Effect of sex-sorting and cryopreservation on the post-thaw sperm quality of Iberian red deer spermatozoa.

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20 **Abstract**

21 This study investigated the effect of sex-sorting and cryopreservation on post-thaw
22 characteristics and fertility of red deer sperm. Semen was collected by
23 electroejaculation from 10 mature stags during the breeding season, and each ejaculate
24 split into four experimental groups: Bulk sorted spermatozoa, sorted but not sexed
25 (BSS); sorted high purity X-spermatozoa (XSS); sorted high purity Y-spermatozoa
26 (YSS); and, control non-sorted spermatozoa (NS). Following, all samples were frozen
27 over liquid nitrogen. Two straws per stag and sample type were analyzed immediately
28 post-thaw and following a 2-h incubation period at 37°C. Post-thaw total motility (TM)
29 as assessed by CASA was not different ($P<0.05$) among NS, BSS and YSS sperm. For
30 XSS, post-thaw TM was lower (39%, $P<0.05$) than that for NS (54%) or BSS (50%),
31 but similar ($P>0.05$) to that of YSS (47%) sperm. The percentage of apoptotic
32 spermatozoa as assessed by PI/YO-PRO-1 and flow cytometry analysis, was higher
33 (17%, $P\leq 0.05$) for XSS sperm than NS (12%), BSS (13%) and YSS (14%) sperm.
34 Following incubation there were no differences ($P>0.05$) in TM or percent apoptosis
35 among treatments. Post-thaw chromatin stability calculated as the DNA fragmentation
36 index (%DFI) was similar among treatments; following incubation %DFI increased in
37 all except YSS, which displayed the lowest value ($P<0.05$). Artificial insemination of
38 synchronized hinds yielded 44, 51 and 70% delivery rates for YSS, NS and standard
39 frozen-thawed sperm, respectively ($P<0.05$). Notably, 93 and 55% of fawns born were
40 males for the YSS and NS spermatozoa, respectively ($P<0.05$). In summary, Y-sorted
41 sperm displayed acceptable post-thaw sperm evaluation parameters and the expected
42 offspring sex ratio. More studies are needed to understand the source of sperm damage
43 that may compromise the fertility of Y-sorted red deer sperm.

44 Key-words: Red deer, sex-sorted sperm, insemination, X and Y-chromosomes

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1. Introduction

Iberian red deer belong to a deer subspecies that only inhabit the Iberian Peninsula. In recent years, there has been an increased trend towards deer farming in Spain, becoming a viable alternative to raising more conventional livestock species. Generally speaking, these are farms in which the main financial profits rely on antler trophies. Since males have the highest economic value, the possibility of sex selection at breeding would result in considerable cost savings and avoid the massive slaughter of females.

Assisted reproduction technologies have provided the opportunity to choose the offspring sex in domestic livestock species. For instance, with embryo production by MOET (multiple ovulation and embryo transfer) this can be achieved by determining the sex of preimplantation embryos and then selecting those of interest for transfer. However, the low efficiency of this technique derived from discarding embryos of unwanted sex limits its use. Therefore, the only accurate and potentially cost effective method for offspring sex selection is sperm sexing by flow cytometry [1]. Indeed, pre-selection of spermatozoa based on the different DNA amount between the X and Y chromosomes has become one of the most important reproductive technology advances in livestock species [1-3].

The main drawback of this technique is the low fertility yield, which has often been attributed to the low insemination dose used with sex-sorted sperm. However, increasing the number of spermatozoa to numbers comparable to those used with non-sexed sperm did not improve pregnancy rates in heifers [4]. In addition, the flow cytometry procedure is not innocuous given that sperm are exposed to stressors such as fluorescent dyes, high dilution rate, mechanical injury, laser illumination and subsequent passage through an electric field for sex-sorting [2]. Capacitation-like changes have been observed after sorting of both ram and boar spermatozoa [5-8]

71 reducing their fertilizing lifespan in vivo [9]. Unlike the capacitation status, however,
72 motility, as well as acrosome and DNA integrity of sorted sperm are usually high
73 because prior to the procedure cells are stained with a food dye that discriminates
74 nonviable cells [10] thus eliminating them from the sorted pool. Nevertheless, some of
75 the negative effects of sorting on sperm quality measures may not be immediately
76 evident, but only after an incubation period [11-13].

77 Because sex-sorted spermatozoa are usually cryopreserved for logistic reasons,
78 this aggravates any existing cell damage, further impacting on fertility [14].
79 Cryopreservation alone may lead to capacitation-like changes, motility impairment and
80 oxidative damage [14]. Hence the low levels of antioxidants present in the sperm
81 sorting medium, paired with the high production of reactive oxygen species during the
82 sorting and freeze-thaw processes may further compromise the fertilizing ability of sex-
83 sorted sperm samples.

84 In deer, few studies have addressed sex-sorting of sperm despite an increased
85 interest by the industry. When Sika deer sperm were sorted prior to insemination of
86 Wapiti hinds, calving rates were close to half of those obtained with non-sorted sperm
87 from the same proven stags [15]. In that study, however, sperm characteristics were not
88 assessed and the reasons for the low fertility were unknown. Another laboratory
89 compared the effect of sex-sorting on DNA fragmentation in cryopreserved sperm from
90 white-tailed deer stags [16]. Interestingly, in this study rates of DNA damage were
91 lower in sorted than non-sorted sperm, although samples were only evaluated
92 immediately after thawing and some detrimental changes might have become evident
93 over time. Therefore, the objective of this study was to assess sperm quality at different
94 steps during the sex-sorting procedure as well as to examine the fertility rates obtained
95 with sex-sorted-frozen-thawed samples.

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97 **2. Materials and Methods**

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99 **2.1. Reagents and media**

100 Fluorescence probes YO-PRO-1, Hoechst 33342 and Mitotracker Deep Red were
101 purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and PNA-FITC
102 were acquired from Sigma (Madrid, Spain), and acridine orange (chromatographically
103 purified) was purchased from Polysciences (Warrington, PA, USA). Fluorescent probe
104 stock solutions were prepared in DMSO (50 μ M YO-PRO-1; 1 mM Mitotracker Deep
105 Red) or water (7.5 mM PI; 0.2 mg/mL PNA-FITC; 1 mg/mL acridine orange; 25
106 mg/mL Hoechst 33342) and stored in the dark at -20 °C, except for Hoechst 33342
107 (H42) and acridine orange which were stored at 5°C. Flow cytometry equipment,
108 software and consumables were purchased from Beckman Coulter (Fullerton, CA,
109 USA) or Becton Dickinson (San Jose, CA, USA).

110 The collecting medium used during sorting was a Tris-Citrate-Glucose (TCG)
111 (pH: 7.3 and pOsm: 380 mOsm/kg) containing: glucose (250 mM), sodium citrate (12
112 mM), EDTA (1.6 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk at 5% (V/V)
113 penicillin (0.7 mM), and streptomycin (1.14 mM). The ejaculate washing medium was
114 the transport extender with the addition of 2.5% (V/V) egg yolk. The transport medium
115 was a Tris-Citrate-Fructose (TCF) (pH: 7.3 and pOsm: 330 mOsm/kg) containing: Tris
116 (213 mM), citric acid monohydrate (71.83 mM), fructose (55.51 mM), egg yolk at 20%
117 (V/V), penicillin (0.7 mM), and streptomycin (1.14 mM). The working medium for
118 running samples through the flow cytometer was the bovine gamete medium (BGM-3)
119 and was composed of: NaCl (87 mM), KCl (3.1 mM), CaCl₂ (2 mM), MgCl₂ (0.4 mM),
120 NaH₂PO₄ (0.3 mM), HEPES (40 mM), sodium lactate (21.6 mM), sodium pyruvate (1

mM), kanamycin (0.017 mM), phenol red (28.22 mM) and BSA (6 mg/mL) (pH 7.5). Solutions for SCSA (Sperm Chromatin Structure Assay) were prepared as follows [17]: TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4); acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4); and, acridine orange solution (0.1 M citric acid, 0.2 M Na₂HPO₄, 1 mM EDTA, 0.15 M NaCl, 6 µg/mL acridine orange pH 6.0). These solutions were kept at 5 °C in the dark.

2.2. Ejaculate collection and sperm sample preparation

Samples were obtained from 10 mature stags during the breeding season (mid-September). Animals were housed in a semi-free ranging regime at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in accordance with Spanish law in regards to the care and use of research animals (RD 53/2013) conforming to European Union regulation 2010/63. Semen collection by electroejaculation was carried out as described Martínez-Pastor et al. [18]. Briefly, males were anesthetized with 0.75 mg/Kg of Xylazine (Rompun[®] 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of feces and the prepucial area was shaved and washed with physiological saline solution. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The electroejaculation regime consisted of a consecutive series of 5 pulses of similar voltage and separated by 5 sec. Initially, 1V was applied that was then progressively increased at the next series until reaching a maximum of 5V. Semen was collected by fractions in graduated glass tubes. We discarded the fractions with urine contamination, that is, positive to Urea Test Strips

(Diagnostic Systems GmbH, Holzheim, Germany). Fractions with total sperm motility under 80% were also discarded.

Semen was diluted 1:3 in TCG 2.5% egg yolk and then centrifuged at 600xg for 5 minutes. The supernatant was removed and sperm concentration of the pellet was assessed using a hemocytometer (Bürker chamber; Brand GmbH, Wertheim, Germany), after diluting an aliquot of the sample in a glutaraldehyde solution (5 µL of sample in 500 µL of 2% glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). Then, sperm aliquots were diluted to a concentration of 800×10^6 sperm/mL in TCF medium supplemented with 20% (v/v) of egg yolk and transported to the sorting facility (about 8 h at 17°C). Upon arrival to the laboratory each of the sperm samples was split into two aliquots, for control (non-sorted sperm or NS) and sperm sorting (bulk sorted sperm or BSS; X-enriched sperm or XSS; and, Y-enriched sperm or YSS). Sperm samples for sorting were further diluted to 100×10^6 sperm/mL with TCG (0% egg yolk) medium and stained with 2.6 µL of H-42 (Stock solution: 25 mg/mL) during 50 minutes at 34°C as previously described by Parrilla et al. [19].

2.3. Flow cytometry sperm sex sorting

Just prior to sorting stained sperm samples were filtered through a 30-µm nylon mesh filter and 1 µL of food dye (0.002% w/v; FD&C #40, Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to each sample to quench the fluorescence of H-42 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process [20]. Spermatozoa were sorted into XSS and YSS according to the Beltsville Sperm Sorting Technology method [21] using a high-speed cell sorter (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) modified for sperm sorting. The

cell sorter was operated at 40 psi and was equipped with a UV-laser set at an output of 175 mW (Spectra Physics 1330, Mountain View, California). The samples were sorted in the presence of a HEPES-buffer based sheath [22] supplemented with 0.1% EDTA (w/v), and were collected in 50-mL tubes prefilled with 2.5 mL of collection medium (TCG medium containing 5% (v/v) of EY). A total of 20×10^6 sorted spermatozoa were collected per tube in an approximate volume of 10 mL with a purity of $\geq 90\%$.

2.4 Sperm cryopreservation

Sorted spermatozoa were centrifuged at 3000xg for 4 min at 21°C. The supernatant was discarded, and the pellets were re-extended to 40×10^6 sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of EY. Then sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21°C to 5 °C over 90 minutes, and left for an equilibration time of 2 h. After this period semen was packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany), placed on a rack 4 cm above liquid nitrogen for 10 min, and then plunged into liquid nitrogen and stored accordingly.

A control group consisting of non-sorted (NS) spermatozoa frozen under the same conditions as the sorted spermatozoa was also included in this experiment. Control sperm aliquots were gradually diluted to 20×10^6 sperm using HEPES-buffer based sheath fluid in the presence of collection medium, mimicking the conditions to which sorted sperm were exposed. Then control sperm samples were stored at room temperature (21–22°C) for approximately 4 h before being processed for freezing together with the sorted samples as described above and at the same sperm concentration.

2.5. Sperm analyses

Two straws per stag and sample type (NS, BSS, XSS and YSS) were analysed after thawing and after a 2-h incubation at 37°C. The goal of carrying out the incubation was to evaluate the resistance of sperm cells to heat stress.

2.5.1. Motility assessment by computer-aided sperm analysis (CASA)

A CASA system was used to assess sperm motility characteristics. Samples were loaded into a Makler counting chamber (10- μ m depth) at 37°C. The CASA system consisted of a triocular optical phase contrast microscope (Eclipse E400; Nikon, Tokyo, Japan), equipped with a warming stage at 37°C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the ISAS software v. 1.2 (Proiser, Valencia, Spain). Sampling was carried out using a X10 negative phase contrast objective (no intermediate magnification). Image sequences were saved and analyzed afterwards. The standard parameter settings were 50 frames/s and 20 to 90 μ m² for head area. For each sperm sample, the following parameters were measured: the percentage of motile spermatozoa (TM; %), curvilinear velocity (VCL; μ m/sec), straightness (STR; %), amplitude of lateral head displacement (ALH; μ m). The total motility (TM) was defined as the proportion of spermatozoa with VCL>10 μ m/s.

2.5.2. Sperm analysis by flow cytometry

Several quality measures were assessed by flow cytometry using fluorescent probes previously validated for red deer sperm [18, 23]. Samples were diluted to 1×10^6 spermatozoa/mL in BGM-3, and stained using the fluorophore combinations PI/YO-PRO-1 to assess membrane permeability and viability, PI/PNA-FITC to assess acrosomal status, and YO-PRO-1/Mitotracker deep red to assess mitochondrial activity.

A CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA), with semiconductor lasers emitting at 405 nm (violet; Hoechst 33342) and 488 nm (blue; YO-PRO-1 and PI) was used for analyses. Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342, 530/40 (green) for YO-PRO-1 and 613/20 (red) for PI. The system and event analyses were controlled using the Summit software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 spermatozoa were recorded, saving the data in flow cytometry standard (FCS) v. 3 files. The analysis of the resulting data was carried out using WEASEL v. 3 (WEHI, Melbourne, Australia). The results of PI/PNA-FITC and YO-PRO-1/PI/Mitotracker deep red combinations were interpreted as previously described for red deer sperm [18,23]. In both cases, Hoechst 33342 (5 mM) was added to weed out debris from sperm. Spermatozoa stained in these two solutions were incubated 10 minutes in the dark before the analysis. Interpretation of the different stain combination results was as follows: YO-PRO-1(-)/PI(-), live spermatozoa with intact plasmalemma (Live_NotApo, %); YO-PRO-1(+)/PI(-), apoptotic spermatozoa (Apoptotic, %); YO-PRO-1(-)/Mitotracker (+), live spermatozoa with active mitochondria (Mito_Act, %); and, PI(-)/PNA-FITC(-), live spermatozoa with intact acrosome (Live_Acro, %).

2.5.3. Chromatin stability

Chromatin stability was assessed following the SCSA (Sperm Chromatin Structure Assay) [17], as performed previously with epididymal sperm samples from red deer [24]. This technique is based on the susceptibility of sperm DNA to acid-induced denaturation *in situ* and the metachromatic staining acridine orange (AO). This stain fluoresces green when combined with double stranded DNA (dsDNA), but red when combined with single stranded (denatured) DNA (ssDNA). Samples were diluted in TNE buffer to a final concentration of 1×10^6 cells/mL, and stored at -80°C . For analysis, samples were thawed on crushed ice. Then, 200 μL of sample were pipetted into a flow cytometry tube, and immediately mixed with 0.4 mL of the acid-detergent solution for denaturation. After 30 s, samples were stained with 1.2 mL of the AO solution. The tube was kept on ice for 3 min before flow cytometry analysis.

We analyzed at least 1000 events per sample, exciting the AO with the Ar-ion 488 nm laser, and using a 530/30 filter for the green fluorescence of dsDNA-bound AO, and a 650 long pass filter for the red fluorescence of ssDNA-bound AO. Data were saved in flow cytometry standard (FCS) v. 2 files and then processed using the R statistical environment.

We calculated the DNA Fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence against the total fluorescence (red and green). High values of DFI indicate chromatin abnormalities. We also calculated %DFI, as the percentage of spermatozoa with $\text{DFI} > 25$, and High DNA Stainability (HDS) as the percentage of the spermatozoa with green fluorescence higher than channel 600 (out of 1024 channels).

2.6. Artificial insemination

Since, males are the main interest in the production system of Iberian red deer and there were no differences between YSS and NS, we carried out an artificial insemination trial

using YSS sperm. We used NS sperm samples diluted down to the same concentration as YSS sorted samples, but not submitted to the sorting process, as a control for YSS. Moreover, artificial inseminations were also performed with standard frozen-thawed sperm from nine different males. Semen from these males was collected by electroejaculation, diluted with Triladyl at 100×10^6 spermatozoa/mL and cryopreserved as described by Anel-López et al. [25].

The trial was conducted at the Medianilla farm in Cádiz (Spain) during the month of September in 2010, 2011, 2012 and 2013. Six hundred seventy-six Iberian deer does (4-6 years old; 75-101 kg body weight) were housed in outdoor enclosures that provide exposure to natural fluctuations in light and temperature. Estrous synchronization of hinds was performed as previously described by Garde et al. [26]. Briefly, single controlled internal drug releasing (CIDR) devices (type G, 330 mg progesterone per device, InterAg Effective Agricultural System, Hamilton, NZ) were inserted vaginally. The CIDRs were replaced in each animal on day 9 to ensure high progesterone concentrations and 0.75 mL of PGf₂ α (Prosolvin®; VIRBAC ESPAÑA S.A.) per hind were administered at this time. Upon CIDR removal on day 12, hinds were given 250 IU of eCG (Folligon, Intervet, Salamanca, Spain) intramuscularly. Females were then transcervically inseminated with NS or YSS sperm at a concentration of 40×10^6 spermatozoa/mL or standard frozen-thawed sperm at a concentration of 100×10^6 spermatozoa/mL. Hinds that had been inseminated were moved to a separate pen to be housed individually one week prior to the expected parturition date. The sex of fawns was identified at birth. Fertility rates were assessed based upon delivery rates.

2.7. Statistical analysis

Data were analysed using the SASTM V.9.1. Package (SAS Institute Inc., Cary, NC, USA). Data are shown as mean $\pm$ standard error of the mean (SEM). Significance level was set at $P \leq 0.05$. Data for sperm evaluation parameters were analyzed using a linear mixed-effects models (MIXED procedure, ML method) test, where sample (NS, BSS, XSS and YSS) and incubation time after thawing (0 vs. 2 hours) were considered fixed factors, and males were considered a random effect. When significant, means were compared using Tukey test. Sperm fertility data were compared using a general linear models (GLM) analysis, including type of sperm sample (NS vs. YSS) and male within type of sperm sample as fixed factors and fertility as a dependent variable. The number of inseminated does was considered as weight variable. Bonferroni test was used for the comparison of means.

3. Results

Post-thaw TM was not different ($P \leq 0.05$) among NS, BSS and YSS sperm. However, for XSS, post-thaw TM was lower (39%, $P \leq 0.05$) than that for NS (54%) or BSS (50%), but similar ($P > 0.05$) to that of YSS (47%) sperm (Figure 1A). Following the two-hour incubation period there were no differences ($P > 0.05$) in sperm TM among treatments, although the values for this parameter decreased with time for all treatments (Figure 1A).

Regarding kinetic sperm parameters, there were no significant differences among the different sperm groups for VCL and ALH (Figure 1B-D). However, STR was lower (55%, $P \leq 0.05$) for NS sperm than BSS (66%), XSS (62%) and YSS (63%) sperm, although this difference disappeared following sperm incubation (Figure 1C).

For three of the sperm parameters assessed by flow cytometry, that is, Live_NotApo, Mito_Act and Live_Acro, averages were similar among treatments for both evaluation times (Figure 2A, C, D). Conversely, the percentage of apoptotic spermatozoa was higher (17%, $P \leq 0.05$) for XSS sperm than NS (12%), BSS (13%) and YSS (14%) sperm (Figure 2B). Following a 2-h incubation period, the percent apoptotic cells was generally lower ($P > 0.05$) among BSS (8%), XSS (9%) and YSS (9%) sperm (Figure 2B).

The post-thaw %DFI was similar ($P > 0.05$) among all types of sperm samples (Figure 3A). Following the incubation period, values for this parameter increased further in all sperm treatments except for YSS in which the %DFI was similar ($P > 0.05$) to that observed immediately after thawing. Moreover, at the 2-h incubation time point the %DFI of YSS sperm was the lowest ($P \leq 0.05$) when compared to all other types of sperm (Figure 3A). Similarly, the %HDS was lower ($P \leq 0.05$) for YSS sperm at both

evaluation times in relation to NS and XSS sperm, although it was not significantly different to that in BSS sperm (Figure 3B) .

Despite the fact that YSS sperm faired best in regards to most sperm quality parameters assessed, fertility for this type of sample was lower ($P \leq 0.05$) than that for NS sperm (44% vs. 51%, respectively; Figure 4). Moreover, these values were lower than those achieved with standard frozen-thawed sperm, which provided live delivery rates of 70% (data not shown). As per accuracy of the sperm sorting procedure, 93 and 55% of fawns born from insemination of YSS and NS sperm, respectively, were males.

4. Discussion

While the commercialization of sexed semen is already a reality for some species such as cattle or horses, this technology is still not well developed for deer. The only study performed in this species showed a low fertility of sex-sorted sperm when compared to non-sexed sperm [15]. However, that study did not address whether low pregnancy rates with sex-sorted sperm were due to the lower sperm numbers used for insemination or to the damage to sperm resulting from the sex-sorting procedure [15]. Therefore, the aim of our study was to evaluate the effect of sex sorting and cryopreservation of deer sperm on its subsequent post-thaw quality and fertilizing ability. For this purpose we evaluated different sperm quality parameters in bulk sorted sperm (BSS), as well as in X- and Y-enriched sperm populations that were sorted following criteria for purities reaching at least 93% of the desired population (XSS and YSS). Moreover, we also compared the in vivo fertility rates between the YSS and NS sperm treatments. Ultimately, we showed that it is possible to obtain a population of Y-sorted frozen-thawed sperm samples yielding satisfactory pregnancy rates and a high percentage of offspring of the desired sex.

Notably, one of the many drawbacks with the sex-sorting technology is that equipment needed to perform the sorting is usually available only in specialized laboratories. Moreover, sorting through flow cytometry is usually very slow, lasting for hours, and hence sperm cell yields are usually low and, invariably there is some degree of sperm cell damage. In addition, for practical reasons, sorted-sperm from livestock species must be cryopreserved for storage prior to its use back at the farm further compromising sperm quality [14]. In our study, red deer sperm displayed appropriate survival rates through the sex sorting procedure, as manifested by sperm quality parameters evaluated in comparison to those in non-sorted sperm. However, the damage produced during passage through the flow cytometer might have been partially counteracted by the fact that non-viable sperm may be removed through the sorting procedure. In fact, sex-sorting yielded a high percentage of motile sperm when cells were stained with viability stains and non-viable sperm were eliminated [27]. By performing this simple step herein we believe we were able to enrich sperm quality of sorted sperm and optimize their ability to further sustain the freeze-thaw procedure.

In this study kinetic values were similar among sperm treatments with the exception of STR in NS immediately post-thaw, which was lowest. Reported results regarding the effect of the sex-sorting procedure on sperm kinetics are generally confusing. While sorting reduced VCL and ALH [28,29] in some studies, others have found no differences [30] or even an improvement [31] in the sperm motility parameters of sorted sperm. Disagreements among studies could be due to the different treatments performed during the sex-sorting procedure or even to species-specific differences. It is known that some species such as rams are highly resistant to the sorting process, exhibiting improved motility compared with non-sorted spermatozoa [32]. Moreover, the addition of substances such as seminal plasma in the collection medium improved

the motility and viability of sex-sorted bull and boar spermatozoa [33-34]. Conversely, the presence of seminal plasma during staining and sorting decreased the percentage of live spermatozoa in bovine [35]. These findings highlight the fact that species-specific studies must be performed to define sex-sorting protocols yielding best results.

We were also interested in assessing any changes potentially linked to capacitation, such as increases in VCL and ALH or a decrease in STR [36,37]. Therefore, post-thaw sperm motility parameters were also evaluated following a 2-h incubation period. Interestingly, all VCL, ALH and STR remained unchanged in our study, suggesting that the sex-sorting procedure did not appear to induce capacitation-like changes in red deer sperm, as reported for ram and boar [5,8]. It is also plausible that the incubation length tested herein (2 h) and/or that the medium in which sperm were incubated, were not conducive to such changes.

The percent apoptotic cells was in general higher for all sperm treatments over NS sperm, while all other sperm quality parameters evaluated by flow cytometry yielded similar values across all treatments. Sex-sorting of stallion sperm also yielded lower percentages of intact spermatozoa and a slight increase in apoptotic sperm [38]. In addition, these authors identified many proteins related to apoptosis in sex-sorted stallion spermatozoa.

Regarding to %DFI, in our study YSS samples showed a lower percentage of this parameter after incubation. Moreover, the percentage of HDS was lowest for YSS sperm. In fact, sex-sorted samples, in general, displayed lower values of HDS than non-sorted samples. This agrees with a previous study using sex-sorted sperm in white tail deer [16]. The authors concluded that the sperm subpopulation obtained after sex sorting displayed greater DNA stability than conventionally prepared samples and

suggested that sorted samples had higher concentrations of highly selected viable spermatozoa with a reduced propensity toward sperm mortality.

Unexpectedly, immediately post-thaw sperm motility was lowest and percent of apoptotic cells as well as %DFI were highest for XSS sorted-sperm, despite the fact that X- and Y- sorted sperm had followed an identical protocol. To date, few studies have compared sperm quality measures from the different populations of spermatozoa obtained following the sex-sorting procedure, and no study has been carried out in red deer. Hence previous studies have focused on the effect of sex-sorting on sperm quality of the all sperm population, independently if they were X- or Y-bearing spermatozoa. The differences found in this study between X- and Y- spermatozoa in some of the sperm parameters evaluated could stem from differences in regards to size, shape or differentially expressed proteins that may render them more susceptible to sex-sorting and/or the freeze-thaw procedures. For instance, Chen et al. [39] showed 14 differentially expressed proteins between X- and Y-spermatozoa linked to energy metabolism, stress resistance, composition of cytoskeleton, activity of serine proteases, binding and fusion of sperm/oocytes, and development of the zygotic embryo. In particular, that research identified in X-bearing spermatozoa lower expression of glutathione S-transferase family (GSTM3) protein, which may protect cells against an acute toxic insult [40]. In addition, N-SMase activation associated factor (NSMAF) displayed higher expression in X-bearing sperm [38]. NSMAF can combine with tumor necrosis factor (TNF) and active neutral sphingomyelinase (N-SMase) [41], inducing cell apoptosis [42,43]. Similarly, mitochondrial Cytochrome b-c1 complex subunit 1 (UQCRC1) is a component of the ubiquinol-cytochrome c reductase complex (complex III) and plays an important role in maintaining the structural stability of complex III. However, complex I/III are major sources of ROS, and the overexpression of UQCRC1

in X-sperm [39] may damage mitochondria and lead to cellular apoptosis [44]. This may explain, for instance, the increased %DFI observed for XSS in our study. Altogether, these findings support a decreased ability for X-bearing spermatozoa to resist stressors such as those stemming from sex-sorting and freezing procedures although further studies are required to understand the potential mechanism leading to the increased percentages of damaged cells observed in the XSS group.

Notably, while Y-bearing spermatozoa displayed the best quality values for many of the parameters evaluated, fertility following artificial insemination was still lower than that for NS sperm. Therefore, the sex-sorting procedure elicited some degree of sperm cell damage. During this process the cells are exposed to different stressors such as fluorescent dyes, high dilution rate, mechanical injury, laser illumination and subsequent passage through an electric field for sex-sorting [2], all of which may cause immediate or latent damage.

In conclusion, we show herein that sex-sorted red deer spermatozoa may display acceptable sperm evaluation measures, in the case of Y-sorted sperm often better than those displayed by non-sorted spermatozoa. Nevertheless, the sex-sorting procedure did negatively affect sperm function as manifested by the lower fertility of Y-bearing vs. NS spermatozoa. More studies are needed to understand the differences in sperm quality between the different groups of sorted (X vs. Y) spermatozoa, as well as the sources of sperm damage that may compromise the fertility of Y-sorted sperm.

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584

585 Figure 1:

586 Sperm motion measures of sex-sorted red deer spermatozoa. Results were compared
587 among non-sorted (NS), bulk sorted (BSS), X-sorted (XSS) and Y-sorted (YSS) frozen-
588 sperm, immediately after thawing or following an incubation period (2 h at 37°C).

589 Data are expressed as Mean $\pm$ SEM.

590 (A) TM (%): Total motility; (B) VCL (μ m/sec): Curvilinear velocity; (C) STR (%):
591 Straightness; (D) ALH (μ m): Amplitude of lateral head displacement.

592 Striped vs. solid bars denote significant differences ($P<0.05$) within sample type,
593 between 0 and 2 h of incubation at 37°C. Different capital letters denote significant
594 differences ($P<0.05$) between samples within incubation time (0 or 2 h).

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598 Figure 2:

599 Sperm viability and mitochondrial activity of sex-sorted red deer spermatozoa. Results
600 were compared among non-sorted (NS), bulk sorted (BSS), X-sorted (XSS) and Y-
601 sorted (YSS) frozen-thawed sperm, immediately after thawing or following an
602 incubation period (2 h at 37°C).

603 Data are expressed as Mean $\pm$ SEM.

604 (A) Live_NotApo (%): Live sperm; (B) Apoptotic (%): Apoptotic sperm; (C) Mito_act
605 (%): Spermatozoa with active mitochondria; (D) Live_Acro (%): Live sperm with intact
606 acrosome.

607 Striped vs. solid bars denote significant differences ($P<0.05$) within sample type,
608 between 0 and 2 h of incubation at 37°C. Different capital letters denote significant
609 differences ($P<0.05$) between samples within incubation time (0 or 2 h).

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611

612 Figure 3:

613 DNA integrity of sex-sorted red deer spermatozoa. Results were compared among non-
614 sorted (NS), bulk sorted (BSS), X-sorted (XSS) and Y-sorted (YSS) frozen-thawed
615 sperm, immediately after thawing or following an incubation period (2 h at 37°C).

616 Data are expressed as Mean $\pm$ SEM.

617 (A) %DFI (%): DNA fragmentation index; (B) HDS (%): High DNA Stainability

618 Striped vs. solid bars denote significant differences ($P<0.05$) within sample type,
619 between 0 and 2 h of incubation at 37°C. Different capital letters denote significant
620 differences ($P<0.05$) between samples within incubation time (0 or 2 h).

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622

623 Figure 4:

624 Fertility of sex-sorted red deer sperm.

625 Fertility was assessed as birth rates. BSS: sorted samples; YSS: samples with high
626 purity of Y-sperm.

627 Different capital letters denote significant differences ($P < 0.05$).

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Figure 1

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B)

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645 D)

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Figure 2:

A)

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Figure 3:

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679 **Figure 4:**

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