Evaluation of motility, membrane status and DNA integrity of frozen–thawed bottlenose dolphin (*Tursiops truncatus*) spermatozoa after sex-sorting and recryopreservation

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Abstract

Artificial insemination (AI) with sex-sorted frozen–thawed spermatozoa has led to enhanced management of *ex situ* bottlenose dolphin populations. Extended distance of animals from the sorting facility can be overcome by the use of frozen–thawed, sorted and recryopreserved spermatozoa. Although one bottlenose dolphin calf had been born using sexed frozen–thawed spermatozoa derived from frozen semen, a critical evaluation of *in vitro* sperm quality is needed to justify the routine use of such samples in AI programs. Sperm motility parameters and plasma membrane integrity were influenced by stage of the sex-sorting process, sperm type (non-sorted and sorted) and freezing method (straw and directional) ($P < 0.05$). After recryopreservation, sorted spermatozoa frozen with the directional freezing method maintained higher ($P < 0.05$) motility parameters over a 24-h incubation period compared to spermatozoa frozen using straws. Quality of sperm DNA of non-sorted spermatozoa, as assessed by the sperm chromatin structure assay (SCSA), was high and remained unchanged throughout freeze–thawing and incubation processes. Though a possible interaction between Hoechst 33342 and the SCSA-derived acridine orange was observed in stained and sorted samples, the proportion of sex-sorted, recryopreserved spermatozoa exhibiting denatured DNA was low ($6.6 \pm 4.1\%$) at 6 h after the second thawing step and remained unchanged ($P > 0.05$) at 24 h. The viability of sorted spermatozoa was higher ($P < 0.05$) than that of non-sorted spermatozoa across all time points after recryopreservation. Collective results indicate that bottlenose dolphin spermatozoa undergoing cryopreservation, sorting and recryopreservation are of adequate quality for use in AI.

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Introduction

The bottlenose dolphin (*Tursiops truncatus*) is protected under Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna), which limits the movement of animals or their products between countries (CITES – (website) Convention on International Trade in Endangered Species of Wild Fauna and Flora, consulted on June 25th, 2011. Available on <http://www.cites.org/eng/resources/species.html>). These limitations have created artificial barriers for international animal exchange and as a result, breeding and genetic management of captive bottlenose dolphin populations are usually limited to individual companies within a country. In addition, transportation of animals across long distances is expensive and potentially dangerous for the animals (Robeck *et al.* 1994).

Assisted reproductive technologies (ART) such as semen cryopreservation, artificial insemination (AI) and

sex-sorting of spermatozoa obviate the need for animal transportation, and provide a modern tool for the genetic and sex ratio management of captive bottlenose dolphin populations across nations. In the bottlenose dolphin, AI was developed after basic research was conducted to characterize the species' reproductive physiology (Robeck *et al.* 2005). Longitudinal studies enabled the prediction of ovulation and characterization of female reproductive cycles through transabdominal ultrasonic evaluation of the ovary (Brook *et al.* 2000) and urinary hormone measurement (Robeck *et al.* 2005). These techniques were combined with sperm cryopreservation (Robeck & O'Brien 2004) and hysteroscopic insemination, which resulted in the first bottlenose dolphin calf being born by way of AI (Robeck *et al.* 2005). Sex-sorted spermatozoa obtained using flow cytometry (Johnson *et al.* 1989) combined with AI led to the first pre-sexed bottlenose dolphin having been born in 2005 at

SeaWorld San Diego (O'Brien & Robeck 2006). To date, 13 bottlenose dolphin calves have been produced using sex-sorted, cryopreserved spermatozoa with a sex pre-determination success rate of 92% (O'Brien & Robeck, personal communication). These techniques optimize population genetics and sex ratio simultaneously; therefore, they are invaluable for species management in captivity where limited population size requires female-skewed sex ratios for maximum reproductive rates. In addition, a bottlenose dolphin population in captivity with more females than males maintains similarities to wild groups (Wells 2000, O'Brien & Robeck 2006), resulting in a more normal social population that reduces inter-male competition and potential associated injuries.

Once semen is collected, sperm samples should undergo sorting after no more than an 18-h transport/storage period to ensure that sexed spermatozoa are not excessively aged at the time of cryopreservation (O'Brien *et al.* 2009). An alternative to having sorting facilities around the world is the development of adequate methods for the sorting and cryopreservation of previously cryopreserved spermatozoa. In this case, semen would be cryobanked and shipped frozen from any location to the sorting facility where it would be thawed, sorted and recryopreserved. Sorting of cryopreserved spermatozoa was first established in sheep (O'Brien *et al.* 2003, Hollinshead *et al.* 2004a) and adapted for humans (O'Brien *et al.* 2005), non-human primates (O'Brien *et al.* 2003, 2005) and domestic cattle (Hollinshead *et al.* 2004b). One dolphin calf had been produced using frozen-thawed, sorted and recryopreserved spermatozoa (FSF; O'Brien *et al.* 2009), but further research is needed in this species to evaluate the *in vitro* quality of spermatozoa after different steps in the cryopreservation/sorting/recryopreservation process. Examination of the *in vitro* quality and the recryopreservation process when

different freezing methods are used would also assist in maximizing the efficient use of spermatozoa from frozen-thawed ejaculates, and determining the number of spermatozoa required at the first cryopreservation step for the subsequent production of a recryopreserved sex-sorted AI dose.

The overall goal of the study was to conduct a comprehensive evaluation of the *in vitro* quality of frozen-thawed dolphin spermatozoa undergoing sorting and recryopreservation using either a straw or a directional freezing (DF) method.

Results

Motility parameters before recryopreservation

Sperm motility parameters were influenced by stage of processing (post-thaw, post-density gradient centrifugation (DGC), post-stain, post-sort, pre-freeze and post-second thaw) sperm type (non-sorted (Control) and sorted (FSF)) and recryopreservation (straw, DF) method ($P < 0.05$). The effect of sperm treatment on motility parameters at various stages of processing following the first thawing step and during flow cytometric sorting is displayed in Table 1. Overall, compared with Control spermatozoa, the processing of FSF resulted in significantly improved motility parameters at the pre-freeze step before recryopreservation (Table 1).

Post-thaw samples from the first cryopreservation retained high proportions of their pre-freeze total sperm motility (TM) and progressive sperm motility (PM) (87.9 ± 7.3 and $92.2 \pm 5.9\%$ respectively). The post-DGC sperm characteristics TM, PM, average pathway velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), straightness of sperm movement (STR) and rapid velocity (RAP) were higher ($P < 0.05$; Table 1) than after the first post-thaw.

Table 1 Motility parameters of bottlenose dolphin spermatozoa after the first post-thaw, during and after flow cytometric sorting, and before freezing (mean $\pm$ s.d., $n = 18$).

Sperm motility characteristics	Processing stage					
	First post-thaw	Post-DGC	Post-stain	Post-sort	Pre-freeze FSF	Pre-freeze control
TM (%)	66.7 $\pm$ 7.0*	89.0 $\pm$ 5.9 [†]	91.0 $\pm$ 4.3 [†]	92.3 $\pm$ 3.2 [†]	83.9 $\pm$ 4.4 [†]	54.1 $\pm$ 13.6 [‡]
PM (%)	55.8 $\pm$ 6.3*	83.0 $\pm$ 4.6 [†]	79.4 $\pm$ 3.6 [†]	90.0 $\pm$ 2.6 [†]	78.8 $\pm$ 3.8 [†]	48.4 $\pm$ 12.9 [§]
VAP (μ m/s)	150.0 $\pm$ 17.0*	196.0 $\pm$ 28.0 [†]	189.1 $\pm$ 18.4 [†]	183.7 $\pm$ 14.7 ^{†,‡}	173.0 $\pm$ 17.9 [†]	149.0 $\pm$ 0.7*
VSL (μ m/s)	138.0 $\pm$ 15.0*	187.0 $\pm$ 28.0 [†]	176.4 $\pm$ 21.1 ^{†,‡}	177.3 $\pm$ 14.5 ^{†,‡}	166.2 $\pm$ 16.5 [†]	141.5 $\pm$ 11.4*
VCL (μ m/s)	197.0 $\pm$ 22.0*	222.0 $\pm$ 24.0 [†]	222.5 $\pm$ 15.9 [†]	238.8 $\pm$ 21.3 [†]	211.1 $\pm$ 26.6 ^{*,†}	182.0 $\pm$ 17.9 [§]
ALH (μ m)	5.3 $\pm$ 0.7 ^{*,‡}	4.3 $\pm$ 0.5 [†]	4.8 $\pm$ 0.6 ^{*,†,‡}	5.5 $\pm$ 0.7 [†]	4.6 $\pm$ 0.7 ^{*,†}	4.2 $\pm$ 0.9 [†]
BCF (Hz)	38.0 $\pm$ 1.2 ^{*,‡}	36.0 $\pm$ 4.6*	36.5 $\pm$ 3.3*	43.9 $\pm$ 1.3 [†]	42.9 $\pm$ 1.4 [†]	40.0 $\pm$ 2.7 [†]
STR (%)	91.0 $\pm$ 2.0*	95.0 $\pm$ 1.0 ^{†,‡}	92.2 $\pm$ 3.1*	96.3 $\pm$ 1.0 [†]	95.8 $\pm$ 0.8 ^{†,‡}	94.0 $\pm$ 2.4 [†]
RAP (%)	64.7 $\pm$ 7.5*	88.4 $\pm$ 6.2 [†]	89.7 $\pm$ 4.4 [†]	92.0 $\pm$ 3.2 [†]	82.7 $\pm$ 4.9 [†]	52.5 $\pm$ 13.6 [‡]
MED (%)	2.2 $\pm$ 1.1*	0.4 $\pm$ 0.7 [†]	1.1 $\pm$ 0.9 ^{†,‡}	0.2 $\pm$ 0.4 [†]	1.1 $\pm$ 0.9 ^{†,‡}	1.4 $\pm$ 0.5 [†]
SLOW (%)	21.5 $\pm$ 5.9*	3.8 $\pm$ 2.4 ^{†,‡}	5.0 $\pm$ 1.2 ^{†,‡}	1.0 $\pm$ 0.1 [†]	7.0 $\pm$ 1.9 [†]	20.2 $\pm$ 7.4*

TM, total motility (%); PM, progressive motility (%); VAP, average pathway velocity (μ m/s); VSL, straight-line velocity (μ m/s); VCL, curvilinear velocity (μ m/s); ALH, amplitude of lateral head displacement (μ m); BCF, beat cross frequency (Hz); STR, straightness of sperm movement (%); RAP, rapid velocity group (%); MED, medium velocity group (%); SLOW, slow velocity group (%). *,†,‡,§ Values with different superscripts within the same row are significantly different ($P < 0.05$). Post-DGC, post-density gradient centrifugation; Post-stain, post-staining with Hoechst 33342 and incubation; Post-sort, post-sorting and centrifugation; Pre-freeze, prior to cryopreservation.

Amplitude of lateral head displacement (ALH), medium velocity (MED) and slow velocity (SLOW) were lower following DGC compared to post-thaw.

With the exception of STR, the process of staining (post-stain) did not significantly affect the motility parameters of spermatozoa selected by the DGC (post-DGC) step (Table 1). Following the sorting process, PM, VCL, beat cross frequency (BCF) and STR were increased compared to those observed post-stain ($P < 0.05$). However, TM, VAP, VSL, ALH, RAP, MED and SLOW remained unchanged between the staining and sorting steps ($P > 0.05$). All post-sort motility parameters except ALH were significantly higher compared to those observed at the first post-thaw step (Table 1).

Pre-freeze characteristics of sorted FSF spermatozoa remained similar to post-DGC, post-stain and post-sort values for TM and MED ($P > 0.05$). FSF spermatozoa PM at the pre-freeze stage was similar ($P > 0.05$) to post-DGC and post-stain, but lower ($P < 0.05$) than post-sort. FSF spermatozoa VAP at the pre-freeze stage was

similar ($P > 0.05$) to post-sort but higher ($P < 0.05$) than post-DGC and post-stain. FSF spermatozoa VSL at the pre-freeze stage was higher ($P < 0.05$) than post-DGC and similar ($P > 0.05$) to post-stain and post-sort. Sorted FSF spermatozoa PM, VCL, ALH and RAP decreased ($P < 0.05$) and SLOW increased ($P < 0.05$) at the pre-freeze stage compared to the post-sort stage. FSF spermatozoa ALH at pre-freeze was similar ($P > 0.05$) to first post-thaw, post-DGC and post-stain, but lower ($P < 0.05$) than post-sort. FSF spermatozoa BCF at pre-freeze was higher ($P < 0.05$) than first post-thaw, post-DGC and post-stain, and similar ($P > 0.05$) to post-sort. FSF spermatozoa STR at pre-freeze was higher ($P > 0.05$) than first post-thaw and post-stain, and similar ($P > 0.05$) to post-DGC and post-sort (Table 1).

Control samples at the pre-freeze step presented a decrease ($P < 0.05$) in the following characteristics when compared to the first post-thaw: TM, PM, VCL, ALH, RAP and MED. At the pre-freeze step, the aforementioned parameters were similarly lower for Control compared to

Figure 1 Total sperm motility (top graph) and progressive sperm motility (bottom graph) of frozen-thawed, non-sorted, refrozen-thawed (Control) bottlenose dolphin spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, refrozen-thawed (FSF) bottlenose dolphin spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. a, b, c, d, 1, 2, 3 Values with different letters are significantly different ($P < 0.05$) across times, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means $\pm$ s.d., $n = 18$.

FSF spermatozoa ($P < 0.05$). However, STR of Control samples was higher ($P < 0.05$) at the pre-freeze compared to the first post-thaw step. The parameters VAP, VSL, BCF and SLOW remained unchanged for Control samples during the first post-thaw and the pre-freeze steps (Table 1).

Motility parameters after recryopreservation and thawing

During the 24 h incubation at room temperature after the second thawing, TM and PM remained unchanged ($P > 0.05$) for FSF and Control spermatozoa frozen with the DF method (Fig. 1). In contrast, FSF and Control spermatozoa frozen with the straw method showed a decrease ($P < 0.05$) in TM and PM after 12 h of incubation.

VAP ($P > 0.05$) for FSF spermatozoa frozen with the DF method presented a decrease ($P < 0.05$) in VAP only at 24 h post-thaw (Fig. 2). For FSF spermatozoa frozen with the straw method, VAP was lower ($P < 0.05$) by 12 h of incubation.

Control spermatozoa frozen with both straw and DF methods underwent decreases ($P < 0.05$) in VAP at 12 and 18 h, and remained unchanged ($P > 0.05$) at 24 h of incubation.

At 18 and 24 h post-thawing, VSL decreased ($P < 0.05$) for FSF spermatozoa frozen with the DF method (Fig. 2). For Control spermatozoa frozen with the DF method, and Control and FSF spermatozoa frozen with the straw method, the decreases in VSL occurred at 12 h and again at 24 h of incubation. The VCL of both sperm types

Figure 2 Average pathway velocity (top graph) and straight line velocity (bottom graph) of frozen-thawed, non-sorted, refrozen-thawed (Control) bottlenose dolphin spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, refrozen-thawed (FSF) bottlenose dolphin spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. a, b, c, d, 1, 2, 3 Values with different letters are significantly different ($P < 0.05$) across times, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means $\pm$ s.d., $n = 18$.

(Control and FSF) frozen with the DF method remained unchanged ($P > 0.05$) across the incubation period (Fig. 3), whereas both sperm types (Control and FSF) frozen with the straw method showed a decrease ($P < 0.05$) in VCL at 24 h post-second thaw.

RAP was similar ($P > 0.05$) throughout the incubation period for Control and FSF spermatozoa frozen with the DF method (Fig. 3). Control spermatozoa frozen with the straw method showed a decrease ($P < 0.05$) in RAP at 12 h post-second thaw, whereas the same sperm type frozen with the DF method did not show a decrease ($P < 0.05$) in RAP during the 24 h incubation.

Overall, FSF spermatozoa frozen with the DF method maintained higher ($P < 0.05$) motility parameters across the 24 h incubation period after the second thawing than all other treatment groups (Figs 1, 2 and 3).

Plasma membrane and acrosome integrity before recryopreservation

Overall, integrity of the plasma membrane and acrosome were influenced by sperm type and stage of processing ($P < 0.05$, Table 2). The effect of sperm treatment on plasma membrane and acrosome integrity at various stages of processing following the first cryopreservation is displayed in Table 2.

Plasma membrane and acrosome integrity after recryopreservation and thawing

The effects of sperm treatment and recryopreservation method on plasma membrane and acrosome integrity during incubation following the second thawing are

Figure 3 Curvilinear velocity (top graph) and rapid velocity (bottom graph) of frozen-thawed, non-sorted, refrozen-thawed (Control) bottlenose dolphin spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, refrozen-thawed (FSF) bottlenose dolphin spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. a, b, c, d, 1, 2, 3 Values with different letters are significantly different ($P < 0.05$) across times, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means $\pm$ S.D., $n = 18$.

Table 2 Plasma membrane status and acrosome integrity of bottlenose dolphin spermatozoa after the first post-thaw, during and after flow cytometric sorting, and before recryopreservation (mean $\pm$ s.d., $n=18$).

Sperm characteristics and staining method	Stage of processing					
	First post-thaw	Post-DGC	Post-stain	Post-sort	Pre-freeze sort	Pre-freeze control
Eosin-nigrosin						
Intact PM (%)	63.4 $\pm$ 8.4*	81.2 $\pm$ 7.7 ^{†,‡}	79.4 $\pm$ 7.5 ^{†,‡}	92.7 $\pm$ 3.2 [§]	84.0 $\pm$ 4.9 [†]	54.8 $\pm$ 10.7
PI/FITC-PNA						
Intact PM, intact acrosome (%)	46.3 $\pm$ 9.1*	87.3 $\pm$ 6.2 [†]	86.3 $\pm$ 5.8 [†]	88.3 $\pm$ 5.9 [†]	80.2 $\pm$ 5.2 [†]	43.2 $\pm$ 8.7*
Intact PM, damaged/reacted acrosome (%)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3
Non-intact PM, intact acrosome (%)	50.1 $\pm$ 9.2*	11.0 $\pm$ 6.3 [†]	13.2 $\pm$ 6.1 ^{†,‡}	10.0 $\pm$ 5.4 [†]	18.9 $\pm$ 4.8 [†]	51.4 $\pm$ 7.6*
Non-intact PM, damaged/reacted acrosome (%)	3.7 $\pm$ 1.6	1.5 $\pm$ 0.5	0.4 $\pm$ 0.7	1.3 $\pm$ 0.9	1.5 $\pm$ 1.3	5.0 $\pm$ 2.9

*,†,‡,§,|| Values with different superscripts within the same row are significantly different ($P<0.05$). PM, plasma membrane; PI/FITC-PNA, propidium iodide and FITC-conjugated *Arachis hypogaea* (peanut) agglutinin staining. Post-DGC, post-density gradient centrifugation; Post-stain, post-staining with Hoechst 33342 and incubation; Post-sort, post-sorting and centrifugation; Pre-freeze, prior to cryopreservation.

displayed in Fig. 4. The percentage of live spermatozoa determined using the eosin-nigrosin stain was correlated ($R^2=0.79$, $P<0.001$) with the percentage of live spermatozoa using propidium iodide (PI, in the

FITC-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA) staining method; Fig. 5).

Plasma membrane integrity for FSF spermatozoa was consistently higher ($P<0.05$) than that of Control

Figure 4 Top graph: bottlenose dolphin spermatozoa with an intact plasma membrane as determined by eosin-nigrosin staining, bottom graph: spermatozoa with an intact plasma membrane and acrosome as determined by PI/FITC-PNA staining. Spermatozoa were assessed before recryopreservation and during a post-thaw incubation for 24 h at room temperature. Values with different letters in superscript row are significantly different ($P<0.05$) across times, and values with different numbers in superscript are significantly different ($P<0.05$) within the same time point. Data are means $\pm$ s.d., $n=18$.

Figure 5 Photomicrographs of bottlenose dolphin spermatozoa after staining with PI/FITC-PNA ($\times 400$ original magnification). Top picture: (a) two spermatozoa with an intact plasma membrane and acrosome (no fluorescence) and (b) one spermatozoon with a non-intact plasma membrane and a damaged/reacted acrosome (red and green fluorescence), bottom picture: (c) two spermatozoa with a non-intact plasma membrane and an intact acrosome (red fluorescence) (d) one spermatozoon with an intact plasma membrane and a damaged/reacted acrosome (green fluorescence).

spermatozoa across the post-thaw incubation period. The percentage of live spermatozoa with a damaged or reacted acrosome was $<2.1\%$ across all treatments.

Sperm DNA denaturation before recryopreservation

Mean_{zt} and COMP_{zt} of sperm samples were similar ($P>0.05$) between the first post-thaw and post-DGC, whereas s.d._{zt} was lower for spermatozoa at post-DGC than at first post-thaw (Fig. 6). Post-stain sperm samples exhibited higher ($P<0.05$) COMP_{zt} and Mean_{zt} than post-DGC, and remained similarly elevated ($P>0.05$) at post-sort and pre-freeze stages (Fig. 6). The s.d._{zt} decreased ($P<0.05$) at post-DGC and remained similar during the post-stain, post-sort and pre-freeze stages (Fig. 6). For pre-freeze samples, Mean_{zt} was similar ($P>0.05$) for both FSF and Control spermatozoa and

s.d._{zt} was higher ($P<0.05$) for Control than FSF samples (Fig. 6). Sorted FSF spermatozoa exhibited higher ($P<0.05$) COMP_{zt} than Control spermatozoa (Fig. 6).

Sperm DNA denaturation after recryopreservation and thawing

Mean_{zt} was similar ($P>0.05$) at 0 h after the second thawing for Control and FSF spermatozoa frozen with the DF and straw methods. After 6 h of incubation, Mean_{zt} remained similar ($P>0.05$) for Control and FSF spermatozoa across all time points (Fig. 7), however, for FSF spermatozoa, Mean_{zt} was consistently lower than that of Control spermatozoa.

Overall, during the 24 h incubation after the second thawing, FSF spermatozoa displayed consistently lower ($P<0.05$) s.d._{zt} and COMP_{zt} than Control spermatozoa, regardless of the freezing method (Fig. 7).

Male effect

While there were no differences in pre-freeze TM and PM among males, significant differences were observed during the incubation period after the second thawing step (data not shown). Overall, male 3 displayed superior TM, PM, and a higher ($P<0.05$) percentage of spermatozoa with an intact plasma membrane and acrosome than those of other males throughout the 24 h incubation period after the second thawing step, for the Control and sex-sorted groups. Despite superior motility, male 3 presented increased ($P<0.05$) COMP_{zt} (at 6, 12, 18 and 24 h post-second thaw) compared to males 1 and 2. Across the 6 to 24 h incubation post-second thaw, COMP_{zt} values were 7 ± 6 , 9 ± 5 and $13 \pm 4\%$, for males 1, 2 and 3 respectively (data pooled across both sperm treatment and each incubation time point).

Discussion

Sex-predetermination has been established in breeding programs of captive populations of bottlenose dolphins using fresh sperm sorting, sperm cryopreservation and AI technologies (O'Brien & Robeck 2006). Integration of sperm sorting using previously cryopreserved semen into such bottlenose dolphin ART programs would allow sorting of banked gametes from deceased animals, or from animals housed for more than 12 h of transportation time away from a semen-sorting facility (O'Brien *et al.* 2009). The present study provides new information on the *in vitro* quality of frozen-thawed, sorted bottlenose dolphin spermatozoa undergoing a second cryopreservation step. In comparison to the conventional straw freezing method, DF was effective in maintaining several motility parameters at superior values over the 24-h period following thawing of recryopreserved sexed spermatozoa. The results

Figure 6 Top graph: Mean_{ol}, middle graph: S.D._{ol} and bottom graph: COMP_{ol} of sperm samples at first thawing, during sorting process and before second recryopreservation of sorted and Control bottlenose dolphin spermatozoa. Post-DGC, post-density gradient centrifugation; Post-stain, post-staining with Hoechst 33342 and incubation; Post-sort, post-sorting and centrifugation; Pre-freeze, prior to cryopreservation. ^a, ^b, ¹, ²Values with different letters are significantly different ($P < 0.05$) across times, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means $\pm$ S.D., $n = 18$.

demonstrate that it is feasible to freeze-thaw, sort, then recryopreserve bottlenose dolphin spermatozoa to obtain good *in vitro* quality for up to 24 h post-second thaw.

Efficient sorting of cryopreserved spermatozoa can only be performed once the frozen-thawed sample is processed by a two-step discontinuous DGC for the removal of cryodiluent components and non-viable spermatozoa, as they interfere with the correct orientation of spermatozoa in the flow cytometer, resulting in lower sorting rates (O'Brien *et al.* 2003, Hollinshead *et al.* 2004a). The improvements in sperm motility parameters (TM, PM, VAP, VSL, VCL, STR, RAP, MED and SLOW) of frozen-thawed bottlenose dolphin spermatozoa following DGC were comparable to those observed in studies with frozen-thawed bull (Hollinshead *et al.* 2004b) and ram (O'Brien *et al.* 2003)

semen. In addition, Maxwell *et al.* (2007) reported a similar increase in TM, VAP, VSL, VCL and ALH in bull sperm samples from the first post-thaw to post-DGC. The DGC processing resulted in the selection of a motile, membrane-intact sperm population able to maintain *in vitro* characteristics after recryopreservation. Notably, frozen-thawed spermatozoa selected by DGC in the present study presented *in vitro* characteristics similar to those seen in fresh-chilled (FC) bottlenose dolphin semen before sorting and cryopreservation (O'Brien & Robeck 2006).

While no direct comparisons were made between the post-thaw quality of sorted dolphin spermatozoa derived from frozen and FC semen, current results show that FSF spermatozoa retained 44% of pre-freeze PM compared with a previous study where sorted spermatozoa from FC semen retained 60% of pre-freeze PM (O'Brien &

Figure 7 Mean_{αt} (top graph), S.D._{αt} (middle graph), and COMP_{αt} (bottom graph) of frozen–thawed, non-sorted, frozen–thawed (Control) bottlenose dolphin spermatozoa using straws and directional freezing (DF), and frozen–thawed, sorted, refrozen–thawed (FSF) bottlenose dolphin spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. a, b, c, 1, 2 Values with different letters are significantly different ($P < 0.05$) across times, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means $\pm$ s.d., $n = 18$.

Robeck 2006). By 6 h of incubation post-thaw, retention of PM by sorted spermatozoa was similar for both studies (FSF: 45%, FC-sorted: 48%, O'Brien & Robeck 2006). The final population of FSF spermatozoa thereby appears to be of high quality as reflected by their sustained longevity *in vitro*. Selection of a superior population of spermatozoa by the combined processes of DGC, sorting and freeze–thawing has also been reported in the sheep (De Graaf *et al.* 2006).

In the present study, FSF spermatozoa frozen with the DF method showed significant superior PM, RAP, across the 24-h post-thaw incubation, and VAP, VCL and VSL from 12 to 24 h post-thaw incubation than Control spermatozoa frozen with the DF method or FSF and Control spermatozoa frozen with the straw method. These results clearly demonstrate that the DF method improves the quality of frozen–thawed semen compared to the straw cryopreservation method. These findings of the beneficial impact of DF on sperm quality are in

agreement with previous studies in the bottlenose dolphin (O'Brien & Robeck 2006), beluga (O'Brien & Robeck 2010), rhinoceros (*Ceratotherium* sp., Reid *et al.* 2009) and killer whale (Robeck *et al.* 2011).

Benefits derived from DF are believed to be due in part to the reduction of mechanical trauma to the sperm membrane during ice crystal formation. This is accomplished through a controlled seeding or nucleation process that results in laminar as opposed to random (straw method) ice crystal formation (Arav *et al.* 2002). In addition, the linear controlled nucleation process eliminates the freezing point plateau typically observed with straw freezing systems, thus reducing the exposure time of sperm cells to potentially harmful supersaturated cryopreservation media (Mazur 1984, O'Brien & Robeck 2006).

Even though TM and PM are clearly indispensable for IVF and *in vivo* fertilization, semen analysis using computer assisted sperm analysis (CASA) equipment

has been shown to provide increased details and improve the accuracy of information collected on sperm motility characteristics. In cattle, several sperm velocity parameters have been shown to be positively associated with fertility rates (Cseh *et al.* 2004, Gillan *et al.* 2008). In the present research, FSF spermatozoa frozen with the DF method displayed higher velocities (VAP, VSL, VCL) at the majority of time points during the 24-h post-thaw incubation than Control spermatozoa frozen with the straw or DF method. Additional AI trials will assist in determining if these positive findings are associated with satisfactory *in vivo* functional capacity of dolphin FSF spermatozoa derived from DF.

In the present study, the percentages of plasma membrane-intact spermatozoa determined by light and fluorescence microscopy techniques were correlated. Simultaneous assessment of plasma membrane and acrosome integrity using the PI/FITC-PNA method, validated herein for use with dolphin spermatozoa, will be useful for future studies on sperm biology in this species. Previous studies have illustrated the robust nature of the bottlenose dolphin acrosome during cryopreservation and sorting processes (Robeck & O'Brien 2004, O'Brien & Robeck 2006). Such findings were confirmed in the present study whereby <5% of spermatozoa, across all treatments, presented a damaged/reacted acrosome.

Though the integrity of sperm DNA is not critical for fertilization of the oocyte, chromatin abnormalities can interfere with further embryo development (Silva & Gadella 2006). In addition to this, severe DNA alterations are related to male infertility in humans (Evenson *et al.* 2002), cattle (Ballachey *et al.* 1988), pigs (Evenson *et al.* 1994) and horses (Love & Kenney 1998). Damage to sperm DNA can be caused by intrinsic factors like protamine deficiency, mutations that compromise DNA packaging, aging, reactive oxygen species and an incomplete process of apoptosis during spermatogenesis (Gatewood *et al.* 1990, Sakkas *et al.* 2003, Singh *et al.* 2003). External factors that result in sperm DNA damage include heat (Paul *et al.* 2008), chemotherapeutics (Delbes *et al.* 2007), radiation (Sailer *et al.* 1995, Aitken *et al.* 2005), pollution (Rubes *et al.* 2005) and age (Singh *et al.* 2003) as well as *in vitro* semen processing factors like type of extender, prolonged incubation time (Krzyzosiak *et al.* 2000) and cryopreservation (Hammadeh *et al.* 2001).

Although DGC did not select dolphin spermatozoa with higher DNA quality than frozen-thawed spermatozoa as observed after DGC of human spermatozoa (O'Connell *et al.* 2003), $s.d._{at}$ was lower for dolphin spermatozoa at post-DGC than at the first post-thaw, indicating that the DGC step selected a sperm population with more homogenous DNA quality than that observed at the first post-thaw.

The effect of sex-sorting on sperm DNA quality has not previously been examined in bottlenose dolphins.

However, processes of sex-sorting including mechanical stress, incubation with Hoechst 33342 (H33342) and 150 mW laser exposure were proved to be safe steps in the sex-sorting of bull spermatozoa, where the damage to the sperm DNA was increased by <3% when compared to Control spermatozoa (Garner 2006).

At the post-stain step in the current study, the samples presented a significant increase in $COMP_{at}$ and $Mean_{at}$ compared to the post-DGC step. In fact, $COMP_{at}$ values after staining and sorting were more than double that observed in the previous step (post-DGC). These findings contrast with those in the bull and boar, where DNA quality was not influenced by the sorting process as assessed by the sperm chromatin structure assay (SCSA; Garner 2006) or the Sperm-Sus-Halomax kit (Chroma-Cell SL, Madrid, Spain; De Ambrogi *et al.* 2006). After sorting, $COMP_{at}$ and $Mean_{at}$ values underwent a decrease between the pre-freeze and 0 h post-thaw steps and continued to decline from 0 to 6 h post-thaw, after which they remained unchanged throughout the rest of the 24-h incubation period. It is accepted that mature spermatozoa lack the ability to repair DNA abnormalities (Genesca *et al.* 1992, Daudoune 2003). Thus, the unusual trend in SCSA variables exhibited by FSF spermatozoa, whereby the proportion of cells with high amounts of denatured DNA was observed to increase and then decrease, suggests that processing of the sperm samples for sex-sorting introduced an artifact during the SCSA.

Acridine orange (AO) forms complexes with ds and ss DNA by intercalating between base pairs, whereas H33342 binds to base pairs in the minor groove of ds DNA (Kapusinski 1990). Though some H33342 will diffuse out of the cell via a concentration gradient during the sorting and post-sorting processes, some H33342 still remains in the cells as demonstrated by the fluorescence of IVF embryos derived from sorted spermatozoa (Garner 2009). In the present study, the amount of AO bound to ds (normal) DNA may have been artificially decreased due to H33342 interference, whereas the extent of AO intercalation between base pairs of ss (abnormal) DNA was not affected, because H33342 only binds to ds DNA. Though it is not clear why $Mean_{at}$ values were similar for all treatments at the pre-freeze stage, it appears that some aspect(s) of chromatin staining of stained or sorted spermatozoa during the SCSA results in an artificially elevated proportion of spermatozoa with denatured DNA. The hypothesized interference of AO staining in sorted spermatozoa lasts as long as the H33342 is present in the sperm nucleus, and it potentially explains the gradual 'normalization' of $COMP_{at}$ values as the H33342 diffused out of the cells during the pre-freeze and post-thaw dilution and incubation periods.

Although further research is necessary to understand the reasons for the possible artifact found herein, and to determine why this has not been reported in other

species where sorted spermatozoa have been examined using the SCSA (Garner 2006), this is the first comprehensive study on the use of the SCSA as an *in vitro* indicator of sperm quality in a marine mammal species. Spermatozoa from the three bottlenose dolphins in the current study displayed a low susceptibility to DNA denaturation after one freeze–thaw cycle (COMP_{dt} : $15 \pm 3\%$), and after the combined treatment of two freeze–thaw cycles, sex-sorting and a 24-h incubation after the second thawing step (COMP_{dt} : $5 \pm 3\%$). These results are comparable to that reported for fresh semen from highly fertile stallions (COMP_{dt} : $13 \pm 6\%$, Love & Kenney 1998). It is very likely that a robust and reliable assay such as the SCSA will be incorporated in further research on bottlenose dolphin spermatozoa as well as other marine mammal species.

Based on initial results from FSF trials, it was estimated that 35 h of sorting time of frozen–thawed spermatozoa were required to produce an AI dose of 200×10^6 progressively motile spermatozoa using one flow cytometer (O'Brien *et al.* 2009). However, with the improved semen processing techniques described herein, the recent acquisition and application of a dual flow cytometer system and the development of a low AI dose methodology (50×10^6 progressively motile spermatozoa, Robeck *et al.* 2010), the time required for an FSF AI dose can be decreased to ~ 4 – 5 h. Although AI trials are needed to ensure that this dose is adequate to achieve the desired conception rate of 50%, this increased efficiency of sex-sorting FSF sperm should allow for the expansion of these techniques to help maintain the diversity and encourage the expansion of small *ex situ* endangered or non-endangered cetacean populations. For example, only 10% of the 119 animals estimated in the world's *ex-situ* Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) population are housed in American aquaria, and the majority of calves from natural breeding are derived from only one male (Robeck *et al.* 2009). Sex-sorting of cryopreserved Pacific white-sided dolphin spermatozoa from other countries combined with the recently developed ART techniques for this species (Robeck *et al.* 2009) would allow a faster increase of the American female population, assuring the existence of an *ex-situ* population in American aquaria and developing a model for the restoration of any cetacean species that may become endangered in the future.

The present study demonstrated that the characteristics of bottlenose dolphin spermatozoa undergoing cryopreservation using DF, sorting and recryopreservation were well maintained *in vitro*, and that such samples are of suitable quality for AI. The successful birth of a bottlenose dolphin calf using sorted, frozen–thawed spermatozoa from previously cryopreserved semen (O'Brien *et al.* 2009), and the high *in vitro* quality of these samples, as confirmed herein with semen from multiple males, validate the feasibility of incorporating

frozen semen into sex-selection AI programs for world-wide management of *ex situ* bottlenose dolphin populations.

Materials and Methods

Experimental design and animals

Three cryopreserved ejaculates from each of the three males were used to compare the effects of sex sorting (FSF and Control – non-sorted) and two recryopreservation methods (conventional and DF) on *in vitro* sperm characteristics (sperm motility, sperm membrane status, sperm acrosome integrity and DNA quality). *In vitro* sperm characteristics were determined using aliquots of sperm suspensions from both recryopreservation methods and sorting method (FSF and Control). Aliquots were obtained from different steps of the sorting process and at fixed times following the second thawing step (0, 6, 12, 18 and 24 h post-second thaw).

Three proven breeding male bottlenose dolphins (Males 1–3; aged 16–21 years in 2009 and weighing 224–230 kg) housed at SeaWorld San Diego (San Diego, CA, USA) were used for semen collection. The dolphins were housed together in an outdoor pool holding 850 m³ of natural processed saltwater at an ambient water temperature of 18–20 °C during the study. Animals were fed a diet of frozen–thawed whole fish (herring, *Clupea harengus*, and Columbia River smelt, *Thaleichthys pacificus*) at ~ 4 – 5% of their body weight per day.

Ethics of experimentation

Semen samples were collected using routine husbandry training and were obtained from unrestrained animals. All procedures described herein were reviewed and approved by the SeaWorld Incorporated Institutional Animal Care and Use Committee, and were performed in accordance with the Animal Welfare Act for the care of Marine Mammals.

Evaluation of semen and *in vitro* sperm characteristics

Males were trained for unrestrained ejaculation as previously described (Keller 1986, Robeck & O'Brien 2004). Ejaculate osmolality, pH, concentration and volume were determined using standardized techniques (Robeck & O'Brien 2004, Robeck *et al.* 2005). Ejaculates were used if PM was $> 85\%$ and osmolality was within the normal range for bottlenose dolphins (325–345 mOsm/kg, Robeck & O'Brien, unpublished data). Ejaculates of low sperm concentration ($< 600 \times 10^6$ spermatozoa/ml) were centrifuged (600 g, 10 min) and an appropriate volume of supernatant was removed to obtain a concentration of 600×10^6 spermatozoa/ml. For ejaculates of high sperm concentration, Part A extender (TYB; modified from Graham *et al.* 1972; 176 mM TES, 80 mM Trizma base (Tris), 9 mM fructose, 50 µg/ml gentamicin sulfate, 20% (v/v) egg yolk, 325–345 mOsm/kg, pH 7.3 ± 0.1 ; O'Brien & Robeck 2006) was added to raw, non-centrifuged semen in an appropriate volume to obtain the same final concentration of 600×10^6 spermatozoa/ml. Part A was added to semen (1:1, v/v) over a 2–3-min period at room temperature.

Motility parameters were evaluated using CASA (Hamilton-Thorne, HTM-IVOS Version 12.2; Holt *et al.* 2007) in a manner similar to that previously described for beluga (*Delphinapterus leucas*; O'Brien *et al.* 2008) and killer whales (*Orcinus orca*; Robeck *et al.* 2011). Semen was diluted with Androhep Enduraguard (AE; Minitube of America, Verona, WI, USA) to a concentration of $15\text{--}25 \times 10^6$ spermatozoa/ml. A total of five to ten microscopic fields representing a minimum of 200 spermatozoa were randomly selected per sample for the calculation of the following motility parameters: VAP ($\mu\text{m/s}$), VSL ($\mu\text{m/s}$), VCL ($\mu\text{m/s}$), ALH (μm), BCF (Hz), STR (%; VSL/VAP), TM (%; VAP > 20 μm) and PM (%; VAP > 50 $\mu\text{m/s}$ and STR > 80%). Preset values for the instrument consisted of the following: 30 frames at a frame rate of 60 frames/s, minimum contrast of 80, minimum cell size (pixels) of five. Three additional groups based on velocity of movement were also determined: RAP (VAP > 50 $\mu\text{m/s}$, %), MED (20 $\mu\text{m/s}$ < VAP < 50 $\mu\text{m/s}$, %) and SLOW (VAP < 20 $\mu\text{m/s}$, %).

Sperm membrane status was determined for raw ejaculates using eosin–nigrosin staining (Robeck & O'Brien 2004). For all other samples, membrane status and acrosome integrity were measured simultaneously using the stains PI (Sigma 287075, Sigma–Aldrich) and FITC-PNA (Sigma L-7381, Sigma–Aldrich; Graham *et al.* 1990, Pena *et al.* 2001). The PI/FITC-PNA staining method used herein was a combination of different dual staining protocols (Graham *et al.* 1990, Szasz *et al.* 2000, Pena *et al.* 2001), as previously described for killer whales (Robeck *et al.* 2011). Briefly, a 12.5- μl aliquot of sperm sample was transferred to a foil-covered microcentrifuge tube and mixed with 1 μl of PI (12.5 mg/ml working stock in PBS). After 30 s, 1 μl of FITC-PNA (1 mg/ml working stock in PBS) was added to the solution and incubated for 1 min. Spermatozoa were then immobilized and fixed by the addition of 1 μl of 2% glutaraldehyde solution (in PBS, pH 7.0–7.4) and 12 μl was placed on a glass slide and covered with a 22 $\times$ 22-mm glass coverslip for evaluation within 5 min. Spermatozoa were observed using a fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a 450–490 nm band pass excitation filter and a 535 nm emission filter. Evaluations were conducted at 400 $\times$ magnification under a low bright field setting to permit visualization of non-fluorescent spermatozoa. A total of 100 cells were classified per sample using the following staining patterns: no stain (intact plasma membrane and an intact acrosome), green staining in the acrosome region including the equatorial segment (intact plasma membrane and a damaged or reacted acrosome), red staining (non-intact plasma membrane and an intact acrosome), red and green staining (non-intact plasma membrane with a damaged or reacted acrosome). The dual staining method was validated for dolphin spermatozoa by observing the fluorescent labeling of samples containing non-viable (killed) and purportedly acrosome damaged spermatozoa. Killed, damaged spermatozoa were obtained by two cycles of snap-freezing in liquid nitrogen and thawing at room temperature. Spermatozoa were snap-frozen in AE (Minitube of America) without cryoprotectant, and > 95% of cells were classified as plasma membrane non-intact and acrosome damaged/ reacted after the second thawing.

An aliquot was frozen at each evaluation step and retrospectively analyzed after thawing for susceptibility of

spermatozoa to DNA denaturation using the SCSA (Love & Kenney 1998, Evenson *et al.* 2002). Individual samples were thawed in a water bath at 38 °C for 15–30 s. An aliquot of 5–20 μl of semen was mixed with 180–195 μl of a TRIS buffer (1 mM disodium EDTA, 0.01 M Tris–HCl, 0.15 M NaCl in 500 ml deionized water, pH 7.4) to a final volume of 200 μl M. Four hundred microliters of acid-detergent solution (0.08 N HCl, 0.1% Triton X-100 (Sigma–Aldrich), 0.15 M NaCl) were immediately added and after 30 s the solution was quenched with 1.2 ml of an AO solution (0.1 M citric acid monohydrate, 0.2 M Na_2HPO_4 , 0.15 M NaCl, 1 mM disodium EDTA, 4 $\mu\text{g/ml}$ AO (Polysciences, Inc., Warrington, PA, USA), pH 6). The samples were immediately placed into the flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) and allowed to equilibrate for 30 s before data acquisition. The samples, tubes and reagents were kept on ice during processing. Sample volume varied to accommodate a flow rate of 100–200 cells/s. Five thousand events were accumulated per sample. Flow cytometer settings were calibrated using spermatozoa from a known fertile bottlenose dolphin. Using this Control sample, settings were adjusted so that mean green fluorescence was at 500 channels (FL-1@500) and mean red fluorescence at 150 channels (FL-3@150). Data were recorded and stored in List Mode and SCSA values were calculated using WinList software (Verity Software House, Topsham, ME, USA). Quantification of DNA denaturation in each spermatozoon was determined by the term alpha-t (αt), the ratio of red/(red + green fluorescence) for each individual spermatozoon analyzed. Alpha-t (αt) describes the relationship between the amounts of green (double-stranded DNA) and red (single-stranded DNA) fluorescence. Endpoints included the percentage of Cells Outside the Main Population (%COMP- αt), Mean- αt and S.D.- αt . The COMP- αt was determined by selecting those spermatozoa located to the right of the control main population, and represents a percentage of the total number of spermatozoa with denatured DNA.

Semen processing, cryopreservation and sorting

Cryopreservation of fresh ejaculates using directional solidification

Semen was diluted (1:1, v/v) with Part A extender (TYB; modified from Graham *et al.* 1972); 176 mM TES, 80 mM Trizma base (Tris), 9 mM fructose, 50 $\mu\text{g/ml}$ gentamicin sulfate, 20% (v/v) egg yolk, 330 ± 5 mOsm/kg, pH 7.3 ± 0.1 ; O'Brien & Robeck 2006) over a 2–3-min period at room temperature. The sperm suspension was placed in a container with 200 ml of room temperature water and cooled to 5 °C over a 1.5–2-h period (-0.2 °C/min), and then diluted with Part B extender (2:1, semen:Part B) previously cooled to 5 °C in a stepwise manner over 30 min (three steps of 25, 25 and 50% volume) to obtain a final concentration of $200\text{--}300 \times 10^6$ spermatozoa/ml and 3% glycerol. One hour after the last addition of Part B, the sperm suspension was transferred to 9-ml hollow glass tubes (at 5 °C, IMT International, Chester, UK) for cryopreservation using a directional solidification machine (MTG-516, IMT, Ness, Ziona, Israel) as described previously for dolphins (O'Brien & Robeck 2006, Robeck *et al.* 2009), beluga (O'Brien & Robeck 2010) and

killer whales (Robeck *et al.* 2011). Briefly, the hollow tubes moved through the first block of the machine (5 °C) for 45 s at a constant velocity (1 mm/s) before reaching a 2-mm distance inside of the second block (−50 °C), where it was held for 60 s for initiation of ice crystal formation (rapid induction of ice nucleation from the seeding point throughout the length of the glass tube). The tubes were then moved at the same velocity across the second block for 3 min before entering the collection chamber (−100 to −110 °C) followed by transfer to liquid nitrogen.

Thawing and preparation of frozen-thawed spermatozoa before sorting

Hollow tubes were held in air for 45 s, and then transferred to a 35 °C water bath equipped with modifications to enable uniform sample thawing over 90 s (Harmony CryoCare Activator; IMT International). Frozen-thawed semen was divided into two 50-ml polystyrene tubes (BD Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) used for non-sorted and sorted spermatozoa. Polystyrene tubes (BD Falcon, 15 ml) were prepared with 2 ml of 45% density gradient medium (DGM) and 2.5 ml of 90% DGM. DGM was a Percoll-based (Percoll Plus, GE Healthcare Biosciences, Pittsburgh, PA) in-house preparation (330 ± 5 mOsm/kg, pH 7.2–7.4). The 45 and 90% DGM solutions were prepared by dilution of 100% DGM with staining medium. Staining medium was a Tyrode's salt solution containing BSA (0.3%, v/v; Sigma A-4697, Sigma-Aldrich), lactate, pyruvate and HEPES (HEPES-TALP staining medium; modified from Parrish *et al.* 1986) supplemented with gentamycin (50 µg/ml), 330 ± 5 mOsm/kg and pH 7.3 ± 0.1 (O'Brien & Robeck 2006).

Four milliliters of semen were placed over the gradient and the tubes were centrifuged at 800 *g* for 20 min. The sperm pellets were aspirated, combined and resuspended with staining medium supplemented with 4% egg yolk (1:3 v/v, sperm sample: staining medium). The sample was filtered (35 µm; CellTrics, Görlitz, Deutschland) into another 15-ml tube (polystyrene, BD Falcon), centrifuged (800 *g* for 10 min) and the new pellet was then resuspended with the same volume of fresh staining medium that was removed post-centrifugation.

Sorting of frozen-thawed spermatozoa

Sperm suspensions from each male were diluted to 200×10^6 spermatozoa/ml with staining medium and incubated with H33342 (Sigma-Aldrich; 89–107 µM) as previously described (O'Brien & Robeck 2006). A high-speed flow cytometer (SX MoFlo, Dako Colorado, Inc., Fort Collins, CO, USA) modified for sperm sorting (Rens *et al.* 1998, Johnson & Welch 1999, Sharpe & Evans 2009) operated at 207 kPa (30 psi) was used to analyze and sort spermatozoa. Spermatozoa were sorted into 3 ml of Part A (with homologous 1% seminal plasma, v/v, prepared as previously described; O'Brien & Robeck 2006) for a maximum of 2 h, and then centrifuged at 850 *g* for 25 min at room temperature. The supernatant was removed and the pellet was resuspended with the appropriate volume of Part A (containing 1% of seminal plasma, v/v) to a final concentration of 30×10^6 spermatozoa/ml. An aliquot of 0.1×10^6 sorted spermatozoa was re-stained (9 µM H33342), sonicated and

re-analyzed by the flow cytometer in order to determine the proportions of X- and Y-bearing spermatozoa (Welch & Johnson 1999).

Recryopreservation of non-sorted and sorted spermatozoa using a conventional straw method or DF

After thawing, the non-sorted sperm suspension was diluted with Part A extender at room temperature to obtain a final concentration of 100×10^6 spermatozoa/ml. Part A contained 1% (v/v) seminal plasma and 25% (v/v) sheath fluid (as used during sperm sorting). Non-sorted and sorted sperm suspensions were cooled to 5 °C over a 1.5–2-h period (−0.2 °C/min), and then diluted with Part B extender in a stepwise manner as previously described herein to achieve a final concentration of 1.5% glycerol and 50×10^6 spermatozoa (non-sorted) or 15×10^6 spermatozoa/ml (sorted). All samples were then equilibrated for 1 h.

Straws (0.25 ml, Minitube of America) were filled with spermatozoa, sealed with pre-cooled metal sealing balls (Minitube of America), placed on a block of dry ice for 10 min and then transferred to liquid nitrogen. For the DF treatment groups, spermatozoa were frozen as described for fresh ejaculates except that 2-ml hollow tubes were used (instead of 9-ml), and the tubes were held for 30 s (instead of 60 s) to permit seeding. After entering the collection chamber, the tubes were transferred to liquid nitrogen.

Thawing and dilution of sperm samples after recryopreservation

Straws were thawed in a water bath at 35 °C for 20 s. Small hollow tubes (2 ml) were thawed in air for 45 s, and then transferred to a 35 °C water bath equipped with modifications to enable uniform sample thawing over 45 s (Harmony CryoCare Activator; IMT International). Large hollow tubes were thawed in air for 90 s, and then placed at 35 °C in the equipped water bath for 50 s. Thawed samples were transferred to microcentrifuge tubes (Eppendorf, Westbury, NY, USA) and diluted (1:0.1, v/v) with AE (Minitube of America).

Statistical analyses

Data for sperm motility parameters, plasma membrane integrity, acrosome integrity and sperm chromatin integrity across the different time points were analyzed using repeated-measures ANOVA (RMANOVA, SigmaStat, Version 3.5, SSPS, Inc., San Rafael, CA, USA). All pairwise multiple comparison procedures between means were conducted using the Student–Newman–Keuls (SNK) test. The relationship between eosin–nigrosin and PI membrane integrity methods was examined using the Pearson Product Moment Correlation Test. $P < 0.05$ was considered significant. Data are presented as mean $\pm$ s.d.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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