**Optimization of protocols for Iberian red deer (*Cervus elaphus hispanicus*) sperm
handling before sex sorting by flow cytometry**

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Short Title: Sperm Sex Sorting in Iberian Red Deer

23 **Abstract**

24 Currently, sperm reproductive biotechnologies such as sex sorting and
25 cryopreservation are undoubtedly valuable tools for improving the economic and biological
26 efficiency of red deer production systems. In this context, and because of the particular
27 characteristics of this species (extensive exploitation typically far from laboratory facilities), a
28 key goal is to optimize the design of an adequate handling protocol of sperm samples before
29 samples are subjected to sex sorting and cryopreservation procedures to obtain better outputs
30 from the application of these technologies. The main aim of this paper was to design an
31 adequate protocol for Iberian red deer sperm handling before sex sorting by flow cytometry to
32 obtain optimal yields when sex sorting is used in this species. Semen samples from 11 adult
33 males were obtained by electroejaculation during the breeding season. In this study, we tested
34 different protocols for the handling of Iberian red deer spermatozoa before sorting by using
35 different concentrations of sperm (400 or 800×10^6) and adding or not Hoechst 33342 before
36 the transport of samples to the sorting facilities. Based on the results, the most adequate
37 method used to handle samples before sorting was transportation at a high sperm
38 concentration (800×10^6) without Hoechst 33342, and transportation with these conditions
39 resulted in better sorting yields ($P < 0.05$) with splitting abilities of: $99.9 \pm 9.2\%$; $83.9 \pm$
40 10.8%) than other treatment with lower splitting ability such as those samples stained before
41 transport and using lower sperm concentration (400×10^6) (Splitting ability; $2,6 \mu\text{L H-42}$;
42 $15.4 \pm 10.4\%$ or $5.2 \mu\text{L H-42}$; $75.7 \pm 10.2\%$), based on sorting speed and sperm quality after
43 sorting and cryopreservation. Moreover, the sorting yields were compatible with the practical
44 application of these reproductive biotechnologies.

45 Keywords: sperm, sex sorting, red deer, cryopreservation

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49 **1.- Introduction**

50 The captive deer breeding industry has experienced an important period of growth
51 worldwide within the past 35-40 years [1,2]. The increasing demand for meat and velvet has
52 been a decisive contribution to this rapid increase in red deer farming [3]. This demand
53 combined with the great interest in producing more doses of semen from males with highly
54 valuable genes to obtain more trophy bucks [2] is a clear indication that products derived
55 from male deer are in the greatest demand. Therefore, as suggested by Gao et al. [4], the
56 application of sex preselection of offspring in reproductive management schemes would
57 greatly improve the economic benefits derived from deer production because this would allow
58 the production of animals of only the desired sex, which avoids the production of by-products
59 with reduced economic value. Currently, the only effective methodology for obtaining
60 progeny of a predetermined gender is sperm sex sorting by flow cytometry, which is based on
61 the differences in DNA content between X and Y chromosome-bearing spermatozoa [5]. This
62 biotechnology has been successfully applied in Sika deer (*Cervus nipon*), and offspring of the
63 desired sex have been produced by using frozen-thawed, sex-sorted spermatozoa in
64 combination with laparoscopic insemination and embryo transfer of in vivo produced
65 embryos [4,6]. For Iberian red deer (*Cervus elaphus hispanicus*), the only commercial benefit
66 is that of hunting, with the trophy males preferred as a game piece [1]. Therefore, the
67 application of sperm sex sorting in combination with cryopreservation and AI in the farming
68 of Iberian red deer to obtain high proportions of males could have many advantages, which
69 include not only aspects related to economics but also those regarding biological and genetic

management that avoid the massive slaughter of females to obtain optimal proportions of males/females.

The establishment of species-specific methodologies for all aspects related to sperm sorting, including liquid storage (for the transport of seminal samples to sorting facilities), sperm staining, sorting and cryopreservation, is a basic requirement to obtain optimum results from the application of this biotechnology in wildlife [7]. As previously described, a high percentage of nonviable sperm in the samples to be sorted compromises the efficiency of the sorting process by increasing the time required to sort any particular sample, which impairs the quality of the sorted sample and further decreases the efficiency of the technique [8]. The influence of the initial quality on the suitability of a semen sample for sorting has been also described for endangered species in captivity, such as elephants and rhinoceros [9]. With consideration of the importance of the initial semen quality and the probability that sorting facilities will be far from the semen donors when working with wildlife species such as red deer, the development of a protocol for the transportation of sperm samples before sorting that maintains optimal quality parameters is essential to achieve the successful application of sex sorting technology in red deer. Similarly, combining sperm sex sorting by flow cytometry with sperm cryopreservation is essential for the practical application of sorting technology in this species because it facilitates the producers access to sorted sperm, allowing greater flexibility in the breeding programs and/or productive systems of red deer.

The overall aim of this paper was to design an adequate protocol for Iberian red deer sperm handling before sex sorting by flow cytometry to obtain optimal yields when sex sorting is used in this species. To achieve this aim, two experiments were performed. First, the effect of different transportation conditions on sperm quality and sortability (defined as the ease in the identification of X and Y chromosome-bearing sperm populations) was assessed in red deer semen samples. Second, whether the sperm transport conditions before

sorting influenced the post-thawing quality and functionality of sex-sorted Iberian red deer sperm was determined.

2.- Materials and Methods

2.1.- Reagents and media

All the products used in this study were at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise stated. For fixing the sperm samples, we used a glutaraldehyde solution (2% glutaraldehyde in 146 mM glucose, 34 mM sodium citrate tribasic and 24 mM NaHCO₃). The base extender was a Tris-Glucosa base solution (TGC: 45 gr/L glucose, 3.4 gr/L sodium citrate dihydrate, 0.6 gr/L EDTA, 0.4 gr/L Tris, 18.2 gr/L β -lactose, 0.03 gr/L penicillin and 0.83 gr/L streptomycin sulfate; pH: 7.7, 380 mOsm/kg). The base solution was double-filtered through a cellulose acetate membrane (0.22- μ m pore) and, when indicated, was supplemented with 20% or 40% egg yolk (EY, v/v) and centrifuged (3000 x g, 30 min); the sediment was discarded.

2.2.- Animals

Samples were obtained during the beginning of the breeding season (rut), which spans from early September to mid-October on the Iberian Peninsula [10]. Ejaculates were obtained from 11 Iberian red deer stags (*Cervus elaphus hispanicus*) during two consecutive years (2009-2010). Animals (2.5-7.5 years old; 165 $\pm$ 30 kg, mean $\pm$ S.D.) lived in a semi-free-range area of a private reserve in the province of Cadiz (Spain) under natural conditions at a latitude of 36°20'N, 5°48'O. Until 24 h before semen collection, the stags were kept with the females in meadows. Then, the stags were separated from the females and housed in a large

outside enclosure located within the property and near the facilities in which semen extraction was performed.

2.3.- Electroejaculation procedure

Animal handling and electroejaculation were performed in accordance with the Spanish Animal Protection Regulation, RD53/2013, which conforms to European Union Regulation 2010/63/UE and adheres to the guidelines established in the Guide for Care and Use of the Laboratory Animals. The ethical committee of the University of Castilla-La Mancha (Spain) and of the University of Murcia (Spain) approved the procedures.

Electroejaculation was performed as previously described by Martínez-Pastor et al. [3]. Briefly, prior to electroejaculation, the stags were restrained and anesthetized with an intravenous injection of 0.75 mg/kg xylazine (Rompun® 2%; Bayer AG, Leverkusen, Germany) and 1.5 mg/kg ketamine (Imalgene 1000®; Rhone-Mérieux, Lyon, France). The rectum was cleared of feces, and the prepuce area was shaved and washed with physiological saline serum. Electroejaculation was conducted using a rectal probe measuring 375 mm in length and 40 mm in width; the length of the electrodes was 80 mm. The probe was connected to a power source that allowed the voltage and amperage to be controlled. Ejaculation occurred at average values of 4.5 V and 90 mA (average time to ejaculation 3 min 44 s). Anesthesia was reversed with 0.3 mg/kg yohimbine hydrochloride (prepared at 0.9%; Sigma, Madrid, Spain). The ejaculates were collected by fractions in graduated glass tubes covered by a warming bag (35-37°C). Urine free fractions were diluted 1:1 (v/v) in TGC extender supplemented with 2.5% (v/v) EY and were centrifuged (600 x g/5 min). The pellets from the identical ejaculate were mixed, and after diluting 5 µL of sample in 500 µL of fixing

solution, the sperm concentration (spermatozoa/mL) was calculated using a hemocytometer (Bürker chamber; Brand GmbH, Germany). The ejaculated samples were used in this study when the individual motility was $\geq 60\%$.

2.4.- Sperm sex sorting

Immediately before flow cytometric sorting, the sperm samples, processed as described hereafter (see experimental design section), were filtered through a 30 μm nylon mesh filter, and 1 μL of food coloring dye (0.002% w/v; FD&C #40; Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to each sample for quenching the fluorescence of Hoechst-33342 (H-42) in the sperm with compromised cell membranes, which allowed these sperm to be gated-out during the sorting process [11]. The Y chromosome-bearing sperm were separated according to the Beltsville Sperm Sorting Technology method [11] using a high-speed cell sorter (SX MoFlo; DakoCytomation Inc., Fort Collins, CO, USA) modified for sperm sorting. Sorting gates were placed to achieve $>90\%$ purity in the Y-sperm population. 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, Terra Bella Ave., Mountain View, CA, USA). The samples were sorted using HEPES-buffer-based sheath fluid [12] supplemented with 0.1% EDTA (w/v) and were collected in 50 mL tubes prefilled with 2.5 mL of Tris-Glucose medium containing 5% (v/v) EY. A total of 20×10^6 sorted spermatozoa were collected per tube in an approximate volume of 25 mL.

2.5.- Sperm cryopreservation

Sorted spermatozoa were centrifuged at 3000 x g for 4 min at 21°C. The supernatant was discarded, and the pellets were re-suspended to 20×10^6 sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) EY. Then, sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612; PolyScience, Niles, IL, USA) and were slowly cooled from 21°C to 5°C over 90 min and left for an equilibration time of 2 h. After this period, the semen was packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen vapors (4 cm above liquid nitrogen) for 10 min and then plunged into liquid nitrogen for storage. After at least 1 year in storage, two straws from each group were thawed in a circulating water bath at 65°C for 6 s.

For the preparation of the control groups, semen aliquots from the identical stags were gradually highly diluted using a HEPES-buffer-based sheath fluid to 1×10^6 sperm/mL in the collection media, mimicking the conditions at which sorted sperm were exposed. After dilution, sperm samples were stored at flow cytometer room temperature (21–22°C) for approximately 1.5 h, before being processed for freezing at the identical time as that of the sorted samples.

2.6. - Sperm assessment

2.6.1.- Sperm assessment before and after sorting

Immediately upon arrival at the sorting facilities at the University of Murcia, sperm samples were evaluated for motility and viability. Similarly, motility and viability of sorted samples were evaluated immediately after sorting and before cryopreservation at the end of the equilibration time at 5°C.

Sperm motility was objectively evaluated using a computer-aided sperm analysis system (ISAS®; Proiser R+D, Paterna, Spain), according to the procedure described by Martínez-Pastor et al. [3]. Before the assessment of sperm motility, spermatozoa were diluted

to 20×10^6 spermatozoa/mL in PBS (pH 7.5, 320 mOsm/kg) and were equilibrated for 5 min at 37°C. Aliquots of 4 μ L from each sample were loaded in a prewarmed (38°C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and were immediately transferred to the prewarmed stage (38°C) of a light microscope equipped with phase contrast optics. At least 5 fields and 200 cells were recorded using the 10x negative phase-contrast objective. Software settings were adjusted to deer spermatozoa (25 frames/s; 20 to 90 μ m for head area; VCL > 10 μ m/s to classify a spermatozoon as motile). Only the percentage of total motile spermatozoa was measured.

Sperm viability was assessed using a LIVE/DEAD® Sperm Viability Kit (L-7011; Invitrogen Europe, Paisley, Scotland), following the instructions provided by the manufacturer. Semen samples were diluted 1/100 (v/v) in PBS and stained with propidium iodide (PI; 12 μ M final concentration) and SYBR-14 (100 nM final concentration). The samples were mixed and incubated in the dark at 37°C for 10 min. The samples were remixed before they were run through a flow cytometer (EPICS XL; Coulter Corporation Inc., Miami, FL, USA). All dyes were excited using an argon-ion 488 nm laser. SYBR-14 fluorescence (particles containing DNA, with intact plasma membranes) was detected using a 525 nm band-pass (BP) filter, and the PI signal (used to indicate nonviable cells) was detected using a 620 nm BP filter. The acquisition and analyses were performed using EXPO 2000 (Coulter) software. A double-gating system was used to remove debris based on scattering properties and SYBR-14 or PI fluorescence, and 15,000-gated events were recorded per sample. Only the percentages of spermatozoa with an intact plasma membrane (SYBR-14+/PI-) were included in the results.

2.6.2.- Sperm assessment after cryopreservation

After cryopreservation, a more complete analysis of sperm parameters, which included total and progressive motility, kinematic parameters, viability and apoptosis, was performed with sorted and control frozen-thawed samples to detect any potential accumulative damaging effect of sperm handling protocol before sorting combined with that of sex sorting and cryopreservation procedures.

Sperm evaluations were performed immediately after thawing (0 h) and after 6 h of post-thawing incubation at 37°C. As described above, sperm motility after thawing was objectively evaluated using a computer-aided sperm analysis system (ISAS®; Proiser R+D, Paterna, Spain). For sperm motility, the following parameters were recorded for further analysis: the percentage of motile spermatozoa (Total Motility), the percentage of spermatozoa showing a progressive motility (Progressive Motility), and the kinematic parameters of VAP, LIN, STR, ALH, and BCF. Detailed explanations of these descriptors of sperm motility patterns have been previously provided [13]. For the conditions of assessment, the number of fields and cells evaluated were identical to those described above.

Sperm viability and “apoptosis” were evaluated using flow cytometry as previously described by Dominguez-Rebolledo et al. [14]. In brief, samples were diluted to 1×10^6 spermatozoa/mL in PBS and were stained with 5 μ M Hoechst 33342, 0.1 μ M YO-PRO-1 and 10 μ M PI. After 20 min in the dark, the samples were processed using a flow cytometer (Becton Dickinson LSR-I flow cytometer; BD Biosciences) equipped with a 325 nm He-Cd laser (excitation for Hoechst 33342) and a 488 nm Ar-ion laser (excitation for YO-PRO-1 and PI). YO-PRO-1 is a probe used to stain early apoptotic cells (with intact plasmalemma not stained by PI but showing increased permeability). The membrane-impermeant PI was used to identify spermatozoa with damaged plasmalemma, and H-42 stained all nuclei, which aided in discarding noncellular debris from the analysis. Only the percentages of live (YO-PRO-/PI-) and apoptotic (YO-PRO+/PI-) spermatozoa were included in the results.

235 2.7.- Experimental Design

236 2.7.1.- Experiment 1

237 This experiment was performed to define a suitable protocol for Iberian red deer
238 spermatozoa transport and handling before sorting. For this purpose, different sperm
239 transportation conditions were tested using semen samples from 9 stags, obtained as described
240 above, from which aliquots were processed as follows.

241 Samples stained before transport: Sperm aliquots from each stag were diluted to a
242 concentration of 800×10^6 sperm/mL in TGC medium, and aliquots of 500 μ L of the
243 extended semen were stained with 2.6 μ L or 5.2 μ L of H-42 stock solution (25 mg/mL). After
244 the addition of H-42, the samples were incubated at 34°C for 50 min and then diluted 1:1 (v/v)
245 with TGC medium supplemented with 40% (v/v) EY. Therefore, the transport conditions
246 were 400×10^6 sperm/mL, 20% EY and 2.6 μ L or 5.2 μ L of H-42. The samples were
247 evaluated for motility and viability immediately upon arrival at the sorting laboratory.

248 Immediately before sorting, sperm samples were diluted 1:4 (v/v) with TGC medium
249 to prepare samples that contained 100×10^6 stained sperm/mL, 5% (v/v) EY and 2.6 μ L
250 (Group 1) or 5.2 μ L (Group 2) of H-42.

251 Unstained samples: Sperm aliquots from each stag were diluted to a concentration of
252 400×10^6 sperm/mL or 800×10^6 sperm/mL in TGC medium supplemented with 20% (v/v)
253 EY and were transported to the sorting facilities without staining. As with the stained
254 samples, the motility and viability of each sample were immediately evaluated upon arrival at
255 the sorting laboratory. After sperm evaluation, 400×10^6 sperm/mL samples were re-diluted
256 to 100×10^6 sperm/mL with TGC medium and stained with 1.3 μ L (Group 3) or 2.6 μ L
257 (Group 4) of H-42 for 50 min at 34°C. The identical protocol was used for the samples
258 containing 800×10^6 sperm/mL (Groups 5 and 6 were stained with 1.3 and 2.6 μ L of H-42,

respectively). Therefore, the characteristics of the unstained samples for sorting were 100 x 10⁶ stained sperm/mL containing 5% or 2.5% (v/v) EY in Groups 3 and 4 and in Groups 5 and 6, respectively.

All samples were transported (between 7 and 10 hours) at 17-18°C, and in addition to the motility and viability evaluation of each sample upon arrival at the sorting facilities, the following parameters related to the quality of the sorting procedure were recorded and analyzed: nonviable sperm represented by the percentage of spermatozoa showing FDA-positive staining (% FDA +); splitting ability calculated as the percentage of samples that were successfully resolved into X and Y populations (see *Figure 1*); percentage of properly oriented spermatozoa; percentage of sorted Y population; and sorting rates defined as the number of Y-chromosome spermatozoa sorted per second.

2.7.2.- Experiment 2

The purpose of this experiment was to evaluate whether different sperm handling protocols before sorting influenced the post-thawing quality of Iberian red deer spermatozoa sorted by flow cytometry. The sperm solely from those experimental groups with a good splitting ability (i.e., more than 70% of the specimens showing a clear differentiation between X and Y sperm populations) were sorted and frozen-thawed, as described above. A control group consisting of non-sorted spermatozoa frozen under the identical conditions as the sorted spermatozoa was included in this experiment. A total of 18 samples were assessed for sorted and control groups.

Sperm evaluation was conducted on the sorted and control samples (1) post-sorting (after the centrifugation step for sperm concentration), (2) at 5°C at the end of the cooling

step, (3) immediately post-thawing (0 h post-thawing), and (4) after 6 h of incubation at 37°C (6 h post-thawing).

2.8.- Statistical analyses

Statistical analyses were performed using the SPSS statistical software package version 15 (SPSS Inc., Chicago, IL, USA). The data from all replicates were assessed using ANOVA with a MIXED procedure, including the sperm treatments (sperm transportation conditions) as a fixed effect and stags as a random effect. The normality of residuals was tested using the Kolgomorov-Smirnov test, applying the Lilliefors' correction (Lilliefors, 1967). When a significant effect was detected by ANOVA, the values were compared using the Bonferroni test. The threshold for significance was set at $P < 0.05$. The results are expressed as the LS means $\pm$ SEM.

3.- Results

3.1.- Experiment 1

The results for sperm motility and viability obtained in all groups upon arrival at the sorting facilities are summarized in Table 1. Unstained samples transported at 800×10^6 sperm/mL had the best values ($P < 0.05$) for sperm motility. No differences ($P > 0.05$) among samples, regardless of the transport conditions, were observed for sperm viability.

Sortability data for Y-sperm populations were recorded in all groups (Table 2). The results showed that the handling method significantly influenced the percentage of samples showing a split (splitting ability), and the best values were obtained ($P < 0.05$) in Group 6 with almost 100% of the samples successfully resolved into X and Y populations. The differences were statistically significant ($P < 0.05$) for all groups, except Group 5. For the other sorting

quality parameters, the sperm handling method had no significant effect ($P>0.05$). The mean value of sorting accuracy for the separated Y-chromosome bearing spermatozoa was within 90-93%, with no significant influence ($P>0.05$) of the sperm handling procedure before sorting.

3.2.- Experiment 2

Only spermatozoa from groups 2, 5 and 6 with a splitting ability higher than 70%, were used for sorting and cryopreservation in this experiment. When the sorted and control samples were evaluated before freezing (after centrifugation and at the end of equilibration at 5°C), no differences of motility and viability ($P>0.05$) were observed among the sorted samples or between those samples and controls (see *Figure 2 A and B*). After centrifugation, sperm motility ranged from 69.2% to 80.7%, whereas at 5°C immediately before freezing, the values ranged from 61.3% to 74.1%. For the viability, the percentages of live spermatozoa ranged from 70.5% to 78.0% after centrifugation and from 67.7% to 72.8% at the end of the equilibration step.

After thawing, the results for total and progressive motility showed no differences ($P>0.05$) between control and sorted groups or among sorted, cryopreserved sperm regardless of the incubation time post-thawing (0 and 6 h; see Table 3). The analyses of kinematic parameters (Table 3) demonstrated that sorted samples at 0 h post-thawing yielded higher values for VAP, LIN, and STR than those in the control group; however, these differences were statistically significant ($P<0.05$) only between the control and sorted samples from groups 2 and 5. After 6 h of incubation, the results were similar, but significant differences ($P<0.05$) were observed only for VAP between the control and sorted group 2.

No effects ($P>0.05$) of the sperm handling method or sorting procedure on the percentages of viable and apoptotic sperm were observed, and no differences for these

parameters were observed between the sorted and control samples at either of the times of post-thawing evaluation (Table 3).

4.- Discussion

To optimize the efficiency of the flow cytometric sperm sex sorting procedure in Iberian red deer, we evaluated the usefulness of different before sorting sperm handling procedures, primarily the transport conditions, by analyzing the effects on the sperm sample quality before sorting, on the sample sortability and on the sperm quality after sorting and cryopreservation.

In the first experiment, optimal values for motility and viability at the end of the transportation period (above 70% and 80%, respectively) were obtained for all groups. However, although the percentage of viable spermatozoa was not affected by transport conditions, the sperm number in the sample influenced motility, and better values for this parameter were obtained in the samples transported at the higher sperm concentration. Similar results with better preservation of motility at a higher sperm concentration have been described for electroejaculated ram-lamb spermatozoa [15]. As suggested by those authors, greater amounts of antioxidant substances from remaining seminal plasma in highly concentrated samples could possibly explain these findings.

Based on the results from the first experiment, the staining procedure before transport did not apparently affect the sperm quality, and more importantly, the presence of H-42 at concentrations of 115 and 231 μ M (groups 1 and 2, respectively) for a relatively long period (6-7 h at 17°C) in the environment surrounding spermatozoa did not lead to a significant decrease in the sperm quality. The H-42 dye is strongly temperature-dependent with minimal uptake below 16°C [16], and H-42 detrimental effects on sperm motility have been described as time dependent [17]. Therefore, under our transport conditions after staining (6-7 h at 17-

18°C), a reasonable conclusion was that the H-42 did not penetrate into the sperm cells at a sufficient concentration to exert a negative effect on the sperm quality as a result of the excessive uptake of H-42. In the same study, Balao da Silva et al. [17], using adequate media to extend the sperm, the damaging effect of H-42 on sperm motility was minimized. Therefore, the extender used in our study, containing 20% egg yolk, might also have contributed and helped to avoid the possible damaging effect of H-42 on the motility of stained-transported spermatozoa.

Regarding the sorting parameters, better splitting ability was obtained for those samples stained after transport with a final egg yolk concentration of 2.5% (groups 5 and 6), whereas with 5% egg yolk, in general, a lower number of samples showed splitting (groups 1 to 4). Most likely, the higher dose of H-42 in samples from group 2 was responsible for the acceptable splitting ability in this group, despite the egg yolk concentration in those samples (5%). Additionally, although no significant differences were observed, the percentages of oriented and separated sperm and the sorting rates were also slightly impaired when more egg yolk was used, which reduced the efficiency of the sorting procedure. These findings were most likely because egg yolk adds opacity to the media [18] that surrounds the sperm cells, which compromises the uniformity of the staining and interferes with the laser excitation of H-42 and with light transmission to the detectors [11,19]. Thus, the resolution for determining the X- and Y-chromosome bearing sperm populations is poor at high concentrations of egg yolk [20]. Before sorting sperm handling protocols that result in low values of splitting ability led to the excessive discarding of valuable samples, which decreases the efficacy and the applicability of the technology. With this consideration, only groups 2, 5 and 6, with more than 70% of the samples showing split, were suitable for performing an effective sorting; therefore, only sperm from those groups were sorted and cryopreserved in the second experiment.

In the second experiment, although sperm concentration influenced the initial sample quality, no influence of concentration occurred after sorting, and the protocol used for sperm handling before sorting did not influence the quality of sorted spermatozoa at any evaluation time before freezing. Additionally, no differences between sorted and control samples for sperm motility and viability were observed either after centrifugation or at 5°C immediately before freezing. These results are consistent with those published by Kjelland et al. [2] for the sex-sorted, frozen-thawed spermatozoa of white-tailed deer and showed that with the sorting procedure, a sperm population with good quality was obtained; therefore, further processing by other technologies such as cryopreservation was possible. Similar results have been described for different species of domestic mammals [21–23]. The most likely primary explanation for these results was the adequate manipulation of the sperm samples combined with the removal of dead and damaged sperm from the sorted population by gating-out those stained by food dye during sex sorting [11].

After thawing, the results were similar, and no influence of sperm handling before sorting on either type of sperm processing (sorted or control) on sperm quality parameters was observed. Based on these results, a damaging, cumulative effect of sorting and cryopreservation procedures did not occur, and the combination of these two reproductive technologies yielded a sorted red deer sperm population with high quality. In previous studies, the results are similar for the effects of sorting and freezing sperm in cervids, which suggest that this species is apparently resistant to the stressors that occur during sorting and cryopreservation procedures [2].

Regarding kinematic parameters post-thawing, the samples stained with the highest concentrations of H-42 (Group 6) after transport showed similar results on motility, ALH and STR to the control, being in concordance with those obtained in our previous study [24]. Obtained values for VAP, LIN and STR, suggest a faster and more linear motility pattern of

sorted-cryopreserved spermatozoa, regardless of the handling protocol used before sorting and freezing, than the control spermatozoa. As noted above, these findings could be most likely due to the efficient elimination of damaged sperm during sorting that renders in a sorted sperm population with optimal characteristics that, as demonstrated herein, are maintained after cryopreservation.

No evidence of hyperactive motility, which is revealed by a sharp decrease in LIN accompanied by an increase in ALH [25], was observed in our study, although this type of motility could have been expected as a result of the well-known capacitation-like changes induced by sperm sorting and/or freezing-thawing procedures [26–28]. Based on the values for VAP, LIN, STR, ALH and BCF obtained in this study and following the criteria described by Ramón et al. [29] for the characterization of red deer sperm populations after thawing, the sex-sorted, frozen-thawed spermatozoa obtained in our second experiment, specially in the group 2 and 5, could be classified as sperm with a vigorous, progressive movement. Moreover, high fertility might be expected from these sperm populations that exhibited, among other characteristics, high values for swimming velocity parameters, including VAP [29,30]. Because high VAP values were obtained in our study for sex-sorted, frozen-thawed spermatozoa, optimal fertility rates could be expected using these spermatozoa in AI procedures. This hypothesis is consistent with the proposal of de Graaf et al. [31] for sheep, which suggests that in certain species, the process of sex sorting may select a sperm population with enhanced sperm function and fertility. However, recent results from our group [24] regarding field fertility of Y-sex-sorted-frozen-thawed red deer spermatozoa after AI suggest a compromised fertilization ability of these spermatozoa. Although these findings are more probably related with the number of sperm used per insemination and/or with individual factors related with the males used in the studies, additional experiments should be

427 performed to clarify the potential damaging effects of sorting and freezing procedures on the
428 fertilizing ability of red deer spermatozoa.

429 In conclusion, our results demonstrated that transportation of red deer sperm before
430 sorting at a high sperm concentration without staining resulted in a good efficiency of sorting
431 procedure in terms of sorting outputs and in terms of sperm quality after sorting and
432 cryopreservation. Moreover, the results of this study are an important contribution to the
433 practical application of these reproductive technologies. Our findings pave the way for further
434 experiences mainly focused on optimizing fertility outcomes.

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Table 1: Percentage of motile and viable sperm in the different experimental groups upon arrival at the flow cytometry laboratory

Data are expressed as the LS MEAN ± SEM.

Sample Group	% Motile Sperm	% Viable Sperm
Samples stained before transport*		
400x10 ⁶ /mL+2.6 µl of H-42	73.9 ± 4.3 ^a	83.6 ± 2.4
400x10 ⁶ /mL+5.2 µl of H-42	74.0 ± 3.6 ^a	81.7 ± 2.3
Unstained samples**		
400x10 ⁶ /mL	80.8 ± 4.0 ^a	81.4 ± 2.6
800x10 ⁶ /mL	89.5 ± 3.8 ^b	87.3 ± 2.0

* Samples stained at the farm
** Samples transported without staining
^{a,b} Different superscripts indicate significant differences (P<0.05) within the same column

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Table 2: Quality parameters for Y-chromosome bearing sperm population sorting in each experimental group.

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% FDA+: Percentage of dead spermatozoa identified as those spermatozoa counter-stained with food dye (FDA);
 Splitting Ability: Percentage of samples showing split after staining treatment; Oriented Spermatozoa: Spermatozoa
 correctly oriented; Separated Sperm: Percentage of Y-chromosome bearing spermatozoa selected and sorted from the
 correctly oriented; Sorting Rates: Number of Y-chromosome bearing spermatozoa sorted per second. Data are expressed
 as the LS MEAN $\pm$ SEM.

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Experimental Group	% FDA +	Splitting Ability (%)	Oriented Sperm (%)	Separated Sperm (%)	Sorting rate
Samples stained before transport*					
Group 1: 400x10 ⁶ /mL+2.6 μ l of H-42	10.9 $\pm$ 0.9	15.4 $\pm$ 10.4 ^d	57.2 $\pm$ 1.4	45.7 $\pm$ 1.4	4649.7 $\pm$ 454.5
Group 2: 400x10 ⁶ /mL+5.2 μ l of H-42	11.3 $\pm$ 0.8	75.7 $\pm$ 10.2 ^{b,c}	58.8 $\pm$ 1.3	42.1 $\pm$ 0.7	4287.0 $\pm$ 256.8
Unstained samples**					
Group 3: 400x10 ⁶ /mL+1.3 μ l of H-42	8.5 $\pm$ 1.0	55.2 $\pm$ 11.0 ^c	60.4 $\pm$ 1.5	44.0 $\pm$ 0.9	4605.2 $\pm$ 303.5
Group 4: 400x10 ⁶ /mL+2.6 μ l of H-42	9.1 $\pm$ 1.0	51.2 $\pm$ 11.4 ^c	60.3 $\pm$ 1.5	43.3 $\pm$ 1.0	4363.2 $\pm$ 327.5
Group 5: 800x10 ⁶ /mL+1.3 μ l of H-42	8.8 $\pm$ 0.9	83.9 $\pm$ 10.8 ^{a,b}	60.7 $\pm$ 1.4	46.2 $\pm$ 0.7	4656.6 $\pm$ 267.5
Group 6: 800x10 ⁶ /mL+2.6 μ l of H-42	8.6 $\pm$ 0.7	99.9 $\pm$ 9.2 ^a	61.1 $\pm$ 1.1	44.8 $\pm$ 0.5	4578.9 $\pm$ 214.1

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* Samples stained at the farm.

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** Samples transported without staining and stained at the flow cytometer laboratory.

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^{a,b,c} Different superscripts indicate significant differences (P<0.05) within the same column

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Table 3. Post-thaw values of parameters assessed in sorted and control sperm samples

Group 2: Sorted sperm from samples stained before transport (400 x 10⁶ sperm/mL; 5.2 µL of H-42; 20% EY during transport; 5% EY during sorting); Group 5: Sorted sperm from unstained samples (800 x 10⁶ sperm/mL; 1.3 µL of H-42; 20% EY during transport; 2.5% EY during sorting); Group 6: Sorted sperm from unstained samples (800 x 10⁶ sperm/mL; 2.6 µL of H-42; 20% EY during transport; 2.5% EY during sorting). VAP: Average path velocity; LIN: Linearity; STR: Straightness; ALH: Amplitude of lateral head displacement; BCF: Beat cross frequency; Viability (YO-PRO-1-/PI-): Viable, intact plasmalemma, normal permeability; Apoptosis (YO-PRO-1+/PI-): Apoptotic-like, increased membrane permeability. Data are expressed as the LS MEAN ± SEM.

0 h Post-thawing	Sorted Samples			Control
	Group 2	Group 5	Group 6	
Total Motility (%)	62.8±10.4	59.3±9.7	49.1±10.1	57.9±7.8
Progressive Motility (%)	34.1±4.69	36±4.3	23.1±4.6	22.9±3.4
VAP (µm/s)	76.3±4.6 ^a	73.5±4.2 ^a	70.3±4.6 ^{ab}	53.8±3.4 ^b
LIN (%)	63.5±2.4 ^a	66.9±2.5 ^a	58.2±2.4 ^{ab}	52±1.8 ^b
STR (%)	75.3±1.8 ^a	78.2±1.7 ^a	72 ±1.8 ^{ab}	68.9±1.4 ^b
ALH (µm)	2.3±0.2	2.3±0.2	2.5±0.2	2.5±0.1
BCF (Hz)	6.6±0.3	6.9±0.3	6.3±0.3	5.8±0.2
Viability (%)	40.31±6.1	31.24±5.8	38.9±6.1	29.7±4.9
Apoptosis (%)	13.52±3.5	11.49±3.2	13.8±3.5	18.2±2.6
6 h Post-thawing				
TM (%)	24.7±5.6	27.4±5.2	15.9±5.6	18.5±4.6
PM (%)	14.3±2.9	12.2±2.7	6.4±2.9	7.9±2.4
VAP (µm/s)	62.1±5.6 ^a	54.8±5.2 ^{ab}	45.1±5.6 ^{ab}	35.1±4.6 ^b
LIN (%)	59.3±4.1	57.5±3.8	48.4±4.1	44.1±3.3
STR (%)	74.1±2.6	72.6±2.4	69.2±2.6	69.4±2.1
ALH (µm)	2.3±0.2	2.2±0.2	2.5±0.2	2.1±0.1
BCF (Hz)	6.±0.6	6.5±0.5	6.6±0.6	5.8±0.5
Viability (%)	7.9±2.4	4.0±2.1	3.9±3.5	7.3±2.7
Apoptosis (%)	8.5±2.1	5.3±1.8	5.9±3.3	14.1±2.3

^{a, b} Different superscripts indicate significant differences (P<0.05) among groups.

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608 **Figure captions**

609 **Figure 1: Splitting Ability.** Histograms, created by SUMMIT computer software after
610 flow cytometric detection of Hoechst 33342-stained spermatozoa fluorescence,
611 corresponding to samples from group 1 (A) and 6 (B), showing a poor and good
612 resolution of X and Y sperm populations (Splitting Ability), respectively.

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614 **Figure 2: Sperm quality prior freezing:** Percentages of motile (A) and viable (B)
615 spermatozoa in sorted (Groups 2, 5 and 6) and control samples, evaluated after sorting
616 and at 5°C (at the end of the equilibration step). Data are LS MEAN $\pm$ SEM

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