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Sex-sorted bovine spermatozoa and DNA damage: I. Static features

J. Gosálvez[a,*], M.A. Ramirez[a], C. López-Fernández[a], F. Crespo[b], K.M. Evans[c], M.E. Kjelland[c], J.F. Moreno[c]

[a] *Departamento de Biología, Unidad de Genética, Universidad Autónoma de Madrid (UAM). 20849-Madrid, Spain*
[b] *Centro Militar de Cría Caballar de Avila, (FESCCR-Ministerio de Defensa) Ávila, Spain*
[c] *Sexing Technologies, Navasota, TX 77868, USA*

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Abstract

This study examined the static response of Spermatozoa DNA Fragmentation (SDF) after sex selection in bulls using a MoFlo® SX (Beckman Coulter, Miami FL) spermatozoa sorter to produce three different subpopulations: 1) Spermatozoa bearing X-chromosomes with a purity of 95%, 2) Spermatozoa bearing Y-chromosomes with a purity of 95%, and 3) non-viable spermatozoa. The static response of SDF refers to the baseline values observed for DNA damage when analyzed pre- and post sex-sorting. Results showed that while the baseline level SDF in pre-sorted bull spermatozoa samples ranged from 5.3% to 11% with an average of 7.9% ± 2.1%, the level of SDF obtained in X- and Y-chromosome sorted samples was much lower (3.1% ± 1.9%) and statistical differences were obtained after comparing both groups ($P < 0.01$). Spermatozoa containing a fragmented DNA molecule tend to be accumulated in the non-viable subpopulation. The baseline SDF level in X- and Y-chromosome sorted subpopulations is reduced, by 63% on average when compared to the values obtained in the neat semen sample. Different bulls exhibit unique SDF reduction efficiencies via the X- and Y-chromosome sex selection process.

Keywords: Bovine reproduction; Andrology; Flow cytometry; Sex-sorting; Sperm DNA fragmentation

1. Introduction

Sex-sorting via flow cytometry is currently the only reliable technique that has been used successfully for the cost effective commercialization of sexed semen in the dairy industry [1,2,3,4]. Particularly in cattle, this praxis offers potential benefits in the logistics of meat and milk production processes [5,6,7]. One of the main constraints of applying this technology for cattle is the lower fertility that has been reported [5,7,8], especially when compared to the use of conventional semen samples for assisted reproductive technologies (ART). However, the reason for the lower fertility that has been associated with sex-sorted spermatozoa is still subject to debate [8,9].

Due to the limited production efficiency of sex-sorting spermatozoa, a normal commercial dose of sexed bull semen contains only 8.4×10^6 spermatozoa per mL (2.1×10^6 spermatozoa per dose in 0.25 cc straws). An insemination dose of only 2.1×10^6 is a low dose for AI. The usual insemination dose for cattle is around 20×10^6 or more cryopreserved sperm [7] in

* Corresponding author. Tel.: +34 91 497 8210; fax: +34 91 497 8344.
E-mail address: jaime.gosalvez@uam.es (J. Gosálvez).

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0.5 cc straws or 40×10^6 spermatozoa per mL. Additionally, the samples used for sex-sorting are handled under para-biological environments such as the interaction of the DNA with fluorophores, laser exposure, spermatozoa separation in micro-droplets, acceleration of spermatozoa through geometrically-pressured fluid channels and centrifugation. All of these para-biological spermatozoa-media or mechanical interactions would theoretically have the potential to produce changes in cell structures, including the DNA molecule. While taking these stressors into account, the decrease in fertility rates could also be related to the amount of spermatozoa containing a certain level of DNA damage generated by the individual. Thus, while spermatozoa concentration could be one of the factors in reducing the capacity for fertilization of sorted samples, other external factors may also be affecting the ability of these samples to produce viable offspring.

The main objective of this investigation was to study the level of DNA damage that could be putatively produced after sex selection in the different subpopulations. The main hypothesis is that during spermatozoa sex-sorting, the level of DNA damage decreases in the spermatozoa fraction to be used for insemination because of a step included in the sorting methodology that removes nonviable and non-flow orientated spermatozoa. Non-flow orientated sperm may result if abnormal morphological issues prevent them from aligning properly in the flow stream as discussed by Dean et al [10]. In general, poor-quality semen including increased morphological abnormalities have been shown to have a higher degree of DNA fragmentation associated with them [11].

2. Material and methods

All bulls included in this analysis are of the Jersey (n = 5) and Holstein (n = 15) breeds. They were selected at random from a collection of bulls maintained for semen production at Sexing Technologies (Navasota, TX, USA). The bulls providing semen samples used in these experiments are regularly used for artificial insemination on a routine basis or were prepared for these experimental purposes. For this particular investigation bulls ranged from 3 to 9 years of age.

Preparation of the semen samples for sex-sorting was similar to that discussed in Seidel and Garner [12]. In this case, X- and Y-chromosome bearing, sorted spermatozoa samples were selected based on differences in fluorescence signals using 16.2 mM Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA), diluted 1 : 1 (Hoechst : Nanopure water, v/v), and Red Food Dye (FD&C #40; Sensient Technologies Corporation, St. Louis, Missouri, USA). Although the DNA content of spermatozoa for individual mammalian species is highly conserved, DNA content differences occur and can even be observed among breeds of cattle [4,12]. For example, the X- and Y- chromosome-bearing sperm DNA content difference is 4.22% for Jersey and 4.01% for Holstein breeds [4]. Therefore, the X-chromosome-bearing sperm have about 4% more dye bound to their DNA than Y-sperm and a greater fluorescence signal can be observed as a result [13].

Live spermatozoa that have intact cell membranes exclude Red Food Dye from entering and quenching the Hoechst fluorescence signal thus exhibiting a higher fluorescence signal than the nonviable spermatozoa. Sorting of X- or Y-chromosome bearing spermatozoa was conducted similar to the procedures previously described by Seidel and Garner [14], using a MoFlo® SX (Beckman Coulter, Inc., Miami FL) spermatozoa sorter and Summit v4.0 software.

For these experiments, the MoFlo® SX was set to a sorting pressure of 40 psi. The sheath fluid used for these spermatozoa sorting experiments was SortEnsure™ (Catalogue number 77-86-1, XY Inc. Fort Collins, CO), a Tris-based sheath fluid [15,16] The laser source was a NdYag, mode locked pulsed Vanguard™ HMD350 (Spectra Physics Lasers-North America, Santa Clara, CA, USA) operating at 355 nm. The operating power was light regulated at 350 mW with a drop drive amplitude of 45–50 volts. Being a dual headed sorter, the laser beam was split using a CVI Melles Griot (Albuquerque, New Mexico, USA) high energy beam splitter (BSI-355-50-1025-45S) providing 175 mW of power to each head. Power was confirmed at 160 mW at the flow cell using a Power/Energy Meter, model 841-PE. A 70 μm Orient-tip™ (XY Inc, Fort Collins, CO) was used to generate the sorting stream while using a drop frequency of 68 000 Hz for sorting. Event rates were held at 30,000 events per second while gating on living and properly oriented spermatozoa, or ~55–65% of the total cells, while taking ~40–45% of the X- or Y-chromosome bearing region to optimize for that purity, subsequently confirmed using the STS Sexed Semen Purity Analyzer (Sexing Technologies, Navasota, TX). The aforementioned settings resulted in sorting speeds of 5,000 to 6,000 sexed spermatozoa per second. After the spermatozoa had been sex-sorted they were collected in 50 mL tubes (Axygen Scientific Inc., Union City, CA, USA)

containing catch fluid consisting of a ~20% egg yolk-TRIS extender [14,15].

The same standards for routine semen preparation and cut-off values for standard semen characteristics for selecting the ejaculates for processing were applied. In all experiments, the bull ejaculates for processing either conventional or sex-sorted straws of semen were used if they met the following criteria: 1) minimum motility of ≥ 55%, 2) minimum concentration of ~ 900 × 10^6/mL, determined using the SP1-Cassette, Reagent S100, and NucleoCounter® SP-100™ system (ChemoMetec A/S, Gydevang 43, DK-3450 Allerød, Denmark), and 3) primary morphologies ≤ 15%, secondary morphologies ≤ 15%, and a total morphology count not to exceed 25%. Further, samples used in the post-thaw analyses had to meet standard quality control conditions of progressive motility ≥ 45% at 0 h and ≥ 30% at 3 h, including intact acrosomes ≥ 50% at 3 h. For three hour post-thaw motility and acrosome measurements, all samples were incubated for 3 h at 37 °C in a humidified chamber. For all semen quality evaluations, 75 × 25 mm glass microscope slides (Andwin, Addison, Illinois, USA) and 22 × 22 mm #1.5 coverslips (Thomas Scientific, Swedesboro, New Jersey, USA) were used. All motility assessments were made using brightfield microscopy and post intact acrosomes and morphology assessments used differential interference contrast (DIC) microscopy with a magnification of X 400.

Each sex-sorted sample was produced according to the standards for semen production dose in Sexing Technologies (Navasota, TX, USA). All extenders used in the experiments were of the same formulation having a pH of 6.8 and an osmolarity balanced at 300 mOsm for the TRIS extender. For cryopreservation, sorted and conventional sperm samples were processed using a two step extension with glycerol. All frozen-thawed sex-sorted samples used in the experiments contained ~2.1 × 10^6 spermatozoa/straw (0.25 cc) while conventional samples had ~25 to 30 × 10^6 spermatozoa/straw (0.5 cc).

The first experiment was conducted to analyze possible differences in the level of spermatozoa DNA fragmentation (SDF) before and after X- and Y-chromosome sex selection. Five Jersey bulls, one ejaculate each (n = 1 ejaculate/bull), were included in this analysis. Neat semen from each individual was divided into two aliquots, one aliquot was sex-sorted and thereafter the spermatozoa were frozen using an automated freezing device, IMV Digitcool® (IMV, Cedex, France) and stored in liquid nitrogen. The second aliquot was directly cryopreserved for subsequent analysis of spermatozoa DNA fragmentation after thawing. The SDF analysis was performed for the different subpopulations, after X- and Y-chromosome sex selection, while comparing both aliquots for each respective bull. One subpopulation included exclusively those spermatozoa which were dead (R2 in Fig. 1) and the other two groups consisted of live spermatozoa (R1 in Fig. 1) subpopulations containing X-chromosome bearing (R3 in Fig. 1) and Y-chromosome bearing spermatozoa (R4 in Fig. 1) at a purity ≥ 95%. Sex ratio purities of the samples were determined using an STS Sexed Semen Purity Analyzer (Sexing Technologies, Navasota, TX), which provides high resolution peaks of X- and Y-chromosome bearing spermatozoa populations and basing each analysis on 2.000 spermatozoa. All of these subpopulations were compared with the level of SDF obtained in the respective pre-sort sample, a sample taken after staining and incubation but before sorting. A total of ~2 × 10^6 spermatozoa for each sample were sorted. Dead spermatozoa were sorted based on Region 2 in Fig. 1 while excluding all other cells except those that fall into that region. The proportion of dead cells in the semen samples averaged 13%, thereby providing an average sort speed of 800 to 900 dead spermatozoa per second.

The second experiment was conducted to analyze the effectiveness of X- and Y-chromosome sex-sorting in reducing the baseline level of SDF and the level of intra-individual variation of SDF. For this purpose, one hundred frozen-thawed X- and Y-chromosome sorted samples from 10 individual Holstein bulls were assessed for DNA fragmentation. Ten semen samples from each bull, each from a different ejaculate collected throughout the year, were used.

2.1. Spermatozoa DNA fragmentation assessment

To determine the degree of DNA damage in each sample, the bull Sperm-Halomax® kit (Halotech DNA, Madrid, Spain) was used. This technique is an adapted version of the sperm chromatin dispersion test (SCD; [17]). Details of this technique have been previously reported for human and other mammalian species [17–20].

In order to increase the spermatozoa concentration per slide, we modified the standard protocol as recommended by the manufacturer. This methodology was used because the spermatozoa concentration obtained after X- and Y-chromosome sex selection, before further centrifugation, was relatively low (~0.8 to 1.0 × 10^6 spermatozoa/mL). In this case, 10 μLs of the sorted

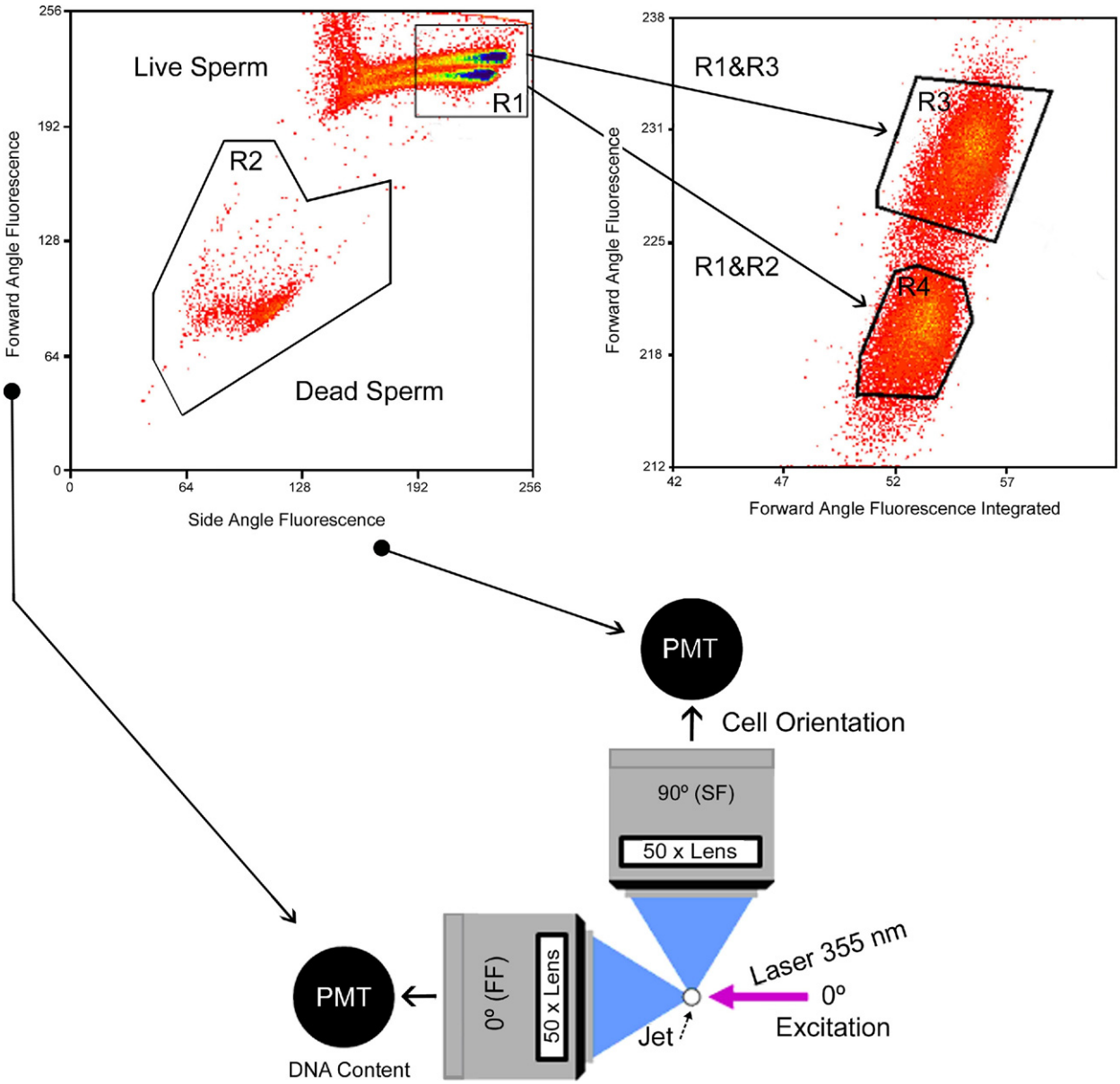

Fig. 1. Illustration of live (R1) and dead (R2) sperm cell populations further separated into X- (R3) and Y-chromosome (R4) bearing sperm cell populations using forward angle fluorescence for detecting DNA content and side angle fluorescence for detecting sperm cell orientation (R3 and R4 are gated on R1). Boolean sort logic is as follows: sorting region (R) for X-chromosome bearing sperm cells = R1 and R3 and not R2, while sorting region for Y-chromosome bearing sperm cells = R1 and R4 and not R2.

sample were diluted in 10 μLs of low melting agarose and one 10 μL microdroplet of the mix was spread onto the surface of the pretreated slide. This was a modification with respect to the manufacturer′s recommendation because under the regular protocol, the final concentration of the agarose is higher which may make spreading the spermatozoa agarose mixture more difficult. Based on our experience, the agarose must be maintained at 38 °C once the spermatozoa have been mixed with agarose to avoid any such issue. Under temperatures lower than 38 °C the spreading of the agarose spermatozoa mixture could be more difficult.

For visualizing spermatozoa chromatin after processing, slides were stained using SYBR® Green I (10X) (Invitrogen, Molecular Probes, Eugene, Oregon, USA) or GelRed (10X) (Biotium, Hayward, CA, USA)

in Vectashield® Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) for DNA staining. This staining method permits manual scoring by allowing for the distinction of spermatozoa nuclei containing either fragmented DNA (large and spotty halo of chromatin dispersion) or unfragmented DNA (small and compact halo of chromatin dispersion). A percentage of fragmented versus non-fragmented spermatozoa was determined by examining 300 spermatozoa for each sample. Samples were viewed and captured using a Nikon Eclipse 80*i* microscope with fluorescence capabilities and NIS Elements BR 3.00 imaging software with a CCD (Photometrics® CoolSNAP™ *EZ*).

2.2. Statistical analysis

The results for purposes of statistical comparison are reported as mean and standard deviation, while the distributions of the different values are presented as box and whisker plots using Statgraphic Plus 5.1 software (Academic Enterprise, StatPoint Inc., Herndom, Virginia, USA). The non-parametric Wilcoxon-Mann-Whitney test was used to test the hypothesis that two samples being compared do not show significant differences.

3. Results

High resolution images were obtained when SYBR® Green I or GelRed staining was combined with the SCD protocol (Fig. 2). Spermatozoa heads presenting compact haloes of dispersed chromatin are indicative of spermatozoa without fragmented DNA, while those presenting large and expanded haloes are identified as containing fragmented DNA (arrows in Fig. 2b and c).

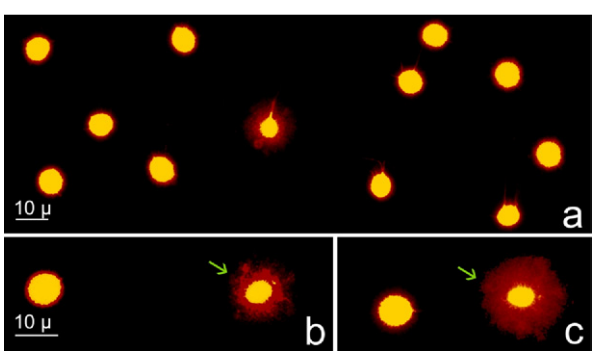

Fig. 2. Bull sperm DNA fragmentation after the sperm chromatin dispersion procedure. (a) Fragmented (large red fluorescent halo) and unfragmented (small red fluorescent halo) in a sex-sorted spermatozoa sample. Selected unfragmented and fragmented spermatozoa (arrows) in (b) sex-sorted and (c) conventional spermatozoa to show differences in the halo size.

Table 1
Whole distribution of sperm DNA fragmentation (percent) observed pre- and post X/Y sex sorting.

Reference	Pre-sort	XY
JE1	7.00	1.10
JE2	7.50	1.10
JE3	11.00	4.00
JE4	9.00	5.00
JE5	5.30	4.60
Average ± SD	7.96 ± 2.15	3.16 ± 1.91

It is interesting to note that in all of the samples analyzed herein, the haloes of chromatin dispersion after sex-sorting are smaller than those obtained in conventional spermatozoa samples. In Figure 2a, a sex-sorted spermatozoa sample showing a regular small sized halo of chromatin dispersion is shown. In general, the halo size of a spermatozoon identified as containing fragmented DNA after sorting is half the size of one identified in a conventional spermatozoa sample (compare Fig. 2b with 2c).

The baseline level of DNA damage in the five pre-sorted bull semen samples ranged from 5.3% to 11% with an average 7.9% ± 2.1% (Mean ± SD; Table 1). The level of spermatozoa DNA fragmentation obtained in X- and Y-chromosome sorted samples was much lower (3.1 ± 1.9) than that obtained in pre-sorted samples. Statistical differences were obtained after comparing both groups ($P < 0.01$). On average, the efficiency for SDF reduction was about 63%, but in some cases the reduction could be as high as 85% (compare values in Table 1). The level of SDF recovered after X- and Y-chromosome sex-sorting was notably reduced in the X and Y subpopulations when analyzed separately (Fig. 3; X-subpopulation: mean ± SD: 1.8% ± 1.5%; Y-subpopulation: mean 1.2% ± 0.6%). The tendency observed in all of the bulls was to accumulate spermatozoa containing a fragmented DNA molecule in the sorted non-viable subpopulation (Fig. 3; Mean ± SD: 12% ± 4.4%). This general tendency of SDF accumulation for each subpopulation was observed for each individual (Fig. 3).

To study the intra-individual consistency of this reduction in SDF, X-chromosome sorted straws obtained from 100 X- and Y-chromosome sex-sorted samples were assessed and the results are shown in Table 2. On average the level of SDF obtained after X- and Y-chromosome sex-sorting is low and similar to the level obtained in experiment 1 with values for SDF ranging from 0.03% to 2%. The level of spermatozoa DNA damage obtained after X- and Y-chromosome sex-sorting within each individual is quite similar when straws

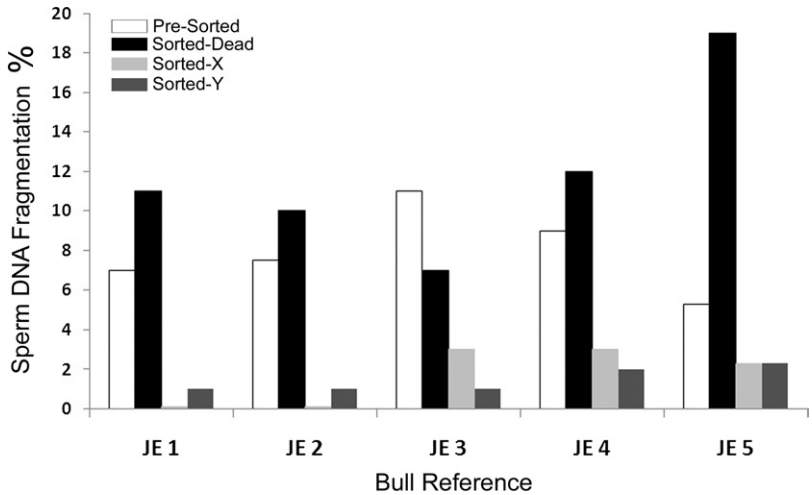

Fig. 3. Diagram to show the subpopulation distribution of SDF pre-sorting and after spermatozoa sex-sorting in five Jersey (JE) bulls, including the dead sperm subpopulation, X-sorted spermatozoa sub-population, and Y-sorted spermatozoa sub-population.

belonging to different dates and belonging to the same individual are compared (see values in Table 2). Interestingly, differences among individual bulls, regarding the efficiency in decreasing the SDF after X- and Y-chromosome sex-sorting, exist (F: 3.27; $P < 0.05$). The descriptive statistics for the values of SDF after sex-sorting are represented in Figure 4. As observed in Figure 4, it is apparent that some bulls (HO-04) harbour more DNA damaged spermatozoa than others (HO-07). In 6 out of the 10 bulls analyzed, outliers which double the mean for SDF are detected (Fig. 4).

4. Discussion

The results presented in this investigation show that spermatozoa sex-sorting, using a MoFlo® SX and di-

Table 2
Distribution of SDF (percent) in X-sorted spermatozoa within 10 individual bulls using 10 different ejaculates per individual. Dates of sperm collection are given.

Reference	Semen sample										Average
	1	2	3	4	5	6	7	8	9	10	
HO-01	1.00	0.66	1.00	0.66	0.66	0.00	0.33	1.66	0.66	0.66	0.73
	01/07/08	01/12/08	03/09/08	06/06/08	06/13/08	07/26/08	10/06/08	10/12/08	11/02/08	12/11/08	
HO-02	0.00	0.00	0.66	0.33	0.00	0.30	0.00	0.66	0.66	0.50	0.31
	04/30/02	12/15/08	12/18/08	12/20/08	12/28/08	12/30/08	01/04/09	01/06/09	01/08/09	01/11/09	
HO-03	1.00	0.66	0.33	0.66	1.00	1.00	1.33	0.33	1.00	1.00	0.83
	03/09/05	03/15/05	08/27/05	08/29/05	09/13/05	09/22/05	03/07/06	06/26/06	06/29/06	07/03/06	
HO-04	0.66	0.33	0.33	0.33	0.66	1.00	0.66	0.66	0.66	0.00	0.53
	11/07/07	11/13/07	11/26/07	11/30/07	12/03/07	12/06/07	12/07/07	12/11/07	12/17/07	12/19/07	
HO-05	0.33	0.00	0.30	0.66	0.00	2.00	0.30	0.00	0.33	1.00	0.49
	03/22/08	04/02/08	04/17/08	06/03/08	06/07/08	06/11/08	06/18/08	06/30/08	07/15/08	07/29/08	
HO-06	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.03
	04/09/08	04/29/08	05/02/08	06/03/08	06/29/08	07/08/08	07/11/08	07/15/08	07/18/08	07/27/08	
HO-07	0.00	0.66	0.33	0.33	0.66	0.00	0.00	0.00	0.00	0.66	0.26
	08/30/06	11/09/07	12/05/07	12/06/07	12/13/07	12/17/07	12/20/07	12/30/07	01/03/08	01/04/08	
HO-08	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.03
	11/06/06	04/07/08	04/16/08	05/09/08	05/19/08	06/01/08	06/23/08	06/26/08	07/24/08	07/30/08	
HO-09	1.66	0.00	0.33	1.66	0.33	0.00	0.00	0.00	0.00	0.00	0.40
	09/15/08	10/22/08	10/30/08	11/17/08	12/14/08	12/17/08	12/28/08	01/02/09	01/09/09	01/11/09	
HO-10	0.33	0.66	0.00	1.66	0.33	0.33	0.00	1.00	0.00	0.00	0.43
	02/02/09	02/11/09	02/23/09	02/28/09	03/02/09	03/09/09	03/18/09	03/24/09	03/27/09	03/31/09	

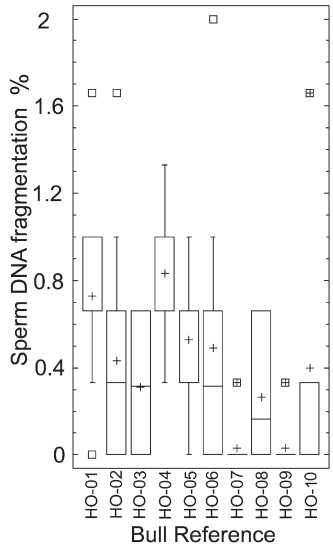

Fig. 4. Box–whisker plots for the distribution of SDF in sorted spermatozoa within each bull using ten different ejaculates per individual.

viding the net sample into three different subpopulations is a process that not only increases the percentage (i.e. purity) of X- or Y-chromosome bearing spermatozoa in a sample, but also considerably reduces the level of DNA damage in the samples being used for artificial insemination (AI) or *in vitro* fertilization (IVF). On average, a 69% in the reduction of SDF should produce beneficial effects on the final spermatozoa quality. However, in terms of sample efficiency for ART, levels of SDF lower than 2% should not have a major impact on offspring when the sample is used for insemination purposes. In fact, although the impact on fertility of SDF in bulls has not been thoroughly assessed, existing data indicate that changes in chromatin packing may play a major role in infertility [21,22] and SDF may have a negative impact of about 10% on pregnancy when the DNA damage is higher than 20% [23].

At present, variations in the level of SDF after spermatozoa sex-sorting have not been conclusive mainly because of the scarce information available. Persistence in the level of SDF after flow cytometry, without a dramatic increase in the baseline level, has been reported in mammalian species [4,24]. However, damage to sex-sorted spermatozoa has been shown to occur during sorting, since interaction of these cells with non-orthodox biological conditions is especially high [25,26,]. For example, under certain conditions the chemical and mechanical stress of staining spermatozoa combined with centrifugation increased the percentage of dead and damaged spermatozoa by 18.6% [27], but a simple unstained sorted sample increased the level of DNA damage by only 2% [4,27]. Variations in the level of spermatozoa stressing resulting in spermatozoa damage have also been reported using high or low pressure with a MoFlo® SX sorting system [25]. In general, for bull and stallion lowering the pressure for spermatozoa sorting from 50 psi, considered as the standard pressure, to 40 psi improved spermatozoa quality without a significant decrease in the sorting efficiency [25].

In the present study, the methodology for spermatozoa sex-sorting includes a step which is conducted to eliminate the dead spermatozoa subpopulation. This particular methodological step demonstrated that the level of SDF does not increase but could be notably reduced in the sample of interest. This strategy is commonly used in bull X- and Y-chromosome sex-sorting and appears to be effective in reducing the level of spermatozoa DNA damage indirectly, as previously suggested by Boe-Hansen et al [28]. These authors found a significant difference between the conventional level of SDF established for bulls and the SDF found in commercial sex-sorted semen when DNA integrity was measured using the Sperm Chromatin Structure Assay (SCSA) and neutral comet assay. The authors suggested that this effect was linked to the sorting process by excluding nonviable spermatozoa. In this work, we demonstrate that the damaged spermatozoa are effectively accumulated in the sorted dead subpopulation and this is the reason why an increase of SDF is always observed in this fraction after X- and Y- chromosome sex-sorting. Although, the previous finding could be assumed as obvious, the reality is not so simple because we have found that not all non-viable spermatozoa contain fragmented DNA and not all spermatozoa selected as being viable are absolutely free of DNA damage. Although a negative correlation with viability and SDF is usually found [17,29], a correlation is not always easy to establish [29] since both parameters may behave in an independent manner [30]. In this experiment we have additional and clear information about this aspect of spermatozoa quality since not all dead spermatozoa account for the level of spermatozoa containing a fragmented DNA molecule in the sorted dead subpopulation. This also points to the fact that, in cattle, a large proportion of spermatozoa containing fragmented DNA is injured, presenting major alterations in the spermatozoa membranes.

It should be stressed that the reduction in the level of DNA damage is fully dependent on the methodology used for flow cytometry sorting, in some instances DNA damage could remain the same or increase, e.g., De Ambrogi et al [24] and Garner [4], respectively.

Notably, the spermatozoa damage is likely to be the result of a synergistic process, whereby the level of damage in each step is probably reinforcing the damage produced in the next one. Therefore, the most parsimonious research plan for improving X- and Y-chromosome spermatozoa sex-sorting would be an attempt to identify and then minimize the damage in each single step of the sex-sorting process and ultimately optimize the reproductive potential. Decreasing DNA damage may involve decreasing laser strength, decreasing sorting pressure, decreasing stains, spermatozoa extender (e.g., TRIS or TALP) modification or some other means; however more research is needed.

The other item of interest to emerge from this investigation is the fact that not all bulls are equally efficient in achieving the desired reduction in the baseline level of SDF when samples from different ejaculates are processed. For example, in terms of SDF reduction, bulls H07 and H09 appear to be more efficient and consistent irrespective of the ejaculate. On the contrary, the DNA data from the rest of the bulls analyzed demonstrated both a higher level of SDF and a higher level of variance based on the values obtained from different ejaculates. The protamines are one of the candidates which could be participating in this differential spermatozoa behaviour. One of the main functions proposed for these spermatozoa protein residues is to preserve the DNA integrity. For example, human spermatozoa express two different protamines, protamine 1 (P1) and protamine 2 (P2), in almost equal amounts. An altered protamine 1 to 2 ratio (P1/P2) has been described previously in infertile patients, and also a correlation between this ratio and DNA fragmentation has been found [31,32]. In human cases, correlation between baseline spermatozoa DNA damage and protamine content has been clearly established [33,34,35].

The idea that SDF is linked to the genetic background of an animal needs to be seriously considered for Bovine. Identifying individuals having sperm with more resistance to the rigorous process of sex-sorting would allow for increased use of this technology in ART. The capabilities of using the sexing technology may thereby be applied further, e.g., reverse sorting techniques, consisting of thawing conventional semen doses for subsequent sex-sorting and re-freezing for future AI or IVF.

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