Response of Thawed Epidi dymal Red Deer Spermatozoa to Increasing Concentrations of Hydrogen Peroxide, and Importance of Individual Male Variability

ARTICLE *in* REPRODUCTION IN DOMESTIC ANIMALS · JUNE 2011

Impact Factor: 1.52 · DOI: 10.1111/j.1439-0531.2010.01677.x · Source: PubMed

9 AUTHORS, INCLUDING:

Felipe Martínez-Pastor
Universidad de León
145 PUBLICATIONS **1,395** CITATIONS
SEE PROFILE

O. Garcia-Alvarez
University of Castilla-La Mancha
39 PUBLICATIONS **259** CITATIONS
SEE PROFILE

Ana J. Soler
University of Castilla-La Mancha
88 PUBLICATIONS **1,387** CITATIONS
SEE PROFILE

Julian Garde
University of Castilla-La Mancha
153 PUBLICATIONS **2,394** CITATIONS
SEE PROFILE

Running Title: Oxidative stress in red deer spermatozoa

Title: Response of thawed epididymal red deer spermatozoa to increasing concentrations of hydrogen peroxide, and importance of individual male variability

AE Domínguez-Rebolledo [*,d], F Martínez-Pastor [†], AF Bisbal [*], JL Ros-Santaella [*], O García-Álvarez [ψ], A Maroto-Morales [*], AJ Soler[*], JJ Garde [*‡], MR Fernández-Santos[*‡]

[*] Reproductive Biology Group, National Wildlife Research Institute (IREC), UCLM-CSIC-JCCM. Albacete, Spain, [†] ITRA-ULE, INDEGSAL, University of Leon, León, Spain, [ψ] Regional Center of Animal Selection and Reproduction (CERSYRA), JCCM, Valdepeñas, Ciudad Real, Spain and [‡] Institute of Regional Development (IDR), UCLM, Albacete, Spain.

Corresponding author: Dr. Fernández-Santos. IREC, Campus Universitario sn, 02071 Albacete, Spain. Tel: +34-967-599200+2581; fax: +34-967-599238.

E-mail address: MRocio.Fernandez@uclm.es (Dr. M.R. Fernandez-Santos)

Contents

Oxidative stress represents a challenge during sperm manipulation. We have tested the effect of increasing hydrogen peroxide (H_2O_2) levels on red deer spermatozoa after cryopreservation, and the role of male-to-male variation in that response. In a first experiment, eight thawed samples were submitted to 0, 25, 50, 100 and 200 µM H_2O_2 for 2 h at 37 °C. Intracellular ROS (H_2DCFDA-CM) increased with H_2O_2 concentration, but we only detected a decrease in sperm function (motility by CASA and chromatin damage by SCSA) with 200 µM. Lipoperoxidation (TBARS) increased slightly with 50 µM H_2O_2 and above. In a second experiment, samples from 7 males were submitted to 0 and 200 µM H_2O_2 for 2 h, triplicating the experiment within each male. Males differed at thawing and regarding their response to incubation and H_2O_2 presence. We found that the kinematic parameters reflected male-to-male variability, whereas the response of the different males was similar for lipid peroxidation and viability. A multiparametric analysis showed that males grouped differently if samples were assessed after thawing, after incubation without H_2O_2, or after incubation with H_2O_2. Red deer spermatozoa are relatively resilient to H_2O_2 after thawing, but it seems to be a great male-to-male variability regarding the response to oxidative stress. The acknowledgement of this individual variability might improve the development of optimized sperm work protocols.

Key words: Red deer, reproductive technology, oxidative stress, hydrogen peroxide, individual variability.

Introduction

Oxidative stress is one of the major threats to sperm functionality, both *in vivo* and *in vitro*. Reactive oxygen species (ROS) have a fundamental role in sperm physiology, but in excess they can damage spermatozoa (Agarwal and Saleh, 2002). ~~During sperm work~~, ROS can be detrimental even within physiological levels, since they may trigger early capacitation and irreversible events, such as acrosome reaction (Hsu et al., 1999). Researchers generally use external sources of ROS to study oxidative stress on spermatozoa such as hydrogen peroxide (H_2O_2), a potent membrane-permeable oxidizing species (Oehninger et al., 1995). Armstrong *et al*. (1999) found that hydrogen peroxide was not only responsible for the loss of motility, but also it caused the loss of mitochondrial membrane potential. Moreover, ROS, including H_2O_2, have dual effects on mammalian sperm. Low concentrations of ROS exogenously added are believed to play a stimulatory role in sperm capacitation (Rivlin et al., 2004), hyperactivation (de Lamirade and Gagnon, 1994), acrosome reaction (Griveau et al., 1995) and sperm-oocyte fusion (Aitken et al., 1995). However, excessive levels of ROS are linked to impaired sperm function and infertility (Sharma et al., 2004). ROS can be also detrimental to sperm DNA integrity (Baumber et al., 2003;Dominguez-Rebolledo et al., 2010).

We have previously reported that different reactive oxygen species generators affected quality parameters differently in red deer, showing that hydrogen peroxide (H_2O_2) was more cytotoxic to red deer spermatozoa than Fe^{2+}/ascorbate (Martinez-Pastor et al., 2009a). Moreover, motility and mitochondrial membrane potential were quickly decreased by H_2O_2 (1 mM and 100 μM), and only H_2O_2 (1 mM) was able to reduce sperm viability. Thus, the present study was designed to deepen on our previous results, analysing a broader range of H_2O_2 concentrations.

Moreover, between-male variability represent a challenge for sperm cryopreservation, since that variability can affect spermatozoa cryosurvival (Soler et al., 2003) and fertility (Gomendio et al., 2006;Malo et al., 2005). That is probably due to differences regarding sperm biochemistry and metabolism (Loomis and Graham, 2008), rooting in the genetic variability of individuals. The male-to-male variability could also affect the resistance of spermatozoa to oxidative stress, for instance through changes in the composition of sperm membranes (Waterhouse et al., 2006). In fact, high polyunsaturated fatty acids levels have been related to higher vulnerability to ROS (Ollero et al., 2001), and previous studies have shown that fatty acid profiles could be modified in deers exposed to heavy metals (Castellanos et al., 2010).

Thus, in the present study we used thawed epididymal spermatozoa of Iberian red deer (*C. elaphus hispanicus*) to test the hypothesis that increasing concentrations of H_2O_2 affected differently to sperm characteristics, seeking for endpoints in which H_2O_2 could noticeably affect spermatozoa. It is well known that epididymal spermatozoa are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognised as the prime source of antioxidant protection (Chen et al., 2003). In this respect, it is needed a better understanding of the spermatozoa behaviour against oxidative damage, since this damage represents a serious challenge for these unprotected cells when they are outside the epididymal environment. Moreover, spermatozoa might be submitted to stressing situations during in vitro procedures (IVF or sorting), which could increase ROS and other oxidative species. Therefore, this study could be useful to simulate the response of epididymal red deer spermatozoa to oxidative stress *in vitro*, allowing to explore procedures to alleviate it.

We also tested if male-to-male variability reflects on the spermatozoa response to H_2O_2, expecting to observe this effect when submitting samples from different males to oxidative stress. Being a wild species, we have the advantage of working with samples coming from unselected populations, thus allowing us to better analyse that kind of variability (Garde et al., 2006).

Materials and Methods

Reagents and media

CM-H_2DCFDA, YO-PRO-1 and TO-PRO-3 were purchased from Invitrogen (Barcelona, Spain). Flow cytometry equipment, software and consumables (including the sheath fluid, BD FACSFlow) were purchased from BD Biosciences (San Jose, CA, USA). Acridine orange (chromatographically purified) was purchased from Polysciences Inc. (Warrington, PA, USA). Other fluorescence probes and chemicals (high grade) were obtained from Sigma Chemical Co. (Madrid, Spain). Stock solutions of the fluorescence probes were as follows: propidium iodide, 7.5 mM in water; CM-H_2DCFDA, 0.5 mM in DMSO; YO-PRO-1 and TO-PRO-3, 50 μM in DMSO. All solutions were stored at -20 ºC and in the dark until needed, except oxidant working solutions, which were prepared the same day. Preparation and staining of samples for flow cytometric analysis were performed by flow cytometer PBS (BD FACSFlow; BD Biosciences).

Animals, spermatozoa collection and cryopreservation

For this study, we used spermatozoa recovered from the epididymides of mature stags (age > 4.5 years, weight > 130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September-October). Gamekeepers collected the complete male genitalia and provided the hour of the death. Hunting was in accordance with the harvest plan of the game reserve, which made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation.

Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at ambient temperature (approximately 22 ºC) within 2 h after being removed. The samples were processed as soon as they arrived at the laboratory. The elapsed time between animal death and sperm recovery ranged from 3 to 6 hours, which is an adequate and reliable time interval for evaluating sperm parameters, as decreases in the quality of sperm traits begin to take place 12 hours after the death of a male (Soler and Garde, 2003). For the collection of epididymal spermatozoa, the testes and epididymides were removed from the scrotal sac. The cauda epididymides, which included 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes (Nunc, Roskilde, Denmark).

Spermatozoa were collected from the distal portion of the epididymis as described by Soler et al. (2003). Epididymal contents from both testicles of the same male were pooled for processing. Then, the sperm mass was diluted to a sperm concentration ~400×10^6 sperm/mL in fraction A of a Tris- Citrate-Fructose (TCF: Tris 27.0 g/L, citric acid 14.0 g/L, fructose 10.0 g/L, and 20% clarified egg yolk) (Fernandez-Santos et al.,

2006). Then, the sperm was further diluted with the same volume of Fraction B of the extender (12%, v/v of glycerol), at ambient temperature (22 °C). Samples were cooled down to 5 °C and, after 2 h of equilibration, were loaded into 0.25 ml plastic straws (IMV, L´Aigle Cedex, France) and frozen in liquid nitrogen vapor (4 cm above liquid nitrogen; -120ºC) for 10 min. The straws remained for a minimum period of 1 year in liquid nitrogen (-196ºC). Thawing was carried out by immersing straws in a water bath at 37 ºC for 30 s.

Experimental Design

Experiment 1. Effects of increasing doses of H_2O_2 on thawed epididymal spermatozoa from red deer.

Experiment 1 was designed to explore the effect of several H_2O_2 concentrations on sperm parameters after thawing, and to evaluate the relation of H_2O_2 with sperm parameters. Thawed semen was washed in TCF (300×g, 5 min), and diluted in the same medium to 30×10^6 spermatozoa/mL. The sperm solution was split among 5 aliquots in microtubes. One of them was left untreated as the control. The other aliquots were subjected to oxidative stress by adding H_2O_2 in four concentrations (25 µM, 50 µM, 100 µM and 200 µM). With this approach we seeked to expand the study initiated previously (Martinez-Pastor et al., 2009a), exploring concentrations between 10 µM (which had no negative effects in that study) and 1 mM (which was patently cytotoxic). All treatments were split into two aliquots. One of them was incubated with 0.5 µM H_2DCFDA (for assessing ROS production) and the other was used to evaluated the rest

of sperm parameters. The microtubes were incubated at 37 ºC and analyzed 120 min after starting the incubation (the control was analyzed at 0 and 120 min). This experiment was replicated 8 times with samples from 8 different males (one straw per male).

Experiment 2. Individual male-to-male variation in the response to oxidative stress.

This experiment evaluated the presence of male to male individual differences on the effect of oxidative stress. Thawed semen was washed in TCF (300xg, 5 min.) and diluted in the same medium to $30x10^6$ spermatozoa/mL. The sperm solution was split among 2 aliquots in microtubes. One of them was left untreated as control and the other was incubated with 200 μM H_2O_2 at 37 °C, evaluating the samples after 120 min. The experiment was replicated with samples from 7 males, with triplicates within each male, using a different cryopreserved straw each time.

Sperm evaluation

Sperm motility

Sperm motility was assessed using a computer-assisted motility analyzer (SCA2002, CASA system; Microptic, Barcelona, Spain) coupled to an optical phase-contrast microscope (Nikon Eclipse 80i), equipped with negative phase-contrast objectives, a warming stage at 37 ºC and a Basler A302fs camera (Basler Vision Technologies, Ahrensburg, Germany). A pre-warmed Makler counting chamber (10 μm

depth) was loaded with 5 ü L of sample and analyzed. The parameters used in this study were: percentage of motile spermatozoa (total motility, TM, %), velocity according to the actual path (VCL, μm/s), linearity (LIN, %) and amplitude of the lateral displacement of the sperm head (ALH, μm). Sample acquisition rate was 25 images/s, and motile spermatozoa were defined as those with VCL>10 μm/s. At least five fields per sample were recorded and analysed afterwards.

Sperm viability

Viability was assessed by the monomeric cyanine nucleic acid stain YO-PRO-1. Samples were diluted down to 10^6 spermatozoa/mL in flow cytometry PBS with 0.1 μM YO-PRO-1 and 10 μM PI. After 20 min in the dark, the samples were run through a flow cytometer. Labelling cells with the apoptotic marker YO-PRO-1 yielded three subpopulations: viable (unstained: YO-PRO-1-/PI-), apoptotic-like membrane changes (YO-PRO-1+/PI-), and non-viable (membrane damaged: PI+). Hoechst 33342 was included at 5.1 μM.

Detection of Reactive Oxygen Species (ROS)

The derivative of fluorescein, CM-H_2DCFDA, was used for the detection of ROS. Oxidation of this probe is detected by monitoring the increase in fluorescence with a flow cytometer, using excitation sources and filters appropriate for fluorescein (green fluorescence). This fluorescence probe was combined with TO-PRO-1, a red-fluorescence analogue to YO-PRO-1. Stock solutions of the fluorescence probe were prepared as CM-H_2DCFDA 0.5 mM in DMSO, TO-PRO-3 50 μM in DMSO, to give a final concentration of 0.5 μM of CM-H_2DCFDA and 0.1 μM of TO-PRO-1. Hoechst 33342 was included at 5.1 μM.

224

225 Flow Cytometry Analyses

226 We used a Becton Dickinson LSR-I flow cytometer (BD Biosciences, San José, CA,
227 USA), furnished with a 325 nm He-Cd (excitation for Hoechst 33342), a 488 nm Ar-Ion
228 laser (excitation for YO-PRO-1 and PI) and a 633 nm He-Ne laser (excitation for
229 Mitotracker Deep Red). Hoechst 33342 fluorescence was read with the FL5
230 photodetector (424/44BP filter), YO-PRO-1 and CM-H_2DCFDA fluorescences were
231 read with the FL1 photodetector (530/28BP filter), and PI and TO-PRO-1 fluorescences
232 were read with the FL3 photodetector (670LP filter). FSC/SSC signals and Hoechst
233 fluorescence were used to discriminate spermatozoa from debris. Fluorescence captures
234 were controlled using the Cell Quest Pro 3.1 software (BD Biosciences, San José, CA,
235 USA). All the parameters were read using logarithmic amplification. For each sample,
236 5000 spermatozoa were recorded at 200 events/s, saving the data in flow cytometry
237 standard (FCS) v. 2 files. The analysis of the flow cytometry data was carried out using
238 WEASEL v. 2.6 (WEHI, Melbourne, Australia). The YO-PRO-1/PI stain was analyzed
239 as previously described for red deer (Martinez-Pastor et al., 2008). From this stain,
240 viability was defined as the percentage of membrane intact spermatozoa (PI–) and the
241 "apoptotic" ratio, as the relation among the YO-PRO-1+/PI– and PI– (YO-PRO-1–/PI–
242 plus YO-PRO-1+/PI– spermatozoa) subpopulations, expressed as percentage. This ratio
243 estimated the proportion of spermatozoa with apoptosis-like membrane changes within
244 the PI– subpopulation.

245

246 Sperm chromatin assessment

247 Chromatin stability was assessed following the SCSA (Sperm Chromatin
248 Structure Assay), based on the susceptibility of sperm DNA to acid-induced

denaturation in situ and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson et al., 1980). Acridine orange (AO) fluorescence shifts from green (dsDNA; double strand) to red (ssDNA; single strand) depending on the degree of DNA denaturation. Samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final sperm concentration of $2x10^6$ cells/mL in cryotubes. Samples were frozen in liquid nitrogen and stored in an ultracold freezer at -80 ºC until needed. For analysis, the samples were thawed on crushed ice. Acid-induced denaturation of DNA in situ was achieved by adding 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) to 200 µL of sample. After 30 seconds, the cells were stained by adding 1.2 mL of an acridine orange solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 6 µg/mL acridine orange pH 6.0). The stained samples were analyzed by flow cytometry exactly at 3 minutes after adding the acridine orange solution.

Samples were run through the LSR-I flow cytometer described above. Green fluorescence was detected using the FL-1 photodetector and red fluorescence with the FL-3 photodetector. Data were collected from 10000 events at 200 events/s for further analysis with Cell-Quest software (Becton Dickinson). A tube with 0.4 mL of detergent-acid solution and 1.2 mL of acridine orange solution was run through the system before running any samples and between samples. At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence to total intensity of the fluorescence (red/[red+green]×100), called DFI (DNA fragmentation index; formerly called αt) for each spermatozoa, representing the shift from green to red fluorescence. High values of DFI indicate chromatin

abnormalities. Flow cytometry data was processed to obtain %DFI (% of spermatozoa with DFI>25) and HDS (High DNA Stainability: % of spermatozoa with green fluorescence higher than channel 600, of 1024 channels).

TBARS assay for quantification of lipid peroxidation (LPO)

The susceptibility of the spermatozoa to lipoperoxidation (LPO) was estimated by the thiobarbituric acid reactive substance (TBARS) method according to Ohkawa et al. (1979). Samples of 100 µL were thoroughly mixed with 200 µL of a stock solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 M HCl. This mixture was heated at 90 °C for 15 min, and then the reaction was stopped by placing the tubes in ice-cold water for 5 min. The tubes were centrifuged at 1500×g for 15 min to pellet the precipitate, and the clear supernatant was collected and transferred to wells (200 µL/well) in a 96-well flat bottom transparent plate (Nunc, Roskilde, Denmark). The plate was completed with a calibration curve prepared from a malondialdehyde (MDA) stock (1,1,3,3-tetramethoxypropane). Sample absorbance at 532 nm was read on a multipurpose microplate reader (Synergy HT, BIO-TEK, Winooski, Vermont, USA). MDA concentration was calculated from a standard curve. The lipid peroxidation index was calculated as nmol of MDA per 10^8 sperm. This assay was duplicated for each sample.

Statistical analysis

Statistical analyses were carried out using the R statistical package (http://www.r-project.org). For the analysis of H_2O_2 (Experiment 1), data were analyzed using linear mixed-effects models, treating the male effect as the random part of the model, and time or H_2O_2 concentration as the fixed part of the model. For the analysis of the male-to-

male variability (Experiment 2), results were arc sine (proportions) or log-transformed (other variables), and male, treatment (values at 0 h, 2 h and 2 h with 200 μM of H_2O_2) and their interaction were analyzed by ANOVA. For the graphical analysis of the data, we used interaction plots and principal component analysis (with TM, VCL, LIN, ALH, LPO and viability). Unless otherwise stated, results are presented as mean±SEM, and statistical significance was accepted for $P<0.05$.

Results

Experiment 1. Effects of increasing doses of H_2O_2 on thawed epididymal spermatozoa from red deer

We evaluated how increasing H_2O_2 concentration affected sperm quality parameters, looking after H_2O_2 concentrations that might induce critical changes on sperm quality during incubation. Intracellular ROS (Fig. 1) spontaneously increased from 0 to 2 h (210±12 at 0 h and 309±9 at 2 h, in mean fluorescence units; $P<0.001$). When H_2O_2 was added to the samples, ROS concentration increased with H_2O_2, comparing with incubation without H_2O_2 (25 μM: 379±13, $P=0.011$; 50 μM: 413±27, $P<0.001$; 100 μM: 428±22, $P<0.001$; 200 μM: 521±37, $P<0.001$).

The effect of H_2O_2 on incubated spermatozoa is showed in Figure 2 as effect sizes respect to the Control at 2 h (0 μM H_2O_2). In general, only the highest H_2O_2 concentration (200 μM) showed an effect in this experiment. Total motility decreased with the incubation (from 36.6±5.7% to 28.6±8.4% at 2 h; $P=0.031$). It did not decreased further with H_2O_2 (effect size not significant; Fig. 2a), except for 200 μM,

which decreased motility down to 21.4±7.5% (P=0.037). While linearity neither changed with incubation nor H_2O_2 treatments (Fig. 2c), velocity and ALH decreased after 2 h of incubation (101.2±6.2 μm/s to 88.5±6.1 μm/s, P<0.031; 4.1±0.2 μm to 3.6±0.2 μm, P<0.020). Their values when they were incubated with 200 μM H_2O_2 were 72.7±9.1 μm/s and 3.0±0.2 μm (P=0.020 and P=0.009, respectively), not changing significantly with other H_2O_2 treatments (Figures 2b and 2d).

Incubation decreased the proportion of viable spermatozoa (55.2±4.4% to 46.1±5.1%; P=0.018), tended to increase the apoptotic ratio (47.9±5.3% to 52.5±5.9%; P=0.060) and slightly increased the lipid peroxidation of the samples, as estimated by the LPO by-product malondialdehyde, but not significantly (3.8±0.7 nmol MDA/10^8 spermatozoa to 3.9±0.6 nmol MDA/10^8 spermatozoa; P=0.051). Addition of H_2O_2 did not modify the proportion of viable spermatozoa or the apoptotic ratio, comparing with 2 h incubation without H_2O_2 (Figures 2e and 2f). LPO levels did not increase in any H_2O_2 treatment comparing with the incubation without H_2O_2 (Fig. 3a); nevertheless, when comparing with the results at 0 h, 50 μM H_2O_2 and above significantly increased MDA concentration (effect sizes of +0.4±0.1 for 50 μM, +0.5±0.1 for 100 μM and +0.4±0.1 for 200 μM, indicating increases above the 0 h levels, P<0.05; the effect size of 2 h incubation without H_2O_2 was +0.3±0.1, P=0.051).

The SCSA test revealed that incubation alone did not cause significant changes to chromatin stability (%DFI: 4.9±1.5% at 0 h and 3.2±0.6% at 2 h, P=0.353; HDS: 2.7±0.8% at 0 h and 4.1±1.3% at 2 h, P=0.198). Only 200 μM H_2O_2 increased %DFI significantly, up to 10.3±2.9% (Fig. 3b), not having effect on HDS (Fig. 3c).

Experiment 2. Individual male-to-male variation in response to oxidative stress.

In this experiment, we tested the effect of male-to-male variation during the incubation, including the response to oxidative stress caused by the higher dose of H_2O_2. In general, males differed at thawing ($P<0.01$ for TM, VCL, ALH and viability). Three groups were differentiated (see group at 0 h in Fig. 4): males 1, 2 and 6 were characterized by higher motility (TM: 40.9±3.0%; VCL: 104.7±5.3 μm/s; ALH: 4.1±0.2 μm) and viability (64.3±2.2%); males 3 and 4 were characterized by low motility (TM: 18.9±2.2%; VCL: 66.7±6.5 μm/s; ALH: 3.0±0.2 μm) while maintaining a relatively high viability (54.1±2.4%); and males 5 and 7 were characterized by low motility (TM: 16.8±1.4%), while maintaining high kinematic parameters (VCL: 94.9±2.0 μm/s; ALH: 4.0±0.1 μm) and lower viability (40.7±2.8%).

Considering the whole experiment, male-to-male variability did not disappear after incubation with or without H_2O_2, but it was a significant factor for all studied parameters ($P<0.001$ for TM, VCL, ALH and viability; $P<0.01$ for apoptotic ratio; $P<0.05$ for LIN and LPO). What is more important, that variability affected how samples from different males responded to the incubation and oxidative stress (male±treatment interaction). We found that interaction significant for VCL ($F_{12,27}=3.036$, $P=0.007$), LIN ($F_{12,27}=3.107$, $P=0.007$) and ALH ($F_{12,27}=2.662$, $P=0.017$). These differences throughout treatments can be appreciated in the interaction plots showed in the Figure 4. It is clear that the behaviour of the samples was similar in the case of LPO and viability (Fig. 4e and 4f, change during incubation and little difference among 2 h and 2 h plus H_2O_2), whereas most of the variability was showed in motility

parameters. Total motility (Fig. 4a) suggested some degree of male-to-male variability on the response to treatments, but not reaching significance ($F_{12,27}$=1.838, P=0.092).

Therefore, most of the variability concerning treatment response was expressed on the kinematic parameters. For VCL (Fig. 4b), males 1, 3 and 5 underwent little change after incubation, but dropped if H_2O_2 was included in the medium, whereas males 2, 6 and 7 were affected by incubation without H_2O_2 (and in a higher degree, specially for 6, in presence of H_2O_2), and male 4 was little affected by the treatments. A similar pattern was detected for ALH (Fig. 4d). For LIN (Fig. 4c), a different grouping developed. In a first group (males 2, 4 and 5), LIN was little affected by incubation or oxidative stress. Contrarily, LIN dropped during incubation in the samples of males 6 and 7, whereas it did not decrease during incubation in the samples of 1 and 3 (in fact, increased for 3), but decreased (considerably for 1) in presence of H_2O_2.

A principal component analysis of averaged results for each male and treatment allowed to show these results in the bidimensional space defined by the first two principal components extracted (Figure 5a). The male-to-male variability (initial characteristics, after incubation characteristics —either in presence or absence of H_2O_2 — and the response to the treatments) are displayed in the Figure 5b. In that plot it is made clear that samples from different males behaved differently, as showed by the different directions and lengths of the vectors joining the points for each sample. According to the direction of change after incubation without H_2O_2, males could be grouped in three groups: one grouping males 1 and 3, other with males 2 and 7 and a third one with males 4, 5 and 6. When H_2O_2 was included, male 2 was just affected by the effect size, while maintaining the same direction. Others showed a different

399 response (males 4, 5 and 7), but only in one or two parameters and not too large; male 6
400 could be included in this group, although the differences were much larger for that male.
401 Finally, males 1, 3 showed a dramatically different response if incubated in absence or
402 presence of H_2O_2.
403

404 ## Discussion

405 Oxidative stress has an important role in sperm physiology. In this study, we
406 have studied this topic on cryopreserved epididymal spermatozoa, therefore, we must to
407 point out that results might be different in fresh or ejaculated doses. Cryopreservation
408 not only reduces sperm quality, but also induces oxidative stress and decreases the
409 antioxidants in semen (Aisen et al., 2005;Peris et al., 2007), and epididymal
410 spermatozoa have not contacted with seminal plasma, which contributes to the
411 antioxidant defence of semen. Furthermore, male-to-male variability also affects to the
412 resistance to cryopreservation-derived damage (Esteso et al., 2006;Loomis and Graham,
413 2008), possibly enhancing post-thawing differences among males. These facts were
414 considered when planning this study, and therefore our analysis and conclusions are
415 within the context of cryopreserved epididymal spermatozoa.
416

417 In our previous study on oxidative agents (Martinez-Pastor et al., 2009a), we
418 found that 10 μM H_2O_2 did not affect thawed spermatozoa, but 100 μM and 1 mM
419 depressed motility within 1 h of incubation (in fact, 1 mM abolished sperm motility
420 almost immediately after adding it to the sample). We determined that a similar effect of
421 xanthine oxidase/hipoxanthine was in fact caused by H_2O_2 generation. In our study, the
422 only significant effects of H_2O_2 were caused by 200 μM, not by 100 μM. Apart from
423 some differences on the experimental design (Dominguez-Rebolledo et al., 2009), it is

424 possible that the limit above which H_2O_2 causes a detectable effect (regarding our
425 experimental tests) would lay in the order of magnitude of 10^{-4} M. Individual males
426 might present a different sensitivity to H_2O_2 within that order of magnitude, as
427 suggested by the male-to-male variability experiment. In our previous study, we used
428 samples from other set of males, which could be the source of the observed differences.
429
430 Although addition of H_2O_2 increased intracellular ROS, no other effects were
431 observed below 200 μM. We found that even 10 μM H_2O_2 could increase intracellular
432 ROS above Control (Martinez-Pastor et al., 2009a), but this increase did not result in a
433 noticeable change of motility or sperm physiology. However, Peris et al. (2007),
434 working with fresh ram sperm, found that 50 μM H_2O_2 decreased motility in only 1 h of
435 incubation. Nevertheless, these authors did not observe capacitation-related changes
436 (chlortetracycline stain) among different H_2O_2 concentrations (0, 50 and 150 μM),
437 except for 300 μM, which caused a significant increase in acrosome-reacted
438 spermatozoa at 1 h of incubation (but not after 4 or 24 h). In the present study, H_2O_2 did
439 not induce changes in the apoptotic ratio of the samples, a parameter depending on YO-
440 PRO-1 stain, putatively related to membrane condition and possibly connected to the
441 physiological status of the sperm cell (Martinez-Pastor et al., 2008;Peña et al., 2007).
442 Previous studies have highlighted the role of ROS on the modulation of sperm
443 physiology, and their role activating capacitation (Awda et al., 2009;Baumber et al.,
444 2003;O'Flaherty et al., 1999). For instance, Roy and Atreja (2008) induced capacitation
445 and associated tyrosine phosphorilation in buffalo spermatozoa by incubating with 50
446 μM H_2O_2. More detailed studies, such us analysis of tyrosine phosphorilation of specific
447 proteins, should be performed in small ruminants, in order to determine if H_2O_2 induces
448 physiological changes beyond those reported by Peris et al. (2007) and us. The detection

449 of these changes is of capital importance, since they might be unnoticed, affecting
450 sperm functionality farther in sperm work protocols.
451
452 Agreeing with previous studies (Aitken et al., 1993;Armstrong et al.,
453 1999;Martinez-Pastor et al., 2009a;Peris et al., 2007), sperm motility was the most
454 sensitive parameter to H_2O_2. Motility loss by H_2O_2 has been primarily attributed to the
455 inactivation of glycolytic enzymes, leading to energetic draining in the flagellum
456 (Armstrong et al., 1999;Baumber et al., 2000). However, the sensitivity of spermatozoa
457 to H_2O_2 varies dramatically among studies. Ramos and Wetzels (2001) found an almost
458 total loss of motility after incubating 5 min human spermatozoa with 25 μM H_2O_2.
459 Bilodeau et al. (2002), testing a wide range of H_2O_2 concentrations on bovine semen,
460 found that 75 μM of H_2O_2 immediately decreased sperm motility, and that just 12.5 μM
461 H_2O_2 decreased motility after 1 h of incubation. This might imply that small ruminant
462 spermatozoa might be more resilient to this effect, as we suggested in a previous study
463 (Martinez-Pastor et al., 2009a).
464
465 We observed a non-significant increase of MDA with time, which seemed to be
466 accelerated by H_2O_2 presence. This increase on LPO was unrelated to motility changes.
467 These observations suggest that red deer sperm might be little prone to H_2O_2-induced
468 lipoperoxidation. Peris et al. (2007) did not found increasing LPO levels when
469 submitting the samples to H_2O_2 levels up to 300 μM, but after incubating their samples
470 for 24 h. However, these authors found correlations among MDA concentration and
471 other sperm parameters, which was not noticed in our study. It seems that there are
472 between species differences regarding susceptibility and consequences of lipid
473 peroxidation. For instance, Alvarez and Storey (1989) could increase LPO and loss of

474 motility in human and mouse sperm by adding H_2O_2 (1 and 5 mM), whereas the same
475 concentrations of H_2O_2 were insufficient to induce LPO in rabbit sperm. Similarly, we
476 could detect an increase on LPO using the TBARS technique in deer spermatozoa after
477 incubating with 1 mM H_2O_2, but we could not detect a significant increase when
478 applying 100 μM H_2O_2 (Domínguez-Rebolledo et al. 2010)
479
480 Subjecting thawed spermatozoa to oxidative stress can affect chromatin integrity,
481 and the SCSA test can be used to detect it (Fernandez-Santos et al., 2009;Martinez-
482 Pastor et al., 2009b). Sperm chromatin integrity was affected by 200 μM H_2O_2.
483 Previously (Martinez-Pastor et al., 2009a), we could not identify such chromatin insult,
484 possibly because of the different set of males used. Again, it is possible that individual
485 sample quality (different stocks of semen doses) could have a role, although we cannot
486 discard variations in the experimental protocol (lack of sperm washing in our previous
487 study). In fact, we found that washed samples were more vulnerable to oxidative stress
488 than unwashed ones (Dominguez-Rebolledo et al., 2009). Other studies have shown
489 apparently lower chromatin damage susceptibility in similar species. For instance, Peris
490 et al. (2007) reported that SCSA showed that 150 and 300 μM H_2O_2 increased the %DFI
491 on ram spermatozoa, but only after 24 h incubation. In human spermatozoa, Ramos and
492 Wetzels (2001) did not detect DNA damage when sperm from normospermic men were
493 incubated for 1 h in the presence of 25 μM of H_2O_2, but damage was observed after 24 h
494 (using TUNEL), alerting that low levels of ROS can be damaging given long incubation
495 times. Another study (Hughes et al., 1996), using the COMET assay, showed that
496 applying 100 and 200 μM H_2O_2 for only one hour caused an important increase on DNA
497 damage, and that only 40 μM H_2O_2 was required to cause a small increase of DNA
498 damage in asthenozoospermic samples (although baseline levels were similar to those of

499 normozoospermic samples). This study highlights the importance of previous
500 susceptibility to oxidative stress and the importance of between sample heterogeneity,
501 regarding ROS resistance.
502
503 In the second part of our study, we aimed at studying the male-to-male
504 variability on the response to H_2O_2. Although we worked with a limited number of
505 males, it was evident that male-to-male variability had an effect, not only regarding the
506 resilience to oxidative stress, but also to incubation without oxidants. In other studies,
507 we have reported that sperm male-to-male variability seems to be high in red deer,
508 possibly due to the unselected nature of the populations from which we obtained our
509 samples (Garde et al., 2006). In fact, working with those wild populations allows us to
510 easily detect and study male-to-male variability, which would be harder to detect
511 working with animals submitted to strong human selection. We have previously showed
512 that red deer present evident male-to-male differences in sperm characteristics and
513 fertility (Malo et al., 2005) and in sperm sensitivity to cryopreservation (Soler et al.,
514 2003). Moreover, we have proposed that that variability could even reflect in biased sex
515 ratios, depending on the fertility of different males (Gomendio et al., 2006).
516
517 In the present study, motility parameters were affected by incubation in some
518 males, whereas in others motility was maintained almost unaltered for the duration of
519 incubation, being only affected if oxidative stress was present. Contrarily, although
520 individual variability was evident considering initial MDA concentration and sperm
521 viability, it did not affect the changes on these variables after incubation with or without
522 oxidative stress. It is known that many factors can affect membrane composition, among
523 them individual variability, and that its composition influences its resistance and

susceptibility to oxidative stress (Lenzi et al., 2002). It is possible that the small increase of LPO observed after incubation and H_2O_2 might have prevented us from detecting the interaction among males and treatments. Another reason could be that the high dilution and the freezing-thawing of the cryopreserved samples would have dimmed membrane-related differences, a hypothesis that could be tested in another study using fresh spermatozoa. It is important to consider that in vivo studies (Reglero et al., 2009) have showed no differences on LPO between deer living in areas contaminated with heavy metals and other living in uncontaminated areas, but the same study found differences among deer living in different estates. These findings suggest that some oxidative stress markers, such as LPO, could indeed depend more on the male than on environmental stressors.

Motility can be affected by multiple factors, and therefore it is a good candidate to detect variability among males or samples (Malo et al., 2005). The resistance of sperm samples to incubation was apparently not dependent on their initial quality. Thus, male 1 and 6 had similar initial motility, but whereas male 1 maintained the same motility after incubation and it was halved when H_2O_2 was present, it dropped dramatically for male 6, and it was abolished by H_2O_2. This example not only shows the impact of between male differences, but also that the initial quality of a sample (just after thawing, in this case) might be not informative of its real potentiality. Therefore, sperm "freezability" (comparison of the pre-freezing and post-thawing quality) might not suffice when characterizing samples from a male in the lab, being necessary to test the real resistance of spermatozoa by challenging them in physiological and non-physiological conditions (Roth et al., 1999;Soler et al., 2008). Furthermore, molecular

548 techniques may be used to predict the performance of spermatozoa beyond
549 cryopreservation (Grunewald et al., 2008;Thurston et al., 2002).

550 In summary, we conclude that oxidative stress caused by H_2O_2 clearly affected
551 kinematic parameters of cryopreserved red deer spermatozoa, but only at relatively high
552 concentrations (considering previous studies, at a magnitude of 10^{-4} M). It did not seem
553 to influence sperm viability or apoptotic markers (as defined with YO-PRO-1). This
554 may be a consequence of membrane resilience to oxidative stress, but also to the effect
555 of cryopreservation, which might have already removed susceptible spermatozoa from
556 the samples. We have to point out that we utilized only epididymal spermatozoa, and
557 that results might vary when using ejaculated samples. In a previous study on red deer
558 (Martínez-Pastor et al., 2006)), we that cryopreservation conditions of epididymal and
559 ejaculated samples might vary. These differences could also affect the response of
560 oxidative stress of ejaculated samples.

561

562 In conclusion, cryopreservation of gametes and embryos and the development of
563 Genetic Resource Banks (GRB) allow us to have a gene resource for an indefinite time
564 (Watson and Holt., 2001). These assisted reproductive technologies (ART) are
565 potentially capable of improving the propagation and conservation of wild and
566 endangered species (Wildt et al., 1997). Of the genetic material in cryobanks, the
567 collection, storage, and subsequent use of spermatozoa has found the most widespread
568 application (Watson and Holt., 2001). According to this, cryopreservation of
569 spermatozoa combined with artificial insemination (AI) has been the method of ART
570 that has been most extensively applied to deer species (Asher et al., 2000). In the
571 present work, male-to-male variability was evident in the response to incubation both
572 with and without H_2O_2. This male-to-male variability is important, since it reflects on

573 fertility and in the outcome of other artificial reproductive techniques (artificial
574 insemination and IVF success). Thus, we must discriminate among samples from
575 different males not only according to their "freezability", but also to their performance
576 after thawing and in stressing situations. We must take into account these differences as
577 much to improve freezing protocols as in the post-thawing protocols, considering
578 protective agents such as antioxidants, and adjusting them to the characteristics of
579 different kind of samples. This is especially important when dealing with valuable
580 specimens of endangered animals, which is usual working with wild species. Actually,
581 there is a remarkable interest in the use of ART for the management of Iberian deer
582 (*Cervus elaphus hispanicus*) populations. Specifically, ART may play an important role
583 for the purpose of ensuring genetic preservation and/or genetic progress. Moreover, our
584 results can contribute to the development of adequate protocols for red deer as a farming
585 species, and also for other small ruminants.
586
587 **Acknowledgements**
588
589 This work has been supported by the Spanish Ministry of Education and Science
590 (Project AGL2004-05904/GAN) and by the Junta de Comunidades de Castilla-La
591 Mancha (Project PAC06-0047). A.E Domínguez-Rebolledo was supported by Consejo
592 Nacional de Ciencia y Tecnología (CONACyT), México). Rocío Fernández Santos and
593 Felipe Martínez-Pastor were supported by the Juan de la Cierva and Ramón y Cajal
594 programs (Ministerio de Ciencia y Tecnología, Spain), respectively. We thank Enrique
595 Del Olmo for helping in field work and cytometry analyses.

596

597 ## References

598

599 Agarwal A, Saleh RA, 2002: Role of oxidants in male infertility: rationale, significance,
600 and treatment. Urol Clin North Am 29 817-827.

601 Aisen E, Quintana M, Medina V, Morello H, Venturino A, 2005: Ultramicroscopic and
602 biochemical changes in ram spermatozoa cryopreserved with trehalose-based
603 hypertonic extenders. Cryobiology 50 239-249.

604 Aitken RJ, Buckingham D, Harkiss D, 1993: Use of a xanthine oxidase free radical
605 generating system to investigate the cytotoxic effects of reactive oxygen species
606 on human spermatozoa. J Reprod Fertil 97 441-450.

607 Aitken RJ, Paterson M, Fisher H, Buckingham DW, Van DM, 1995: Redox regulation
608 of tyrosine phosphorylation in human spermatozoa and its role in the control of
609 human sperm function. J Cell Sci 108 (Pt 5) 2017-2025.

610 Alvarez JG, Storey BT, 1989: Role of glutathione peroxidase in protecting mammalian
611 spermatozoa from loss of motility caused by spontaneous lipid peroxidation.
612 Gamete Res. 23 77-90.

613 Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC, 1999:
614 Characterization of reactive oxygen species induced effects on human
615 spermatozoa movement and energy metabolism. Free Radic Biol Med 26 869-
616 880.

617 Asher GW, Berg DK, Evans G, 2000. Storage of semen and artificial insemination in
618 deer. Anim Reprod Sci. 62:195–211.

619 Awda BJ, Kenzie-Bell M, Buhr MM, 2009: Reactive oxygen species and boar sperm
620 function. Biol Reprod 81 553-561.

621 Baumber J, Ball BA, Gravance CG, Medina V, Vies-Morel MC, 2000: The effect of
622 reactive oxygen species on equine sperm motility, viability, acrosomal integrity,
623 mitochondrial membrane potential, and membrane lipid peroxidation. J Androl
624 21 895-902.

625 Baumber J, Sabeur K, Vo A, Ball BA, 2003: Reactive oxygen species promote tyrosine
626 phosphorylation and capacitation in equine spermatozoa. Theriogenology 60
627 1239-1247.

628 Bilodeau JF, Blanchette S, Cormier N, Sirard MA, 2002: Reactive oxygen species-
629 mediated loss of bovine sperm motility in egg yolk Tris extender: protection by
630 pyruvate, metal chelators and bovine liver or oviductal fluid catalase.
631 Theriogenology 57 1105-1122.

632 Castellanos P, Reglero MM, Taggart MA, Mateo R, 2010. Changes in fatty acid profiles
633 in testis and spermatozoa of red deer exposed to metal pollution. Reprod Toxicol
634 29:346-352.

635 Chen H, Chow PH, Cheng SK, Cheung AL, Cheng LY, WS O, 2003: Male genital tract
636 antioxidant enzymes: their source, function in the female, and ability to preserve
637 sperm DNA integrity in the golden hamster. J Androl 24 704-711.

638 De Lamirade, Gagnon C, 1994: Reactive oxygen species (ROS) and reproduction. Adv
639 Exp Med Biol 366 185-197.

640 Dominguez-Rebolledo AE, Fernandez-Santos MR, Garcia-Alvarez O, Maroto-Morales
641 A, Garde JJ, Martinez-Pastor F, 2009: Washing increases the susceptibility to
642 exogenous oxidative stress in red deer spermatozoa. Theriogenology 72 1073-
643 1084.

644 Dominguez-Rebolledo AE, Martinez-Pastor F, Fernandez-Santos MR, Del Olmo E,
645 Bisbal A, Ros-Santaella JL, Garde JJ, 2010. Comparison of the TBARS Assay
646 and BODIPY C(11) Probes for Assessing Lipid Peroxidation in Red Deer
647 Spermatozoa. (DOI: 10.1111/j.1439-0531.2009.01578.x).

648 Dominguez-Rebolledo AE, Fernandez-Santos MR, Bisbal A, Ros-Santaella JL, Ramón
649 M, Carmona M, Martinez-Pastor F, Garde JJ, 2010 Improving the effect of
650 incubation and oxidative stress on thawed spermatozoa from red deer by using
651 different antioxidant treatments. Reprod Fertil Dev. 22 856-870.

652 Esteso MC, Fernandez-Santos MR, Soler AJ, Montoro V, Quintero-Moreno A, Garde JJ,
653 2006: The effects of cryopreservation on the morphometric dimensions of
654 Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm heads. Reprod
655 Domest Anim 41 241-246.

656 Evenson DP, Darzynkiewicz Z, Melamed MR, 1980: Relation of mammalian sperm
657 chromatin heterogeneity to fertility. Science 210 1131-1133.

658 Fernandez-Santos MR, Dominguez-Rebolledo AE, Esteso MC, Garde JJ, Martinez-
659 Pastor F, 2009: Catalase supplementation on thawed bull spermatozoa abolishes
660 the detrimental effect of oxidative stress on motility and DNA integrity. Int J
661 Androl 32 353-359.

662 Fernandez-Santos MR, Esteso MC, Montoro V, Soler AJ, Garde JJ, 2006:
663 Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal
664 spermatozoa: effects of egg yolk, glycerol and cooling rate. Theriogenology 66
665 1931-1942.

666 Garde JJ, Martinez-Pastor F, Gomendio M, Malo AF, Soler AJ, Fernandez-Santos MR,
667 Esteso MC, Garcia AJ, Anel L, Roldan ER, 2006: The application of
668 reproductive technologies to natural populations of red deer. Reprod Domest
669 Anim 41 Suppl 2 93-102.

670 Gomendio M, Malo AF, Soler AJ, Fernandez-Santos MR, Esteso MC, Garcia AJ,
671 Roldan ER, Garde J, 2006: Male fertility and sex ratio at birth in red deer.
672 Science 314 1445-1447.

673 Griveau JF, Renard P, Le LD, 1995: Superoxide anion production by human
674 spermatozoa as a part of the ionophore-induced acrosome reaction process. Int J
675 Androl 18 67-74.

676 Grunewald S, Said TM, Paasch U, Glander HJ, Agarwal A, 2008: Relationship between
677 sperm apoptosis signalling and oocyte penetration capacity. Int J Androl 31 325-
678 330.

679 Hsu PC, Hsu CC, Guo YL, 1999: Hydrogen peroxide induces premature acrosome
680 reaction in rat sperm and reduces their penetration of the zona pellucida.
681 Toxicology 139 93-101.

682 Hughes CM, Lewis SE, Kelvey-Martin VJ, Thompson W, 1996: A comparison of
683 baseline and induced DNA damage in human spermatozoa from fertile and
684 infertile men, using a modified comet assay. Mol Hum Reprod 2 613-619.

685 Lenzi A, Gandini L, Lombardo F, Picardo M, Maresca V, Panfili E, Tramer F, Boitani C,
686 Dondero F, 2002: Polyunsaturated fatty acids of germ cell membranes,
687 glutathione and blutathione-dependent enzyme-PHGPx: from basic to clinic.
688 Contraception 65 301-304.

689 Loomis PR, Graham JK, 2008: Commercial semen freezing: individual male variation
690 in cryosurvival and the response of stallion sperm to customized freezing
691 protocols. Anim Reprod Sci 105 119-128.

692 Malo AF, Garde JJ, Soler AJ, Garcia AJ, Gomendio M, Roldan ER, 2005: Male fertility
693 in natural populations of red deer is determined by sperm velocity and the
694 proportion of normal spermatozoa. Biol Reprod 72 822-829.

695 Martínez-Pastor F, Martínez F, García-Macías V, Esteso MC, Anel E, Fernández-Santos
696 MR, Soler AJ, De Paz P, Garde JJ, Anel L, 2006. A pilot study on post-thawing
697 quality of Iberian red deer spermatozoa (epididymal and electroejaculated)
698 depending on glycerol concentration and extender osmolality Theriogenology 66
699 1165-1172

700 Martinez-Pastor F, Aisen E, Fernandez-Santos MR, Esteso MC, Maroto-Morales A,
701 Garcia-Alvarez O, Garde JJ, 2009a: Reactive oxygen species generators affect
702 quality parameters and apoptosis markers differently in red deer spermatozoa.
703 Reproduction 137 225-235.

704 Martinez-Pastor F, Fernández-Santos MR, Dominguez-Rebolledo AE, Esteso MC,
705 Garde JJ, 2009b: DNA status on thawed semen from fighting bull: a comparison
706 between the SCD and the SCSA tests. Reprod Domest Anim 44 424-431.

707 Martinez-Pastor F, Fernandez-Santos MR, Del Olmo E, Dominguez-Rebolledo AE,
708 Esteso MC, Montoro V, Garde JJ, 2008: Mitochondrial activity and forward
709 scatter vary in necrotic, apoptotic and membrane-intact spermatozoan
710 subpopulations. Reprod Fertil Dev 20 547-556.

711 O'Flaherty CM, Beorlegui NB, Beconi MT, 1999: Reactive oxygen species
712 requirements for bovine sperm capacitation and acrosome reaction.
713 Theriogenology 52 289-301.

714 Oehninger S, Blackmore P, Mahony M, Hodgen G, 1995: Effects of hydrogen peroxide
715 on human spermatozoa. J Assist Reprod Genet 12 41-47.

716 Ohkawa H, Ohishi N, Yagi K, 1979: Assay for lipid peroxides in animal tissues by
717 thiobarbituric acid reaction. Anal Biochem 95 351-358.

718 Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, Evenson D,
719 Thomas AJ, Jr., Alvarez JG, 2001: Characterization of subsets of human
720 spermatozoa at different stages of maturation: implications in the diagnosis and
721 treatment of male infertility. Hum Reprod 16 1912-1921.

722 Peña FJ, Saravia F, Johannisson A, Wallgren M, Rodriguez-Martinez H, 2007: Detection
723 of early changes in sperm membrane integrity pre-freezing can estimate post-
724 thaw quality of boar spermatozoa. Anim Reprod Sci 97 74-83.

725 Peris SI, Bilodeau JF, Dufour M, Bailey JL, 2007: Impact of cryopreservation and
726 reactive oxygen species on DNA integrity, lipid peroxidation, and functional
727 parameters in ram sperm. Mol Reprod Dev 74 878-892.

728 Ramos L, Wetzels AM, 2001: Low rates of DNA fragmentation in selected motile
729 human spermatozoa assessed by the TUNEL assay. Hum Reprod 16 1703-1707.

730 Reglero MM, Taggart MA, Castellanos P, Mateo R, 2009. Reduced sperm quality in
731 relation to oxidative stress in red deer from a lead mining area. Environ Pollut
732 157 2209-2015.

733 Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H, 2004: Role of hydrogen
734 peroxide in sperm capacitation and acrosome reaction. Biol Reprod 70 518-522.

735 Roth TL, Bush LM, Wildt DE, Weiss RB, 1999: Scimitar-horned oryx (*Oryx dammah*)
736 spermatozoa are functionally competent in a heterologous bovine in vitro
737 fertilization system after cryopreservation on dry ice, in a dry shipper, or over
738 liquid nitrogen vapor. Biol Reprod 60 493-498.

739 Roy SC, Atreja SK, 2008: Effect of reactive oxygen species on capacitation and
740 associated protein tyrosine phosphorylation in buffalo (*Bubalus bubalis*)
741 spermatozoa. Anim Reprod Sci 107 68-84.

742 Sharma RK, Said T, Agarwal A, 2004: Sperm DNA damage and its clinical relevance in
743 assessing reproductive outcome. Asian J Androl 6 139-148.

744 Soler AJ, Garcia AJ, Fernandez-Santos MR, Esteso MC, Garde JJ, 2003: Effects of
745 thawing procedure on postthawed in vitro viability and in vivo fertility of red
746 deer epididymal spermatozoa cryopreserved at -196 degrees C. J Androl 24 746-
747 756.

748 Soler AJ, Garde JJ, 2003: Relationship between the characteristics of epididymal red
749 deer spermatozoa and penetrability into zona-free hamster ova. J.Androl 24 393-
750 400.

751 Soler AJ, Poulin N, Fernandez-Santos MR, Cognie Y, Esteso MC, Garde JJ, Mermillod
752 P, 2008: Heterologous in vitro fertility evaluation of cryopreserved Iberian red
753 deer epididymal spermatozoa with zona-intact sheep oocytes and its relationship
754 with the characteristics of thawed spermatozoa. Reprod Domest Anim 43 293-
755 298.

756 Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV, 2002: Identification of
757 amplified restriction fragment length polymorphism markers linked to genes
758 controlling boar sperm viability following cryopreservation. Biol Reprod 66
759 545-554.

760 Waterhouse KE, Hofmo PO, Tverdal A, Miller RR, Jr., 2006: Within and between breed
761 differences in freezing tolerance and plasma membrane fatty acid composition of
762 boar sperm. Reproduction 131 887-894.

763 Watson PF, Holt WV, 2001. Organizational issues concerning the establishment of a ge-
764 netic resource bank. In: Watson PF, Holt WV, eds. Cryobanking the Genetic Re-
765 source. Wildlife Conservation the Future? London: Taylor & Francis; 86-112.

766 Wildt DE, Rall WF, Critser JK, Monfort SL, Seal US, 1997. Genome resource banks:
767 'Live collection' for biodiversity conservation. Bioscience 47:689–698.

768

769

Figure Legends

Figure 1. Representative histograms from samples stained with CM-H_2DCFDA/TO-PRO-3, showing fluorescence intensity for CM-H_2DCFDA in the TO-PRO-1– subpopulation (viable spermatozoa). A higher fluorescence (given as fluorescence channel number, 1–1024) indicates higher intracellular ROS. The mean fluorescence increased from baseline values at 0 h (a) to 2 h (b), and within 2 h, with increasing H_2O_2 concentrations [100 µM (c) and 200 µM (d) are showed here].

Figure 2. Effect sizes of the H_2O_2 treatments, for the CASA analysis and YO-PRO-1/PI stain (viability and apoptotic ratio). In each case, the Control value at 2 h (0 µM H_2O_2) was used as the intercept of the model (mean±SEM showed), effect sizes being the relative variation of the parameter from the Control value. For each H_2O_2 treatment, P values are given above of the x-axis (H_0: effect not different from 0). Total motility (TM; a), curvilinear velocity (VCL; b) and the mean amplitude of the lateral movement of the head (ALH; d) were significantly reduced after 200 µM H_2O_2 treatment, whereas linearity (LIN; c), viability (e) and the apoptotic ratio (f) were not significantly affected by H_2O_2 addition.

Figure 3. Model effect sizes of the H_2O_2 treatments, showed for the lipoperoxidation analysis (LPO) and SCSA (DNA damage). In each case, the Control value at 2 h (0 µM H_2O_2) was used as the intercept of the model (mean±SEM showed), and the effect sizes are the relative variation of the parameter from the Control value. For each H_2O_2 treatment, P values are given above of the x-axis (H_0: effect not different from 0). Neither LPO (a) nor HDS (high DNA stainability; c) were significantly affected by the tested H_2O_2 concentrations, but the percentage of spermatozoa with high DNA

796 fragmentation index (%DFI; b) significantly increased after incubation with 200 μM
797 H_2O_2.
798
799 **Figure 4.** These interaction plots shows the effect of the individual males (1–7) and
800 treatments (0 h, 2 h incubation and 2 h incubation with 200 μM H_2O_2) on sperm
801 parameters (mean values displayed). Lines do not imply a continuity among 2 h
802 incubation and 2 h incubation plus 200 μM H_2O_2, but they are used to highlight the
803 different values and changes, among the treatments, of samples from different males.
804 Differences among males are evident for motility parameters, while male-to-male
805 differences (regarding different behaviour among treatments) were minimal for LPO
806 and viability.

808 **Figure 5.** Representation of the multivariate data showed in Figure 4 in the
809 bidimensional space resulting from performing a principal component analysis (PCA)
810 with TM, VCL, LIN, ALH, viability (V) and LPO (the first two principal components,
811 PC1 and PC2, were selected). Subfigure (a) shows the variable loadings (linear
812 relationships among the principal components and the variables), represented by the six
813 eigenvectors, in order to help to interpret subfigure (b): for instance, in subfigure (b),
814 samples ”moving” towards the lower-right quadrant would indicate samples with
815 decreasing kinematic parameters, while those “moving” towards the upper-right
816 quadrant would have decreasing motility and viability, while increasing LPO. Subfigure
817 (b) presents the changes underwent by samples from different males (1–7) as
818 translations throughout the PC coordinates. Samples at 0 h are represented by circled
819 numbers, which are the starting point for vectors representing the change underwent by
820 these samples after 2 h of incubation (plain numbers) or 2 h of incubation with 200 μM

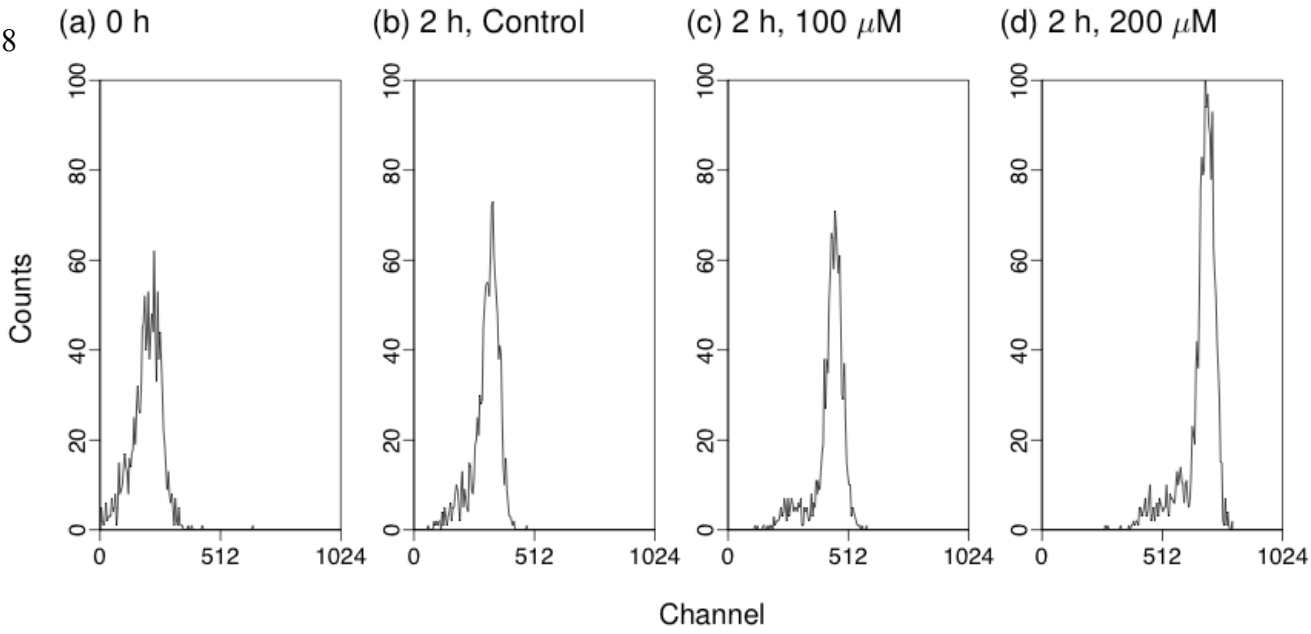
821 of H_2O_2 (italic-bold numbers). The male-to-male differences showed in Figure 4,
822 regarding treatment effects, are evident in this plot.
823
824

825 Figure 1

826

827

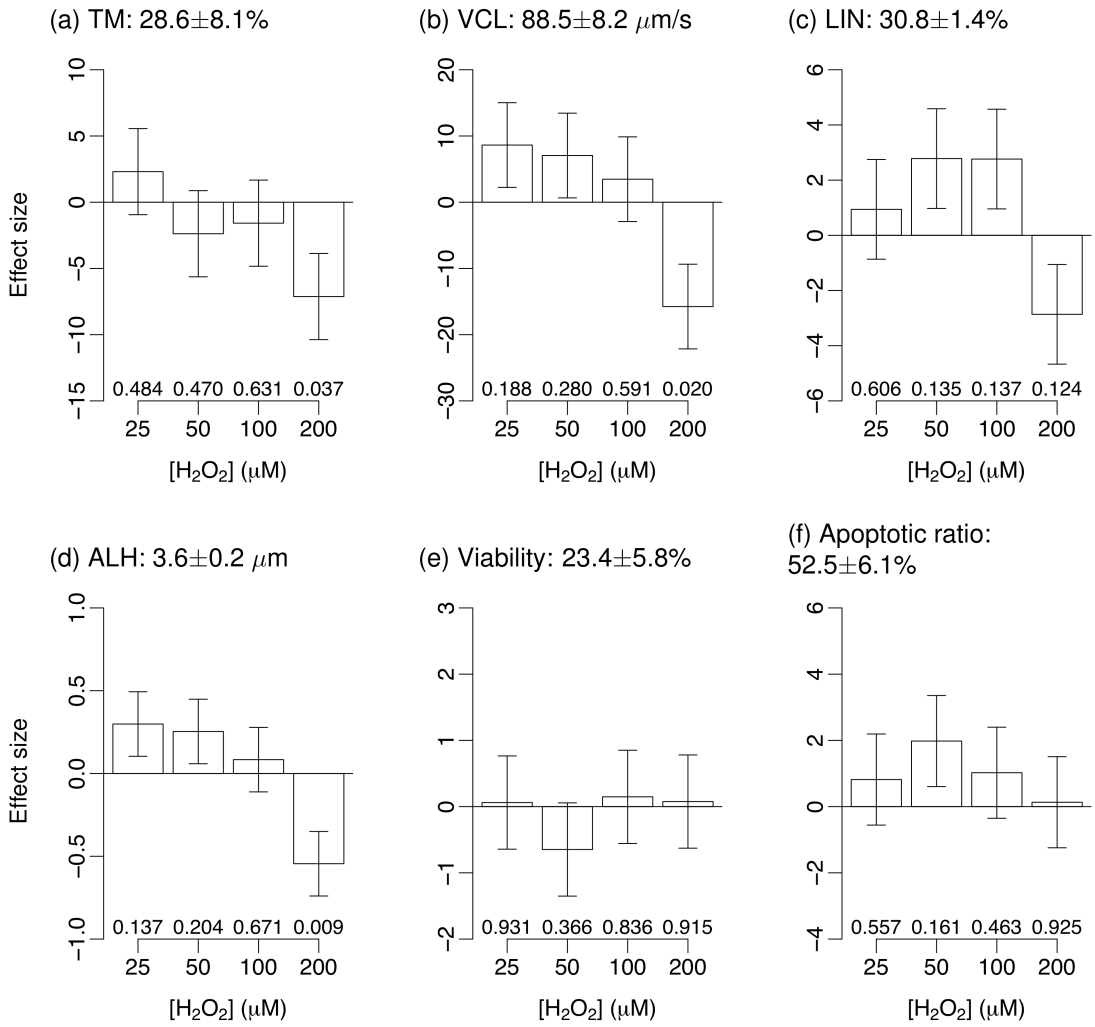
828



829

830 Figure 2

831



832

833

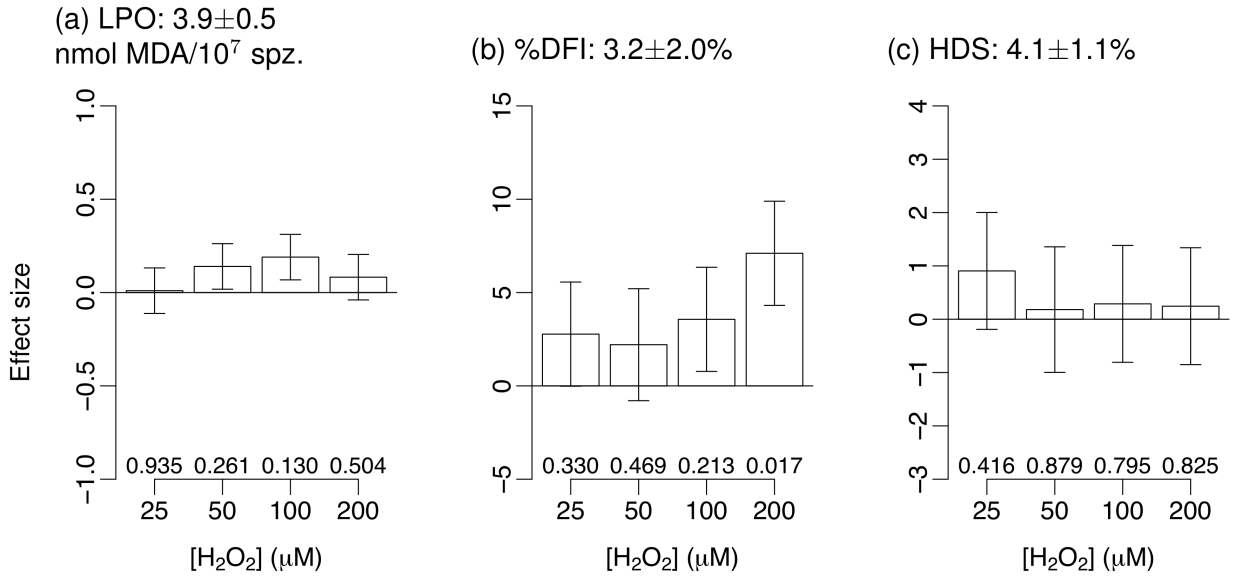
834

835 Figure 3

836

837

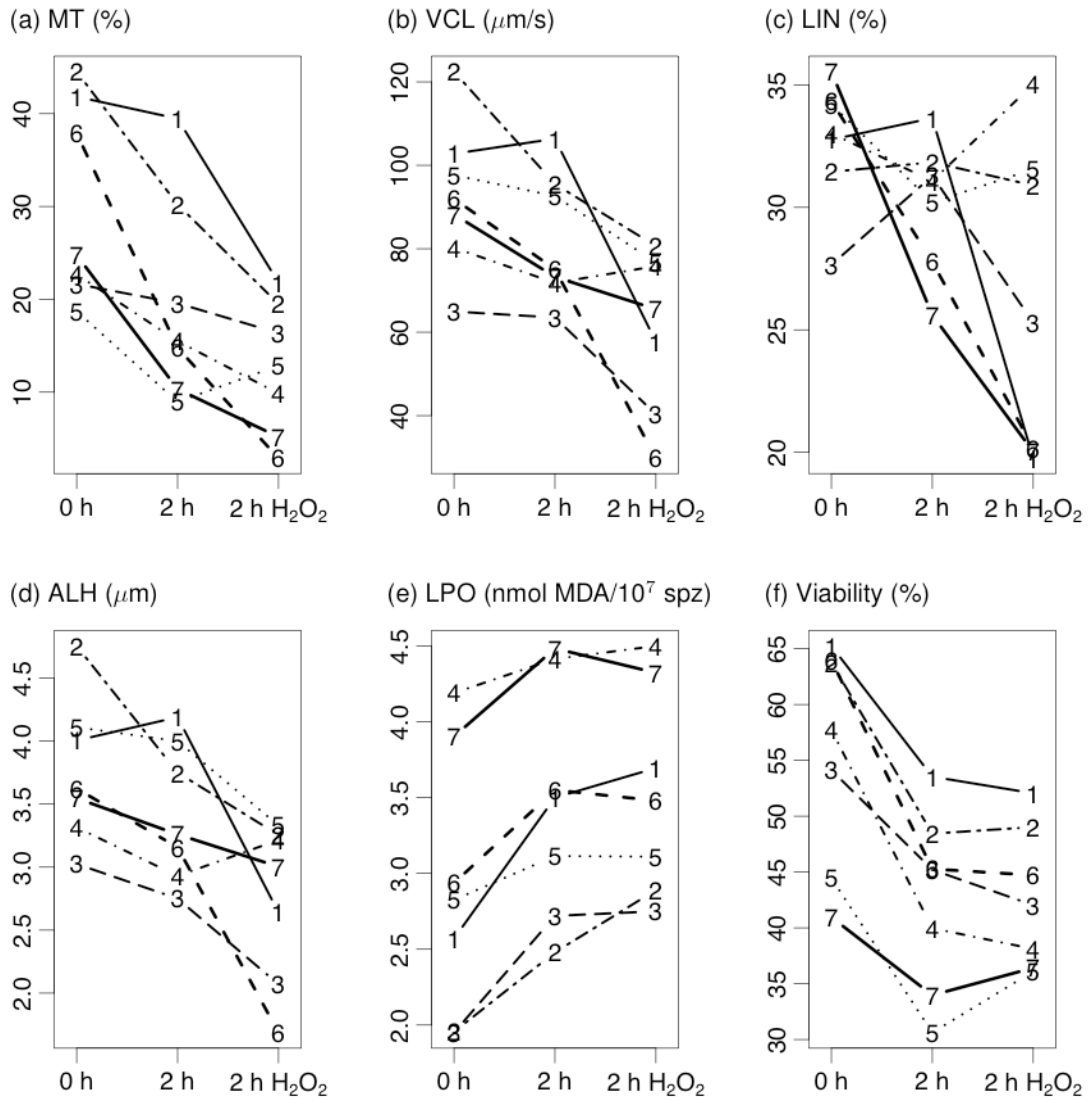
838



839

840 Figure 4

841



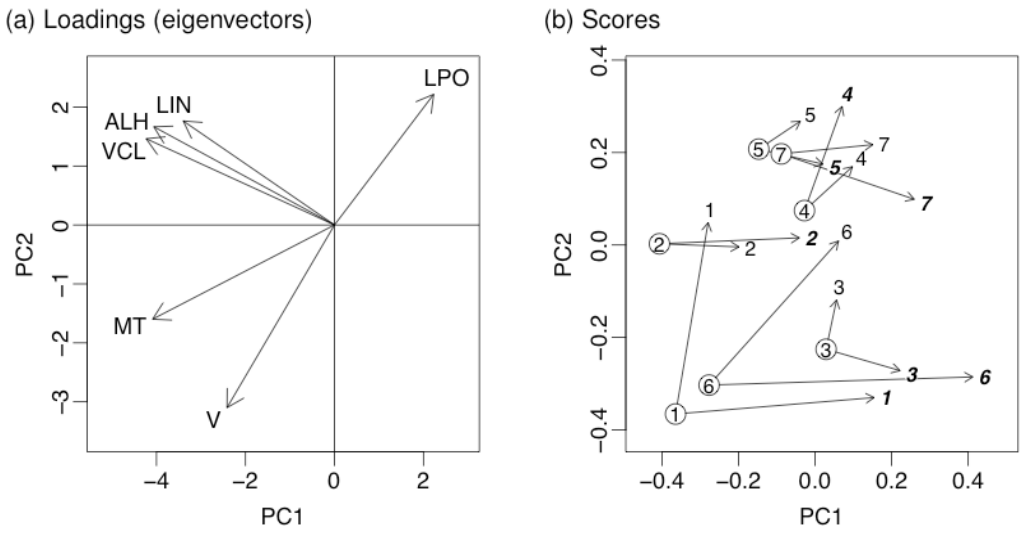
842

843

844

845 Figure 5

846



847

848

849
850
851