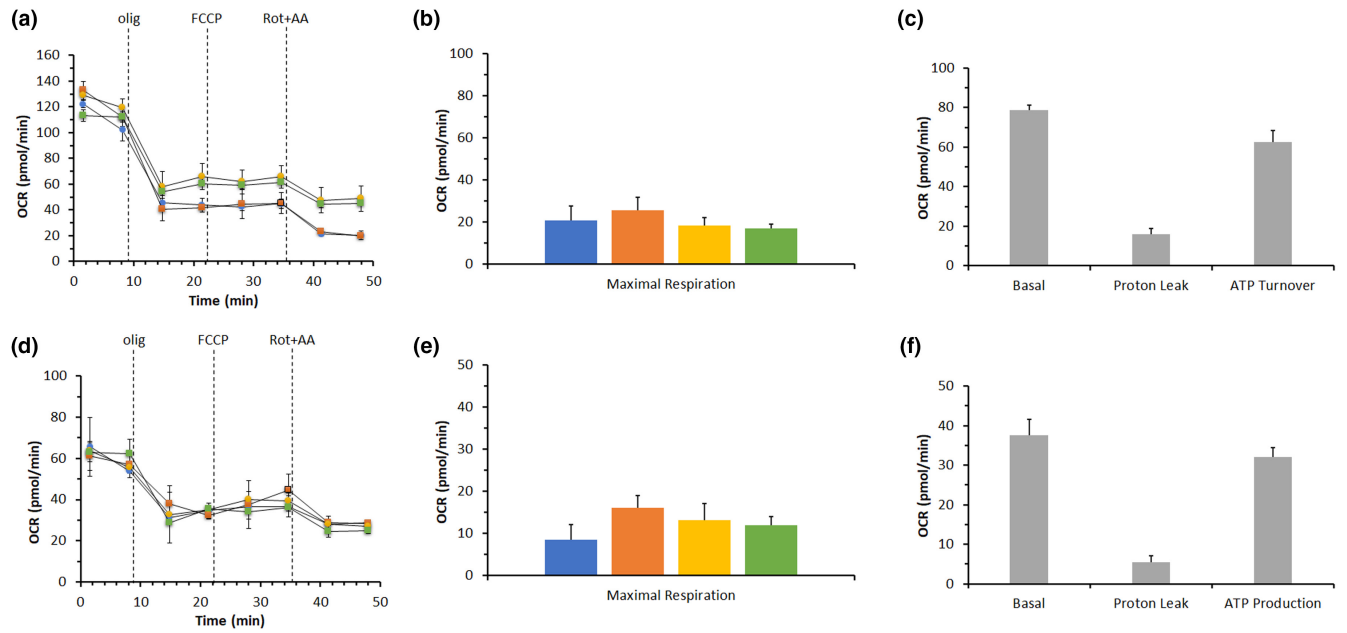

FIGURE 1 Energy profile of bovine spermatozoa. Oxygen consumption rate (OCR) with 1 µM (■), 2 µM (●), 4 µM (■) and 6 µM (●) FCCP (a, c). Maximal respiration in the presence of 1 µM (■), 2 µM (■), 4 µM (■) and 6 µM (■) FCCP with fibronectin (b) and PDL (e). Mitochondrial parameters (basal respiration, proton leak and ATP production) were obtained before FCCP addition in the plot with fibronectin (c) and PDL (f). Data expressed as points (a, c) or columns (b, d) represent the means ± SD (vertical bars) from three experiments carried out on different cells preparation.

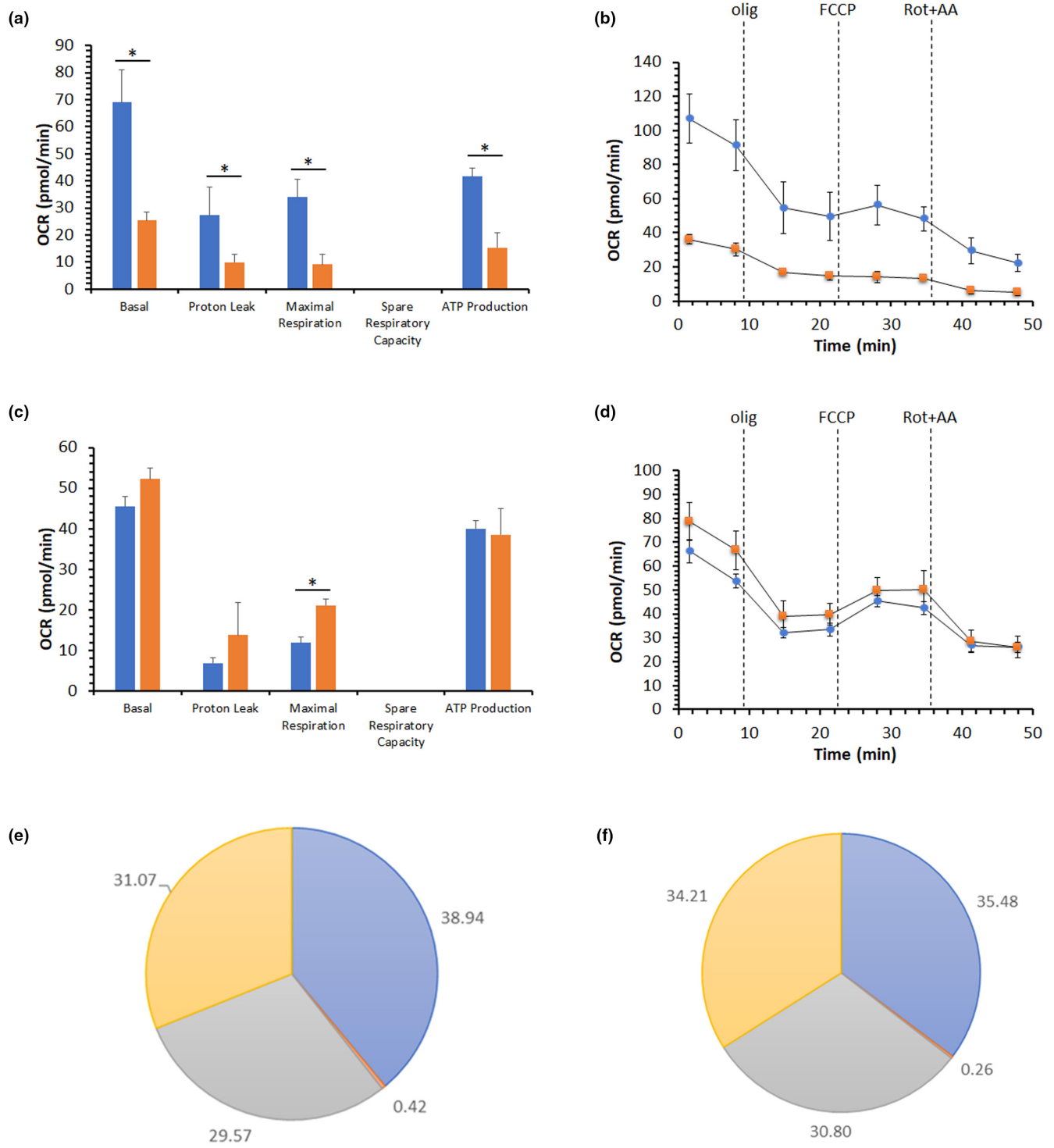

FIGURE 2 Effect of red-light stimulation on the energy profile of bovine spermatozoa. Mitochondrial parameters (basal, proton leak, maximal respiration, spare respiratory capacity and ATP production) without (■) and with (■) red-light treatment (a, c), and oxygen consumption rate (OCR) profile without (●) and with (●) red-light treatment in the presence of fibronectin (b) and PDL (d). Data expressed as points (b, d) or columns (a, c) represent the means ± SD (vertical bars) from three independent experiments. Effect of red-light stimulation on sperm subpopulations following SYBR-14/PI/JC-1 staining on (e) control group, and (f) irradiated spermatozoa ■ $SYBR\text{-}14^+/PI^-/JC1^+$ (viable with active mitochondria), ■ $SYBR\text{-}14^+/PI^-/JC1^-$ (non-viable with inactive mitochondria), ■ $SYBR\text{-}14^-/PI^+/JC1^+$ (non-viable with active mitochondria) and ■ $SYBR\text{-}14^-/PI^+/JC1^-$ (non-viable with inactive mitochondria).

non-irradiated for 1 min and irradiated again for 1 min. Another straw (non-irradiated control) was kept in the dark for 3 min. In all cases, straws were kept at room temperature.

For Seahorse evaluations, all samples were centrifuged as described above and resuspended in Tris–glucose–citrate medium. Afterwards, each sample was split into two aliquots to evaluate

the OCR value or to assess mitochondrial membrane potential and viability, simultaneously, as described in Nesci et al. (2020). Four different sperm populations were identified: viable sperm with active mitochondria ($SYBR14^+/PI^-/JC\text{-}1^+$), viable sperm with inactive mitochondria ($SYBR14^+/PI^-/JC\text{-}1^-$), non-viable sperm with active mitochondria ($SYBR14^-/PI^+/JC\text{-}1^+$) and non-viable sperm with inactive mitochondria ($SYBR14^-/PI^+/JC\text{-}1^-$).

Our results indicated that frozen–thawed bovine sperm, regardless of whether they were examined in fibronectin- (Figure 1a) or PDL-coated plates (Figure 1d), did not respond to the decoupling action of FCCP at any of the concentrations tested (1, 2, 4 and 6 μM). The mitochondrial respiration profile was devoid of maximal respiration. The OCR in the presence of FCCP was below the basal respiration and, consequently, these cells did not have spare respiratory capacity. However, in fresh and frozen sperms, the absence of spare respiratory capacity has already been ascertained in the presence of relatively large glucose concentrations (around 5 mM) in the reaction system (Moraes et al., 2021). Based on these data, one may infer that the damage caused to mitochondrial membranes during freeze-thawing hampers the uncoupling effect of FCCP on oxidative phosphorylation. The reduced maximum respiratory capacity (Figure 1b,e) could result from impaired substrate oxidation, and the lack of spare respiratory capacity could indicate a mitochondrial dysfunction that could not be particularly apparent under basal conditions. The basal respiration, proton leak and ATP production were not found to be dependent on FCCP titration in the OCR profile. While sperm attached to fibronectin-coated plates showed higher mitochondrial respiration than those in PDL-coated plates (Figure 1c,f), mitochondria produced a coupling efficiency fairly high of approximately 0.80 and 0.85 a.u. in the presence of fibronectin or PDL, respectively (the maximum value is 1.0 a.u. under ideal conditions when all the basal mitochondrial oxygen is used for ATP synthesis). Accordingly, proton leak of sperm mitochondria attained a 20% or 15% OCR value of the basal OCR with fibronectin or PDL, respectively.

Under the state of oligomycin-insensitive respiration controlled by proton leak kinetics, the mitochondrial activity was potentially unaffected by changes in substrate oxidation; a modest change in leak rate was likely to be caused by altered substrate oxidation. This hypothesis could corroborate the lack of effect from FCCP.

Light stimulation did not exert beneficial effects on mitochondrial activity of frozen–thawed bovine sperm when they are examined in fibronectin-coated plates (Figure 2a,b). In fact, all mitochondrial parameters after sperm irradiation were drastically reduced compared with the control (Figure 2a). Surprisingly, when the analysis was performed on PDL-coated plates, irradiated sperm showed an increase in mitochondrial parameters (Figure 2c,d), especially the maximal respiration which, despite remaining below the basal activity, increased compared with non-irradiated cells (Figure 2c). The ability of frozen–thawed bovine sperm to counteract the impairment of maximal respiration after red-light stimulation would be explained if the damage to the electron transfer chain is inflicted before reaching the cytochrome *c* during substrate oxidation.

Finally, red-light stimulation did not alter the percentages of sperm subpopulations following SYBR14-14/PI/JC-1 staining (Figure 2e,f).

The loss of response to FCCP would suggest that the alteration of the respiratory complexes structure induced by cryopreservation impaired mitochondrial function, which became bioenergetically inefficient. In line with other authors, this could be explained by the high resilience of bovine sperm to these conditions and their ability to rely on anaerobic metabolism to maintain cell homeostasis and motility (Bulkeley et al., 2021). Those authors showed that the selection of frozen–thawed bovine sperm through swim-up resulted in sperm with higher motility, OCR and ECAR. This would also support the hypothesis that bovine sperm cells have a manageable metabolic interplay between aerobic and anaerobic pathways, thus maintaining their fertilizing capacity even after the stress induced by cryopreservation.

AUTHOR CONTRIBUTIONS

C.A and O.B.P performed the experiments and wrote the first draft of the manuscript; M.L., M.Y., M.S. and G.M. contributed in manuscript revision; D.B. supervised the final version of the manuscript and collaborated in the experimental design formulation; S.N. revised the final version of the manuscript and formulated the experimental design.

ACKNOWLEDGMENTS

CA and OBP performed the experiments; ML, MY, DB and SN developed the experimental design and wrote the final version of the manuscript; MS and GM revised the final version of the manuscript. Open Access Funding provided by Universita degli Studi di Bologna within the CRUI-CARE Agreement.

FUNDING INFORMATION

This work was supported by Fondazione Cassa di Risparmio in Bologna (CARISBO)—Grant number 2020.0381. Open Access Funding provided by Universita degli Studi di Bologna within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Marc Yeste https://orcid.org/0000-0002-2209-340X
Diego Bucci https://orcid.org/0000-0001-6276-2674
Salvatore Nesci https://orcid.org/0000-0001-8569-7158

REFERENCES

Adamkovicova, M., Toman, R., Martiniakova, M., Omelka, R., Babosova, R., Krajcovicova, V., Grosskopf, B., & Massanyi, P. (2016). Sperm motility and morphology changes in rats exposed to cadmium and diazinon. *Reproductive Biology and Endocrinology*, *14*, 42. https://doi.org/10.1186/s12958-016-0177-6

Amaral, A., Lourenço, B., Marques, M., & Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction*, *146*, R163–R174. https://doi.org/10.1530/REP-13-0178

Beauvoit, B., Evans, S. M., Jenkins, T. W., Miller, E. E., & Chance, B. (1995). Correlation between the light scattering and the mitochondrial content of Normal tissues and transplantable rodent tumors. *Analytical Biochemistry*, *226*, 167–174. https://doi.org/10.1006/abio.1995.1205

Bernardini, C., Algieri, C., Mantia, L. D., Zannoni, A., Salaroli, R., Trombetti, F., Forni, M., Pagliarani, A., & Nesci, S. (2021). Relationship between serum concentration, functional parameters and cell bioenergetics in IPEC-J2 cell line. *Histochemistry and Cell Biology*, *156*, 59–67. https://doi.org/10.1007/s00418-021-01981-2

Blanco-Prieto, O., Catalán, J., Trujillo-Rojas, L., Peña, A., Rivera del Álamo, M. M., Llavanera, M., Bonet, S., Fernández-Novell, J. M., Yeste, M., & Rodríguez-Gil, J. E. (2020). Red LED light acts on the mitochondrial electron chain of mammalian sperm via light-time exposure-dependent mechanisms. *Cell*, *9*, 2546. https://doi.org/10.3390/cells9122546

Bulkeley, E. A., Foutouhi, A., Wigney, K., Santistevan, A. C., Collins, C., McNabb, B., & Meyers, S. (2021). Effects from disruption of mitochondrial electron transport chain function on bull sperm motility. *Theriogenology*, *176*, 63–72. https://doi.org/10.1016/j.theriogenology.2021.09.015

Díaz, R., Lee-Estevez, M., Quiñones, J., Dumorné, K., Short, S., Ulloa-Rodríguez, P., Valdebenito, I., Sepúlveda, N., & Farías, J. G. (2019). Changes in Atlantic salmon (Salmo salar) sperm morphology and membrane lipid composition related to cold storage and cryopreservation. *Animal Reproduction Science*, *204*, 50–59. https://doi.org/10.1016/j.anireprosci.2019.03.004

Eells, J. T., Wong-Riley, M. T. T., VerHoeve, J., Henry, M., Buchman, E. V., Kane, M. P., Gould, L. J., Das, R., Jett, M., Hodgson, B. D., Margolis, D., & Whelan, H. T. (2004). Mitochondrial signal transduction in accelerated wound and retinal healing by near-infrared light therapy. *Mitochondrion, Mitochondrial Medicine–Developing the Scientific Basis to Medical Management of Mitochondrial Disease*, *4*, 559–567. https://doi.org/10.1016/j.mito.2004.07.033

Fickel, J., Wagener, A., & Ludwig, A. (2007). Semen cryopreservation and the conservation of endangered species. *European Journal of Wildlife Research*, *53*, 81–89. https://doi.org/10.1007/s10344-007-0089-z

Gao, X., & Xing, D. (2009). Molecular mechanisms of cell proliferation induced by low power laser irradiation. *Journal of Biomedical Science*, *16*, 4. https://doi.org/10.1186/1423-0127-16-4

Hezavehei, M., Sharafi, M., Kouchesfahani, H. M., Henkel, R., Agarwal, A., Esmaeili, V., & Shahverdi, A. (2018). Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive Biomedicine Online*, *37*, 327–339. https://doi.org/10.1016/j.rbmo.2018.05.012

Karu, T. I. (2010). Multiple roles of cytochrome c oxidase in mammalian cells under action of red and IR-A radiation. *IUBMB Life*, *62*, 607–610. https://doi.org/10.1002/iub.359

Khan, I. M., Cao, Z., Liu, H., Khan, A., Rahman, S. U., Khan, M. Z., Sathanawongs, A., & Zhang, Y. (2021). Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance OMICS to assess sperm Cryo-tolerance in farm animals. *Frontiers in Veterinary Science*, *8*, 609180. https://doi.org/10.3389/fvets.2021.609180

Kumar, A., Prasad, J. K., Srivastava, N., & Ghosh, S. K. (2019). Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa. *Biopreservation and Biobanking*, *17*, 603–612. https://doi.org/10.1089/bio.2019.0037

Llavanera, M., Mislei, B., Blanco-Prieto, O., Baldassarro, V. A., Mateo-Otero, Y., Spinaci, M., Yeste, M., & Bucci, D. (2022). Assessment of sperm mitochondrial activity by flow cytometry and fluorescent microscopy: A compartive study of mitochondrial fluorescent probes in bovine spermatozoa. *Reproduction, Fertility, and Development*, *34*(9), 679–688. https://doi.org/10.1071/RD21355

Marchetti, C., Jouy, N., Leroy-Martin, B., Defossez, A., Formstecher, P., & Marchetti, P. (2004). Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. *Human Reproduction*, *19*, 2267–2276. https://doi.org/10.1093/humrep/deh416

Moraes, C. R., Moraes, L. E., Blawut, B., Benej, M., Papandreou, I., Nicholas, C., Denko, N. C., & Coutinho da Silva, M. (2021). Effect of glucose concentration and cryopreservation on mitochondrial functions of bull spermatozoa and relationship with sire conception rate. *Animal Reproduction Science*, *230*, 106779. https://doi.org/10.1016/j.anireprosci.2021.106779

Nesci, S., Spinaci, M., Galeati, G., Nerozzi, C., Pagliarani, A., Algieri, C., Tamanini, C., & Bucci, D. (2020). Sperm function and mitochondrial activity: An insight on boar sperm metabolism. *Theriogenology*, *144*, 82–88. https://doi.org/10.1016/j.theriogenology.2020.01.004

Pastore, D., Greco, M., & Passarella, S. (2000). Specific helium-neon laser sensitivity of the purified cytochrome c oxidase. *International Journal of Radiation Biology*, *76*, 863–870. https://doi.org/10.1080/09553000050029020

Upadhyay, V. R., Ramesh, V., Dewry, R. K., Kumar, G., Raval, K., & Patoliya, P. (2021). Implications of cryopreservation on structural and functional attributes of bovine spermatozoa: An overview. *Andrologia*, *53*(8), e14154. https://doi.org/10.1111/and.14154

Waterhouse, K. E., Gjeldnes, A., Tverdal, A., De Angelis, P. M., Farstad, W., Håård, M., & Kommisrud, E. (2010). Alterations of sperm DNA integrity during cryopreservation procedure and in vitro incubation of bull semen. *Animal Reproduction Science*, *117*(1–2), 34–42. https://doi.org/10.1016/j.anireprosci.2009.04.011

Yánez-Ortiz, I., Catalán, J., Rodríguez-Gil, J. E., Miró, J., & Yeste, M. (2021). Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Animal Reproduction Science*, 106904. https://doi.org/10.1016/j.anireprosci.2021.106904 [In press]

Yeste, M., Castillo-Martín, M., Bonet, S., & Rodríguez-Gil, J. E. (2018). Impact of light irradiation on preservation and function of mammalian spermatozoa. *Animal Reproduction Science*, *194*, 19–32. https://doi.org/10.1016/j.anireprosci.2018.02.004

How to cite this article: Algieri, C., Blanco-Prieto, O., Llavanera, M., Yeste, M., Spinaci, M., Mari, G., Bucci, D., & Nesci, S. (2022). Effects of cryopreservation on the mitochondrial bioenergetics of bovine sperm. *Reproduction in Domestic Animals*, *00*, 1–5. https://doi.org/10.1111/rda.14261