ABSTRACT: This study examined DNA damage and postthaw motility of white-tailed deer sperm (n = 28) before and after sex selection and conventional sorting using MoFlo XDP SX flow cytometry. Semen samples from the same individuals were treated in 4 different ways: 1) chilled-extended sperm samples (without glycerol); 2) cryopreserved conventional samples, samples directly cryopreserved after the addition of extenders; 3) cryopreserved conventionally sorted samples, sorted samples to remove the dead sperm subpopulation; and 4) cryopreserved sex-sorted samples; sorted samples to remove the dead sperm subpopulation and separation of X- and Y-chromosome-bearing sperm. In all the cases (n = 6), conventional samples showed decreased postthaw motilities (43 ± 26%) when compared with X-sorted samples (59 ± 20%; P < 0.05) and Y-sorted samples (54 ± 20%; P > 0.05). The DNA fragmentation baseline was <5% for frozen-thawed conventional samples, but even less after sex sorting and conventional sorting: 2.4 and 1.7%, respectively. On the other hand, conventional samples showed greater (P < 0.05) DNA fragmentation than the sex-sorted sperm (n = 6) at 96 h (average of 4.8 ± 4.5% and 5.3 ± 4%, respectively). Conventionally sorted samples (n = 8) did not have greater (P > 0.05) DNA fragmentation when compared with the sex-sorted samples. Fragmentation of DNA on X-chromosome and Y-chromosome-bearing sorted sperm were not significantly different (n = 10, P > 0.05) after 96 h (2.6 ± 3.6% and 2.2 ± 0.5%, respectively). Future research should be implemented for examining the fertilizing potential of sex-sorted white-tailed deer sperm (e.g., AI fertility trials).

Key words: DNA fragmentation, Odocoileus virginianus, postthaw motility, sex sorting, sperm, white-tailed deer

INTRODUCTION

In mammals, the semen in every ejaculate holds a certain proportion of sperm that does not meet the minimal requirements for fertilization. The reasons for these detrimental sperm alterations can be considered at 2 different levels: 1) primary sperm alterations produced during spermatogenesis and spermiogenesis, and 2) secondary sperm alterations produced after sperm ex vivo semen manipulation (i.e., iatrogenic damage).

Sexed sperm from a flow cytometry sorting process could be candidates for experiencing injury because of a synergistic combination of the sperm with nonbiological reagents, laser exposure, or mechanical stress (Garner, 2006).

The time between insemination and fertilization varies between species, and therefore pregnancy, is influenced by the degree of synchrony between mating and ovulation. Sperm must survive intact and undamaged inside the female tract for a certain period before fertilization. Most mammalian species can accommodate some flexibility by sustaining a reservoir of viable oviducal sperm for several hours or days, but how this biological timetable changes with a different amount of iatrogenic damage is unknown. The objective herein was to examine postthaw motilities and the extent of DNA damage in chilled-extended sperm, cryopreserved conventional sperm, cryopreserved conventional-sorted sperm, and cryopreserved sex-sorted sperm. The hypothesis was that during sperm sex sorting, the ex-
tent of DNA damage in the sorted sperm fraction may 
initially decrease due to the removal of nonviable and 
poorly oriented cells (e.g., Bodmer et al., 2005; Gos-
álvez et al., 2011a), but the stressing features from sex 
sorting may lead to increased sperm DNA damage. For 
example, although baseline sperm DNA fragmentation 
(SDF) may diminish after sex sorting bovine (Bos tau-
rus) sperm, the rate of SDF can increase after a period 
of incubation that emulates the temperature recovery 
episode occurring in the female tract (Gosálvez et al., 
2011a,b).

MATERIALS AND METHODS

All of the animals used in this research were treated 
in accordance with the Federation of Animal Science 
Societies (2010) guide for the use of farm animals in 
research and teaching.

Twenty-eight white-tailed deer bucks (Odocoileus vir-
giniaus) were included in the analysis. The bucks used 
for the sex-sorting experiments were selected based on 
a genetic predisposition for producing large antler char-
acteristics, primarily Boone and Crockett antler score 
measurements ≥200 points. White-tailed deer selected 
for the sex-sorting experiments ranged in age from 1 
to 7 yr of age and came from private ranches in Wis-
consin, Missouri, Arkansas, and Texas. Different sub-
samples were produced from the original electroejacu-
lated and extended sample: 1) chilled-extended sperm 
samples, 2) conventional cryopreserved samples (sam-
pies directly cryopreserved from extended samples), 3) 
cryopreserved conventionally sorted samples (sorted 
samples to remove the dead sperm subpopulation), and 
4) cryopreserved sex-sorted samples (sorted samples to 
remove the dead sperm subpopulation and separation 
of X- and Y-chromosome-bearing sperm).

Using the aforementioned treatments, 2 different ex-
periments were conducted. The first experiment was to 
analyze possible differences in baseline sperm motility 
and SDF between chilled-extended, conventional, con-
ventional-sorted, and sex-sorted sperm. A second ex-
periment was conducted to analyze possible differences 
in the dynamics of SDF between chilled-extended, con-
ventional, conventionally sorted, and sex-sorted sperm. It 
should be mentioned that although 28 white-tailed 
deer were utilized in these experiments, the number 
of bucks being directly compared between treatment 
groups varied for several reasons: 1) a sample may have 
arrived and was not of sufficient volume to process for 
each treatment group, and 2) 10 frozen-thawed conven-
tional semen samples were processed without having a 
neat semen sample saved for comparison.

After sex sorting, all of the sperm cells were collected 
and cryopreserved in 0.25-mL straws using an auto-
mated freezing device, IMV Digitcool (IMV, Cedex, 
France) and stored under liquid nitrogen. The conven-
tional sample was also cryopreserved using similar stor-
age conditions, although semen for 1 buck was cryopre-
served in 0.5-mL straws. After thawing at 37°C, straw 
contents were placed into prelabeled 1.5-mL Eppen-
dorf microcentrifuge tubes (Eppendorf North America, 
Hauppauge, NY), and the sperm baseline (0 h) DNA 
fragmentation index was assessed using the method ex-
plained in the next section.

For the second experiment (Exp. 2), after thawing, 
the sperm from each different sample were placed into 
prellaabeled 1.5-mL Eppendorf microcentrifuge tubes 
and incubated in a water bath at 37°C. The SDF index 
was assessed after 0, 4, 24, 48, 72, and 96 h.

Semen Collection

Semen was collected via electroejaculation, and por-
tions of the ejaculate were collected, kept separate, and 
analyzed based on consistency and color. The portions 
with the best motility (>~80%), based on subjective 
microscope measurements, were extended to approxi-
mately 1 × 10^9 sperm/mL with Biladyl (Mininute of 
America Inc., Verona, WI) or Tris A solution (200 mM 
Tris, 65.7 mM citric acid, 55.5 mM fructose) containing 
20% (vol/vol) egg yolk (Underwood et al., 2009). All 
the samples were shipped or driven to Sexing Technolo-
gies (Navasota, TX) for processing during the first 24 
h after collection. Samples were chilled and maintained 
between 5 to 10°C during transport.

Sperm Preparation for Experimental 
Purposes

Semen samples were divided into 4 different batches. 
One of them was assessed for sperm motility charac-
teristics and SDF before cryopreservation (chilled-ex-
tended). The rest of the subsamples were cryopreserved 
after conventional protocols (samples directly cryopre-
served), conventional sorting (sorting to remove the 
dead sperm subpopulation), or sex sorting (sorting to 
remove the dead sperm subpopulation and separation 
of X- and Y-chromosome-bearing sperm). Therefore, 4 
different types of subsamples were analyzed to assess 
sperm quality: 1) chilled-extended (C-Ext), 2) frozen-
thawed conventional (FT-Conv), 3) frozen-thawed 
conventional-sorted (FT-Conv-Sort), and 4) frozen-
thawed sex-sorted (FT-Sex-Sort).

Conventional Sorting and Sex Sorting

Each conventionally sorted and sex-sorted sample was 
produced according to the standards for semen 
production at Sexing Technologies. Sorting of X- or Y-
chromosome-bearing sperm was conducted similar to 
the procedures described previously by Johnson and 
Welch (1999), Johnson et al. (1999), and Seidel and 
Garner (2002), using MoFlo XDP SX (Beckman Coul-
ter Inc., Miami, FL) sperm sorters and Summit v5.0 
software. For these experiments, the MoFlo XDP SX 
was set to a sorting pressure of 275,790 Pa. The laser 
source was a NdYAG mode locked pulsed Vanguard 
HMD350 (Spectra Physics Lasers-North America,
Santa Clara, CA) operating at 355 nm. The operating power was light regulated at 350 mW. Being a dual-headed sorter, the laser beam was split utilizing a CVI Melles Griot (Albuquerque, NM) 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 sperm, or ~55 to 65% of the total cells, while taking ~40 to 45% of the X or Y region to optimize for that purity, subsequently confirmed using the STS Sexed Semen Purity Analyzer (Sexing Technologies) and STS Analyzer Software v.1.0.0, which provides high-resolution peaks of X- and Y-chromosome-bearing sperm populations. The aforementioned settings resulted in sorting speeds of 5,000 to 6,000 sexed sperm per second. The emphasis on each sort was to achieve either a large percentage (purity) of X- or Y-chromosome-bearing sperm in a given sample with a purity ≥95%. We hereafter refer to sorted X- or Y-chromosome-bearing sperm samples as X-sorted and Y-sorted sperm. The sheath fluid used for these sperm sorting experiments was SortEnsure (catalog number 77-86-1, XY Inc.). Sheath fluid acts to surround the core stream of sorting medium and sperm, thereby guiding them through the flow cytometer for sorting (Seidel and Garner, 2002). After the sperm have been sex-sorted they are collected in 50-mL tubes (Axygen Scientific Inc., Union City, CA) containing catch fluid (Seidel and Garner, 2002). Pre- and post-conventional sort. In both experiments, the deer ejaculates were only used for processing either conventional or sex-sorted straws of semen if they met the following criteria: minimum motility of ≥55%, primary morphologies ≤15%, secondary morphologies ≤15%, and a total morphology count not to exceed 25%. Further, samples used in the postthaw analyses had to meet standard quality control conditions of motility ≥45% at 0 h. For all semen quality evaluations, 25.4 × 76.2 mm glass microscope slides (Andwin, Addison, IL) and 22 × 22 mm #1.5 coverslips (Thomas Scientific, Swedesboro, NJ) were used. All motility assessments were made using brightfield microscopy, and intact acrosome and morphology assessments utilized differential interference contrast microscopy at 400×.

All extenders used in the experiments consisted of the same formulation having a pH of 6.8 and an osmolality balanced at 300 mOsm/kg for the Tris A extender. For testing on a batch-to-batch basis, Tris B fraction extender was diluted 3:1 (A:B) and vortexed with a final osmolality of 830 mOsm/kg. Extender used to dilute deer semen was a 1:1 ratio of A and B fractions of extender, final glycerol content at 6%. A deer semen production dose for fresh semen was ~3 × 10^6 sperm per straw (0.25 mL) and ~6 × 10^6 sperm per straw for cryopreserved conventional and sex-sorted straws of semen.

**SDF Assessment**

Each processed semen sample (i.e., C-Ext, FT-Conv, FT-Conv-Sort, or FT-Sex-Sort) was analyzed after adjusting the concentration obtained postthaw to ~1 to 2 × 10^6 sperm/mL in clear TALP medium. For all samples, SDF was analyzed using the Sperm-Halomax kit (Halotech DNA, Madrid, Spain). The Sperm-Halomax methodology is based on the sperm chromatin dispersion test (Fernández et al., 2003; López-Fernández et al., 2007). Details of this technique have been previously reported for human and other mammalian species (López-Fernández et al., 2008a; Fernández et al., 2009; Zee et al., 2009).
Each experiment was conducted using 25 µL of each diluted aliquot of semen. This volume was mixed with 50 µL of low-melting-point agarose. Ten microliters of the mixture was extended on pretreated slides (Sperm-Halomax kit, Halotech DNA, Madrid, Spain) covered with a 22 × 22 mm #1.5 coverslip (Thomas Scientific) and placed on a cold metallic plate in the refrigerator (4°C) for 5 min. Afterward, the coverslip was removed and each slide was set up horizontally in 10 mL of lysing solution (Sperm-Halomax kit) for 5 min. The nucleoids resulting from the lysing process were dehydrated in a 2-min series of ethanol baths (70 and 100%). Once dried, the slides were stained using a 1:1 mixture of SYBR I (10X; Invitrogen Molecular Probes) in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA) for DNA staining. This staining method permits manual scoring by allowing for the distinction of sperm containing either fragmented DNA (large and spotty halos of chromatin dispersion) or unfragmented DNA (small and compact halo of chromatin dispersion). Samples were viewed and captured using a Nikon Eclipse 80i microscope (Melville, NY) with fluorescence capabilities and NIS Elements BR 3.00 imaging software (Nikon, 2008) with an MLD camera (Photometrics CoolSNAP 164 EZ, Tucson, AZ). Sperm were counted and divided into 2 groups: fragmented and nonfragmented, and a percentage was calculated based on measuring 300 cells.

**Statistical Analysis**

The results are reported as mean and SD for statistical comparison purposes. The comparison among dynamic loss of DNA quality was assessed using the nonparametric maximum likelihood Kaplan-Meier estimator. To compare the survival distributions of the sperm samples from individual bucks and for the different treatments, a log-rank (Mantel-Cox) test was used (SPSS Inc., Chicago, IL). The sperm survival, in this case, is represented as a varying frequency over time for those sperm that were not affected by fragmentation at each time period of analysis. A nonparametric statistical hypothesis test Wilcoxon signed rank was used to

**Figure 1.** Flow cytometry using Summit v5.0 (Beckman Coulter, Miami, FL) software for determining and sorting white-tailed deer sperm: live vs. dead/membrane-compromised sperm populations, X- or Y-chromosome-bearing sperm subpopulations, and a conventionally sorted sperm subpopulation. Color version available in the online PDF.
RESULTS

The results of both the static and dynamic DNA fragmentation experiments demonstrated differences between the sperm samples of individual bucks. Differences in DNA fragmentation rates (Figures 2 and 3) and postthaw motilities (Tables 1 and 2) existed for the different sperm preparation methodologies (i.e., C-Ext, FT-Conv, FT-Conv-Sort, or FT-Sex-Sort). The results of the Breslow (generalized Wilcoxon) test of the nonparametric maximum likelihood Kaplan-Meier estimator survival distributions of the sperm samples in the different treatments are summarized in Table 3.

Static DNA Fragmentation Index

In Exp. 1, baseline DNA damage in the 18 conventional semen samples ranged from 0.3 to 4.9% with an average 1.83 ± 1.47%. On average, the SDF obtained for the FT-Conv-Sort samples (n = 7) was less (Z = −1.476, P = 0.140) than that obtained in C-Ext samples, 0.76 ± 0.74% and 1.33 ± 0.59%, respectively; but greater than in FT-Sex-Sort, 0.4 ± 0.68% (Z = −2.386, P = 0.017). The decrease in SDF for FT-Conv-Sort vs. C-Ext samples (n = 7) was about 45.8% on average, or 0.63 ± 0.69 vs. 1.16% ± 0.44%, respectively. The efficiency in reducing SDF from the sample of interest, comparing C-Ext to FT-Sex-Sort samples from the same bucks (n = 6), was 1.09 ± 0.41% vs. 0.10 ± 0.16%, on average, or approximately a 91% decrease in sperm with DNA fragmentation in the sex-sorted population. The baseline DNA fragmentation for X- and Y-sorted sperm (n = 10) averaged 0.37 ± 0.74% and 0.34 ± 0.48% (Z = −0.175, P = 0.861). The baseline of FT-Conv samples was greater (Z = −2.552, P = 0.011) than in FT-Sex-Sort samples (n = 6), 1.21 ± 1.21% and 0.30 ± 0.67%, respectively. Baseline SDF can be considered low, less than 5% on average for all bucks in the analysis, specifically a maximum of 4.9% for FT-Conv, 2.39% FT-Sex-Sort, and 1.69% FT-Conv-Sort.

The FT-Conv samples showed smaller (Z = −1.203, P = 0.229) postthaw motilities than the FT-Sex-Sort samples from the same individual bucks (n = 6), with
average postthaw motilities of 43 ± 26%, 59 ± 20% (X-sorted; $Z = -1.355$, $P = 0.176$), and 54 ± 20% (Y-sorted; $Z = -0.420$, $P = 0.674$), respectively (based on Table 1), and a similar trend was observed for all of the samples analyzed (Table 2). The FT-Conv-Sort samples had smaller postthaw motilities 53 ± 17% ($Z = -2.497$, $P = 0.013$) when compared with FT-Sex-Sort samples from the same ejaculates of individual bucks ($n = 10$), 60 ± 12% (X-sorted; $Z = -2.077$, $P = 0.038$) and 57 ± 15% (Y-sorted; $Z = -1.686$, $P = 0.092$). The FT-Conv-Sort compared with FT-Conv samples, from the same bucks ($n = 5$), had larger postthaw motilities on average ($Z = -0.813$, $P = 0.416$), 48 ± 24% vs. 36 ± 24%, respectively. The overall X-sorted vs. Y-sorted sperm 0-h postthaw motility results ($n = 13$) were very similar ($Z = -0.982$, $P = 0.326$, i.e., 60.2 ± 11% and 58.2 ± 14%, respectively; Table 2).

**Dynamic DNA Fragmentation Index**

In Exp. 2, the overall DNA damage, after incubation at 37°C for 96 h, in sperm samples from all individual bucks varied more for the C-Ext ($n = 13$, $P < 0.001$) and FT-Conv samples ($n = 18$, $P < 0.001$) (Figure 2a, b), ranging from 1.69 to 61.29% (average = 15.45 ± 17.67%) and 1.35 to 47.06% (average = 13.47 ± 15.56%), respectively. There was much less variation for the FT-Conv-Sort ($n = 8$, $P = 0.159$) and FT-Sex-Sort samples ($n = 13$, $P = 0.006$; Figure 2c, d), after incubation at 37°C for 96 h, ranging from 3.45 to 12.36% (average = 6.7 ± 3.9%) and 0 to 11.11% (average = 3.38 ± 3.17%), respectively.

When comparing sperm samples taken from the same bucks ($n = 6$), the FT-Conv samples showed significantly greater ($P = 0.044$) DNA fragmentation than the FT-Sex-Sort, with average 96-h DNA fragmentation of 4.79 ± 4.48% and 5.32 ± 3.98%. The FT-Conv-Sort samples after 72 and 96 h of incubation had slightly greater (1.68 ± 1.56% and 6.7 ± 3.9%, respectively; $P = 1.00$) DNA fragmentation, compared with the FT-Sex-Sort sperm (2.06 ± 1.56% and 2.83 ± 2.61%, respectively), when comparing samples from the same individual bucks ($n = 8$). The FT-Conv-Sort sperm had significantly less ($P < 0.001$) SDF than C-Ext ($n = 7$; 7.78 ± 3.97% vs. 22.39 ± 21.68%), comparing samples from the same individual bucks (Figure 3), which is
equivalent to a 65.3% decrease in DNA-fragmented sperm after sorting. When comparing sperm samples from the same individual bucks (n = 9), FT-Sex-Sort samples had significantly less ($P < 0.001$) SDF than C-Ext sperm samples at 96 h of incubation (3.25 ± 2.63% and 19.05 ± 19.7%, respectively), translating to a decrease of ~83% SDF after sex sorting. When comparing X-sorted sperm to Y-sorted sperm, with an average difference in purity of ~3% (Table 2), DNA fragmentation was not significantly different (n = 10, $P = 0.714$), averaging 2.59 ± 3.61% and 2.18 ± 0.53% at 96 h of incubation.

### DISCUSSION

In the United States, deer production is growing steadily with more than 200,000 deer of several species being raised commercially on game preserves, farms, and ranches (Shaffer et al., 2005). Successful methods of assisted reproductive technologies (ART) and nonsurgical embryo collection with surgical transfer in captive white-tailed deer have already been developed and are being used (Magyar et al., 1988, 1989; Jacobson et al., 1989). Given the magnitude of white-tailed deer breeding in the United States, sexed semen to produce more

### Table 1. White-tailed deer sperm sample attributes and precryopreservation (Pre-cryo) and postthaw motility (PTM) results for sex-sorted sperm (X-sort and Y-sort), conventionally sorted (Conv-Sort), and conventional (Conv) sperm samples

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<th>Buck number</th>
<th>Volume, mL</th>
<th>Concentration, million/mL</th>
<th>Method</th>
<th>Pre-cryo motility, %</th>
<th>0-h PTM, %</th>
<th>X-sort purity, %</th>
<th>Y-sort purity, %</th>
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<td>65</td>
<td>93</td>
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<tr>
<td>11</td>
<td>3.8</td>
<td>494</td>
<td>Y-sort</td>
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<td>70</td>
<td>91</td>
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<tr>
<td>12</td>
<td>2.7</td>
<td>521</td>
<td>Conv</td>
<td>N/A</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.7</td>
<td>521</td>
<td>Conv-Sort</td>
<td>70</td>
<td>53</td>
<td>50</td>
<td>50</td>
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<tr>
<td>12</td>
<td>2.7</td>
<td>521</td>
<td>X-sort</td>
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<td>55</td>
<td>97</td>
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<td>13</td>
<td>3.2</td>
<td>1,601</td>
<td>Conv</td>
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<td>10</td>
<td></td>
<td></td>
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<tr>
<td>13</td>
<td>3.2</td>
<td>1,601</td>
<td>Conv-Sort</td>
<td>70</td>
<td>62</td>
<td>49</td>
<td>51</td>
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<tr>
<td>13</td>
<td>3.2</td>
<td>1,601</td>
<td>X-sort</td>
<td>73</td>
<td>64</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>9.5</td>
<td>500</td>
<td>Conv</td>
<td>45</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.7</td>
<td>539</td>
<td>Conv</td>
<td>80</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*N/A = not available.*
doses from a given individual to rapidly build herds or to produce more trophy bucks of desired genetics is of high interest. Texas alone has 1,100 licensed breeders with approximately 87,000 deer and a total economic impact of $652 million annually, whereas deer breeding is the fastest-growing industry in rural America, according to a 2007 Texas A&M study (Hylton, 2009), contributing $3 billion annually to the economy of the United States (Cain, 2009).

The present study is the first experimental approach to investigate the sorting capacities of white-tailed deer sperm via flow cytometry and the effect on SDF and motility. Also, the research herein examines the simultaneous use of conventional sorting with sex sorting of sperm to preserve valuable sperm for subsequent assisted reproductive techniques. In fact, the sorting process not only decreased the baseline amount of sperm containing fragmented DNA, but also improved the DNA longevity. Additionally, the FT-Conv sperm samples had reduced postthaw motilities when compared with FT-Sex-Sort samples. The kinetics of sperm DNA damage for the FT-Conv sperm samples showed a significantly greater rate of sperm DNA damage over time than the FT-Sex-Sort. This behavior for the sperm motility and the chromatin of the sperm in deer after sex sorting is unexpected because the most parsimonious hypothesis would be that after a stressing episode such as that experienced during sex sorting, the sperm would have undergone some conditions that negatively influence the chromatin stability.

A decrease in sperm chromatin stability could easily be associated with mechanical injury, enhanced via DNA exposure to Hoechst 33342, laser illumination, or both, as discussed by Garner (2006). In fact, this is what we have observed in bovine where, as in deer, the baseline SDF is greater in FT-Conv than in FT-Sex-Sort samples, but the rate of SDF is greater in FT-Sex-Sort samples than in FT-Conv samples. This is an interesting effect because when both curves are plotted together, a crossover in the tendency of SDF (termed the crossover positioning time; Gosálvez et al., 2011b) for both conventional and sex-sorted samples is produced (Figure 4a). This aforementioned crossover positioning time was not produced in the deer sperm

Table 2. Average white-tailed deer sperm sample attributes and precryopreservation (Pre-cryo) and postthaw motility (PTM) results for sex-sorted (X-sort and Y-sort), conventionally sorted (Conv-Sort), and conventional (Conv) samples

<table>
<thead>
<tr>
<th>Item</th>
<th>Volume, mL</th>
<th>Concentration, million/mL</th>
<th>Total sperm, million</th>
<th>Method</th>
<th>Pre-cryo motility, %</th>
<th>0-h PTM, %</th>
<th>X-sort purity, %</th>
<th>Y-sort purity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (n = 8)</td>
<td>5.45</td>
<td>790</td>
<td>4,215</td>
<td>Conv</td>
<td>68.8</td>
<td>41.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>3.52</td>
<td>402</td>
<td>3,099</td>
<td>Conv</td>
<td>13.6</td>
<td>22.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (n = 10)</td>
<td>4.86</td>
<td>992</td>
<td>4,585</td>
<td>Conv-Sort</td>
<td>68.8</td>
<td>52.7</td>
<td>48.5</td>
<td>51.5</td>
</tr>
<tr>
<td>SD</td>
<td>2.91</td>
<td>399</td>
<td>3,050</td>
<td>Conv-Sort</td>
<td>3.5</td>
<td>17.1</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Average (n = 13)</td>
<td>4.63</td>
<td>941</td>
<td>4,247</td>
<td>X-sort</td>
<td>68.2</td>
<td>60.2</td>
<td>94.3</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2.62</td>
<td>376</td>
<td>2,849</td>
<td>X-sort</td>
<td>5.9</td>
<td>10.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Average (n = 13)</td>
<td>4.63</td>
<td>941</td>
<td>4,247</td>
<td>Y-sort</td>
<td>67.8</td>
<td>58.2</td>
<td>91.3</td>
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</tr>
<tr>
<td>SD</td>
<td>2.62</td>
<td>376</td>
<td>2,849</td>
<td>Y-sort</td>
<td>5.3</td>
<td>14.0</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Breslow (generalized Wilcoxon) results for the Kaplan-Meier survival functions representing the dynamics of sperm DNA fragmentation for sperm samples from white-tailed deer

<table>
<thead>
<tr>
<th>Comparison</th>
<th>n</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Ext¹</td>
<td>13</td>
<td>317.605</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FT-Conv-Sort²</td>
<td>8</td>
<td>10.569</td>
<td>0.159</td>
</tr>
<tr>
<td>FT-Conv³</td>
<td>18</td>
<td>577.718</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FT-Sex-Sort⁴</td>
<td>13</td>
<td>27.635</td>
<td>0.006*</td>
</tr>
<tr>
<td>X-sort vs. Y-sort⁵</td>
<td>10</td>
<td>0.135</td>
<td>0.714</td>
</tr>
<tr>
<td>C-Ext vs. FT-Conv</td>
<td>5</td>
<td>1.149</td>
<td>0.284</td>
</tr>
<tr>
<td>C-Ext vs. FT-Conv-Sort</td>
<td>7</td>
<td>14.189</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FT-Sex-Sort vs. FT-Conv</td>
<td>9</td>
<td>18.549</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FT-Sex-Sort vs. FT-Conv-Sort</td>
<td>6</td>
<td>4.057</td>
<td>0.044*</td>
</tr>
<tr>
<td>FT-Sex-Sort vs. FT-Conv-Sort</td>
<td>8</td>
<td>0.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

¹C-Ext = chilled-extended sperm.
²FT-Conv-Sort = frozen-thawed conventionally sorted sperm.
³FT-Conv = frozen-thawed conventional sperm.
⁴FT-Sex-Sort = frozen-thawed sex-sorted sperm.
⁵X-sort vs. Y-sort = frozen-thawed X-sorted sperm vs. frozen-thawed Y-sorted sperm.

*Significant at the 0.05 α-level.
samples (Figure 4b), so it could be concluded that the sperm subpopulation obtained after sex sorting shows a greater DNA stability than that observed in chilled-extended or conventional prepared samples.

It is difficult to assume that the unique characteristics of sperm are improved via flow cytometry. The plausible explanation is that the sorted sample has decreased concentrations of highly selected viable spermatozoa with a reduced propensity toward sperm mortality. In fact, when ram sperm samples were incubated at equivalent conditions to those used herein to study the dynamic behavior of SDF, there was an increase in sperm DNA stability as concentration of the sperm sample decreased (López-Fernández et al., 2010). In López-Fernández et al. (2010), the rate of SDF in samples diluted to $6.3 \times 10^6$ sperm/mL remained invariable throughout the entire 6-h incubation period, whereas the samples incubated at $100 \times 10^6$ sperm/mL had an increase in the baseline of 40% or more depending upon the individual.

In a parabiological environment designed to conserve sperm quality (i.e., the semen extenders), the accumulation of toxic metabolites, active free enzymes, or both, such as those found in the acrosome, may accelerate sperm degeneration of the accompanying surviving sperm. An efficient elimination of dead sperm, as occurs after sex sorting, can diminish the impact of such stressors. In general, the chromatin in white-tailed deer sperm is stable after sex sorting, and also before sex sorting, when compared with other species analyzed previously, such as human (Gosálvez et al., 2009) and ovine species (López-Fernández et al., 2008a). Notably, DNA fragmentation present in the raw ejaculates in white-tailed deer is also very small when compared with those of other species such as human or equine (López-Fernández et al., 2007; Gosálvez et al., 2009), but is similar to that found in boar or bull (López-Fernández et al., 2008b; Gosálvez et al., 2011a).

The comparative dynamic analysis of sperm DNA damage in different mammalian species indicates that the resistance of the chromatin to being affected by external stressors is different for each species once the sperm is used ex vivo for ART. Whereas in species such as boar or bull, the calculated rate of SDF is of the order of 0.05 (derived from Gosálvez et al., 2011b), this value is 15.2 in the case of ram (derived from López-Fernández et al., 2008a; i.e., around 300 times more intense). Of course, before ejaculation and when mature semen is retained in the epididymis, these rates of sperm DNA damage are not constant for some species (ram or human) but may be maintained by others such as boar, bull, or deer. Explanations for the differential sensitivity of the sperm DNA may be related to the ability of the protamines to efficiently pack the DNA molecule.

Two main types of protamine families (P1 and P2) have been described in mammalian species. The P1 family has been reported in all species of vertebrates studied (McKay et al., 1985; Mengual et al., 2003; Yoshii et al., 2005), whereas the family of P2 proteins (formed by a compendium of other protamines) is present in all primates and most rodents examined thus far. The sperm of stallions, lagomorphs, and proboscids has also been found to contain processed protamine P2 (Balhorn et al., 1977; McKay et al., 1985; Oliva and Dixon, 1990; Yoshii et al., 2005). However, in the case of bull and boar, 2 of the species presenting more resistance to iatrogenic SDF, the gene for P2 is present, but it seems to be dysfunctional or produce an aberrant protein (Balhorn, 2007). Thus, it seems plausible that the abolition of the presence of P2 might cause the sperm chromatin to become more stable.

![Figure 4. Comparison of the dynamic behavior of conventional (unfilled circles) and sex-sorted sperm samples (filled circles) in a) bulls, modified from Gosálvez et al. (2011b), and b) white-tailed deer. CPT = crossover positioning time.](image-url)
Destabilization of the ratio P1/P2 is highly correlated with alteration in the normal pattern of SDF in human (Garcia-Periò et al., 2011). Additionally, different rates of sperm chromatin decondensation have been found for mammalian species with different natural protamine P1/P2 ratios (Perreault et al., 1988). The aforementioned finding suggests that the protamine P2 content of sperm may regulate the rate at which sperm chromatin decondenses and also has implications on how the male genome is reactivated after fertilization. Currently, no information is available about the presence of protamine P2 transcripts in white-tailed deer sperm, but its chromatin stability suggests a similar condition to that found in bull or boar.

Altogether, the results herein also support the idea expressed by de Graaf et al. (2009) that in certain species, such as sheep, the process of sex sorting may select a population of sperm with enhanced sperm function and fertility, whereas in other species there seems to be a reduction in the function of sex-sorted sperm compared with conventional sperm. de Graaf et al. (2009) hypothesized that ram sperm are in some way more resilient to the stressors of sex sorting, based on better postsort results compared with other species. From our research, it would appear that the white-tailed deer sperm chromatin is very tolerant of the sex-sorting process. Altogether, there appears to be both interspecific and intraspecific tolerance by sperm to the sex-sorting process, suggesting that some individuals perform better than others under the stressful and postcollection sex-sorting conditions. The extent of this individual variation needs to be analyzed in greater detail, but it is congruent with the interindividual differences for the rates of SDF reported in other animal species (López-Fernández et al., 2008a; Gosálvez et al., 2009).

As O’Brien et al. (2009) suggested, conservation and captive breeding of deer may be enhanced by sperm sorting and artificial insemination via population structuring (e.g., avoiding surplus males and producing predominantly female offspring via sperm sorting technology, or by preserving and utilizing semen from genetically valuable, underrepresented or deceased males from postmortem collections; Morrow et al., 2009). The Eld’s deer (Cervus eldi thamin) is an example in which the application of conventional ART was applied for the genetic management of an endangered species of deer (Morrow et al., 2009). In such cases, and based on the results herein, it would appear that conventional sorting as well as sex sorting might be applied (i.e., conventional sorting as a means to recover more cells from valuable samples undergoing the sex-sorting process). The basis for this sperm recovery application has now been demonstrated for white-tailed deer (e.g., the baseline SDF obtained for the FT-Conv-Sort samples was similar to that obtained in C-Ext samples and even after 96 h of incubation at 37°C, the SDF of FT-Conv-Sort sperm had less SDF than C-Ext samples). Furthermore, FT-Conv-Sort postthaw motilities were similar to those of FT-Conv samples.

Knowing the demand for cervid sex selection, Gao et al. (2009) successfully produced Sika deer (Cervus nippon) offspring using laparoscopy and 2.3 × 10⁶ frozen-thawed, sex-sorted sperm per insemination. In the near future and dependent on field trial results, sex-sorted semen could be a viable alternative method for the white-tailed deer industry and may serve as a model for the conservation of endangered cervid species such as the Eld’s deer. Future work should be implemented for examining the fertilizing potential of sex-sorted white-tailed deer sperm (e.g., ART field fertility trials).

LITERATURE CITED


