IN VITRO FUNCTION OF FROZEN-THAWED BOTTLENOSE DOLPHIN (*Tursiops truncatus*) SPERMATOZOA UNDERGOING SORTING AND RECRYOPRESERVATION

A Thesis

by

GISELE ANGELICA MONTANO PEDROSO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Animal Science

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Spermatozoa Undergoing Sorting and Recryopreservation

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Co-Chairs of Committee,	Duane C. Kraemer
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ABSTRACT

In Vitro Function of Frozen-Thawed Bottlenose Dolphin (*Tursiops truncatus*) Spermatozoa Undergoing Sorting and Recyopreservation. (December 2010)

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Co-Chairs of Advisory Committee: Dr. Duane C. Kraemer
Dr. Justine K. O'Brien

Artificial insemination (AI) with sex-sorted bottlenose dolphin spermatozoa provides female calves for obtaining more cohesive social groups and optimum genetic management of captive populations. However, distance of animals to the sorting facility represents a limit to the procedure. Although one bottlenose dolphin calf has been born using spermatozoa from frozen-thawed, sorted and recryopreserved spermatozoa, critical evaluation of the steps involved in this process is required to maximize its efficiency for future AIs and expansion of the technology to other species.

Two experiments were designed to determine the efficiency of the sorting process and the quality of frozen-thawed bottlenose dolphin spermatozoa during sorting and recryopreservation. In experiment 1, the effect of two washing media (with and without 4% egg yolk, v/v) following density gradient centrifugation (DGC) on sperm recovery rate and in vitro characteristics of cryopreserved spermatozoa was examined. In experiment 2, cryopreserved semen was used to compare the effects of two recryopreservation methods (conventional straw freezing and directional freezing) on in vitro sperm characteristics of control (non-sorted) and sorted spermatozoa. Egg yolk supplementation of the washing medium in experiment 1 did not influence ($P > 0.05$) the

sperm recovery rate, however, sperm motility parameters and viability were improved ($P < 0.05$). For Experiment 2, motility parameters and viability were influenced by stage of sex-sorting process, sperm type (non-sorted and sorted) and freezing method ($P < 0.05$). After recryopreservation, sorted spermatozoa frozen with the directional freezing method maintained higher ($P < 0.05$) motility parameters over the 24 h incubation period compared to spermatozoa frozen using straws. Quality of sperm DNA of non-sorted spermatozoa, as assessed by the SCSA, remained unchanged throughout the process. However, a possible interaction between Hoechst 33342 and acridine orange was observed in sorted samples. After recryopreservation, viability of sorted spermatozoa was higher ($P < 0.05$) than that of non-sorted spermatozoa across all time points. The percentages of viable spermatozoa determined by light (eosin-nigrosin) and fluorescence microscopy (propidium iodide) techniques were correlated ($R^2=0.79$, $P < 0.001$).

Collective results indicate that bottlenose dolphin spermatozoa undergoing cryopreservation, sorting and recryopreservation are of adequate quality for use in AI.

DEDICATION

I dedicate this work to the most important women in my life, my mom and my sister, for the years we spent together in family and the obstacles we overcame.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and co-chair, Dr. Kraemer and Dr. Justine O'Brien, my committee members, Dr. Charles C. Love for their guidance and support throughout the course of this research. Dr. Kraemer, thank you for the great opportunity you gave me as being your student, thank you for your time, patience and support. Dr. O'Brien, thank you so much for your excellent guidance, patience and friendship. Dr. Love, thank you for your support and helpful suggestions.

Special thanks go to my friends Mariana, Fer, Maribel, Wicha, Ismael and Adrián, whose great friendship across the distance made this work possible.

I thank my colleagues at the RSL and staff for making my time at Texas A&M University a great experience. I also thank Brad Andrews from SeaWorld Inc. for the support and for giving me the opportunity to participate in projects of the company; Karen J. Steinman and Michelle Morrisseau from SeaWorld and Busch Gardens Reproductive Research Center for the help on sperm evaluations during late hours at night, patience, friendship and for making the lab a wonderful place to work.

For sharing with me the wonderful world of marine mammals and accepting me as a student and friend, I thank Dr. Cristian Ugaz and Dr. Jose Luis Solórzano.

I want to thank Daniela Missano for believing in me and supporting me when I lost a job for going to a conference that changed my life, where I met my husband-to-be and when the concretization of a dream started. Important people who were always by my side should be thanked too. They gave me support, help, unconditional friendship and without them I wouldn't be who I am: Brisa, Eric, Otavio, Leila, Irma, Angelica and Karina.

Gaby Foxworth, thank you for your support and the endless hours of conversation. I will really miss you!

Todd, thank you for opening the doors of a new life for me, for your support and endless comforting words during difficult moments. I feel very lucky and flattered to have your company in this new path of my life.

Matthew and Alex, thank you for teaching me so much, every day I learn something wonderful about you both that I hadn't realized before. Thank you for your patience and for accepting me in your life.

I also want to extend my gratitude to the Conacyt (Mexico), for providing the funds and making possible this work and the work of many students.

Finally, thanks to my father, for a great example of a scientist and person, to my brother, who always supported and loved me, to my mother and my sister for being an example of strength and courage.

Everyone mentioned here contributed in some degree to make this work possible. I feel blessed to have had such amazing people around me.

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INTRODUCTION

Although, the bottlenose dolphin (*Tursiops truncatus*) is not reported in the Red List of the IUCN (International Union for Conservation of Nature), it 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 [1]. These limitations, which were originally designed to protect susceptible species from exploitation, combined with country specific regulations targeted toward marine mammals such as the Marine Mammal Protection Act of 1972 (MMPA; 16 U.S.C. 1361 *et seq*.), have created artificial barriers for international animal exchange. As a result, breeding and genetic management of captive bottlenose dolphin populations is usually limited to individual companies within a country. An example of a small cetacean population in captivity in the United States is the Pacific white-sided dolphin (*Lagenorhyncus obliquidens*). Only ten per cent of the estimated 119 animals belong to American aquaria and the majority of the natural breeding has been conducted by one male [2]. For the Pacific white-sided dolphins and other zoological cetacean populations, inter-facility animal exchange could temporarily relieve genetic bottlenecks within organizations. However, variations among inter-institutional management policies, animal and facility location, and behavioral factors related to the integration of new animals into the existing colony have largely prevented these animal exchanges from occurring [3]. In addition, transportation of animals across long distances can be expensive and potentially dangerous for the animals [3]. The ability to apply assisted reproductive technologies (ART) such as semen cryopreservation and AI can reduce or eliminate animal transportation for reproductive reasons, and can provide a modern tool for the genetic management of captive bottlenose dolphin populations across nations and international borders.

Sex pre-selection of spermatozoa using flow cytometric techniques [4] combined with AI offers an unprecedented ability to optimize population genetics and sex ratio simultaneously. These techniques are invaluable for species management in captivity where limited population size requires female skewed sex ratios fchaor maximum

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This thesis follows the style of Theriogenology.

reproductive rates. In the wild, bottlenose dolphin adult males form small groups that typically associate with females only during the breeding season [5,6]. Therefore, a population in captivity with more females than males maintains similarities to wild groups, resulting in a more normal social population that reduces inter-male competition and potential associated injuries.

Production of sexed spermatozoa from fresh semen has been successful in dolphins. However, it is limited by the distance of the sorting facility to the source of the gametes (sperm samples should undergo sorting after a 12 to 18 h transport period [7]). Thus, widespread application of this technology could occur only with the construction of multiple sorting facilities around the world. An alternative strategy would be to develop adequate methods for the sorting and cryopreservation of previously cryopreserved spermatozoa. In this case, semen could be cryobanked and shipped frozen from any location to the sorting facility where it would undergo a second cryopreservation process following sorting. The method of sorting cryopreserved spermatozoa was first established in sheep [8,9] and then adapted for sorting of frozen-thawed spermatozoa from humans [10], non-human primates [8;10]) and domestic cattle [11]. One dolphin calf has been produced using frozen-thawed, sorted and recryopreserved spermatozoa [7], but efforts are required to ensure that both the efficiency of use of spermatozoa and sorted sperm quality are maximized for this species. It is estimated that sperm losses during sorting and cryopreservation processes can exceed 30%, depending upon the species and the number of cryopreservation processes performed; in the case of dolphins, less than 5% of spermatozoa originating from one ejaculate can be obtained after the frozen/thawed-sexed-frozen/thawed cycle [7].

The first step where a significant loss of spermatozoa occurs is after the first post-thaw. Non-viable (dead) spermatozoa and cryoprotectants must be removed before staining and sorting, as they interfere with the correct orientation of spermatozoa in the flow cytometer, resulting in lower sorting rates [8,9]. In order to identify the best method for cryodiluent removal and the efficient recovery of viable, progressively motile spermatozoa for sorting, three pilot trials were performed prior to the two experiments. Pilot trials were conducted to identify the most appropriate volume of thawed semen for

density gradient centrifugation, tube size for centrifugation, and sperm washing medium.

In this study, it is hypothesized that egg yolk, included in the staining medium, improves in vitro characteristics of dolphin spermatozoa after the DGC step. A second hypothesis is that the sorting and recryopreservation processes significantly affect in vitro sperm characteristics. During the processing of frozen-thawed spermatozoa for sorting and recryopreservation, cells must undergo cryopreservation using a directional freezing method, thawing, staining with Hoechst 33342, flow cytometric sorting, centrifugation, and recryopreservation/thawing.

Experiment 1 was conducted to test if egg yolk, as one of the components of stain medium, could improve the recovery rate and sperm characteristics after density gradient centrifugation by protecting the cell membranes without compromising the staining process and subsequent sorting parameters. The following in vitro assays were used: (i) computer assisted sperm analysis of motility, (ii) plasma membrane integrity (viability) using light microscopy (eosin-nigrosin staining) and (iii) simultaneous assessment of viability and acrosome integrity using fluorescence microscopy (propidium iodide and FITC-PNA staining).

In an effort to identify the main steps that cause damage to sperm cells, Experiment 2 was performed to evaluate in vitro characteristics of spermatozoa throughout different steps of the cryopreservation and sorting processes, with comparison to control (non-sorted) spermatozoa undergoing only recryopreservation and thawing. The following in vitro assays were used at fixed time points during the processing of control and sorted spermatozoa, prior to the second cryopreservation: (i) computer assisted sperm analysis of motility parameters, (ii) plasma membrane integrity (viability) using light microscopy (eosin-nigrosin staining), (iii) simultaneous assessment of viability and acrosome integrity using fluorescence microscopy (propidium iodide and FITC-PNA staining) and (iv) DNA denaturation using the flow cytometric-based sperm chromatin structure assay (SCSA). For the purpose of determining the in vitro longevity of spermatozoa post-thawing, the same assays were used at fixed times, over a 24 h period, following the second thawing step.

The overall goal of this research was to optimize methodologies for the sorting and recryopreservation of frozen-thawed bottlenose dolphin spermatozoa, and to use

such methodologies as a base for future research in other wildlife species. The specific objective of this study was to maximize both the recovery rate and the in vitro quality of frozen-thawed spermatozoa during sorting and recryopreservation to facilitate the efficient production of functional spermatozoa for future use in AI.

OBJECTIVES

The objective of this study was to improve methodologies for the sorting and re-cryopreservation of frozen-thawed bottlenose dolphin spermatozoa. Specifically, experiments were designed to determine in vitro quality of dolphin spermatozoa after: density gradient centrifugation, staining with Hoechst 33342, sorting and centrifugation, cooling and equilibration with the cryodiluent, and at fixed times post-thaw (0 h, 6 h,12 h,18 h and 24 h).

The overall goal of the study was to identify the steps that cause the most damage to spermatozoa during processes required for the sorting and recryopreservation of frozen-thawed spermatozoa, and thereby allow for the development of modifications to the methods which will result in an increased availability of sorted spermatozoa for future inseminations.

LITERATURE REVIEW

Male reproduction

In accordance with male reproductive anatomical descriptions of bottlenose dolphins, the genital slit, which contains the penis in the male and vaginal opening in the female, is cranial to and separated from the anal orifice. The fibroelastic penis, which has a sigmoid flexure that can be retracted with the aid of the retractor penis muscle, is retained in an intra-abdominal position, lying within the body wall, caudally to the kidneys [12,13]. The penis can be extended voluntarily from the genital slit during erection with the aid of the retractor penis muscles [3].

Sexual maturity in males typically occurs from 9 to 13 years of age [14], although one male has sired an offspring at 5 years of age [15]. Serum testosterone concentrations increase during puberty from less than 1 ng/mL to greater than 10 ng/mL [14]. However, data from ultrasonographic examinations of testes indicate that the degree of echogenicity of testicular tissue may provide a more reliable indicator of maturation than testosterone concentrations [16]. As evidence, Brook et al, [16] described a male which had sonographically compatible adult testes (relative to size and echogenicity) while simultaneously having testosterone levels below 1 ng/mL.

Although the effect of seasonality on male reproduction requires more investigation, it is evident that in some bottlenose dolphins, seasonality is a primary factor responsible for variations in testosterone concentrations and breeding activity [17]. As an example of seasonal trends in sperm production, several bottlenose dolphin males housed at SeaWorld San Diego (CA, USA) produce ejaculates with significantly higher numbers of spermatozoa during the spring and summer months compared to fall and winter months [O'Brien JK and Robeck TR, unpublished data]. Other factors like presence or absence of cycling females or other males, hierarchy, group size, environment and nutrition can also be involved in the control of spermatogenesis in bottlenose dolphins. Thus, in a managed population, fecundity and ejaculate quality can improve during predictable periods of the year [14,18].

Artificial insemination

The application of ART, specifically AI, to marine mammals has been successful in an increasing number of cetacean species, such as the bottlenose dolphin [6,19], Pacific white-sided dolphin [2], killer whale (*Orcinus orca*) [20] and beluga (*Delphinapterus leucas*) [21,22].

Artificial insemination was developed in the bottlenose dolphin after basic research had been undertaken to characterize the species' reproductive physiology. Longitudinal studies enabled prediction of ovulation and characterization of female reproductive cycles through transabdominal ovary ultrasound [23] and urinary hormone monitoring [19]. Optimum methods for sperm cryopreservation were also devised [13] followed by the development of a hysteroscopic insemination technique culminating in the production of calves [19].

The ability to predict the timing of ovulation in relation to the LH surge (i.e. 9.4 ± 3 h from LH surge onset to LH peak, 24.3 ± 7 h from LH peak to ovulation [19]) led to the successful development of hysteroscopic inseminations performed once within hours prior to ovulation with an AI dose as low as 270 $x10^6$ progressively motile spermatozoa [19]. In a subsequent study with sexed spermatozoa, O'Brien and Robeck [6] achieved pregnancies using a lower dose of 150 $x10^6$ progressively motile sexed frozen-thawed spermatozoa. The number of progressively motile sexed spermatozoa that constitutes a bottlenose dolphin AI dose is considerably high compared with sexed AI doses for cattle (1 $x10^6$) [7,24] or horses (25 $x10^6$) [25]. However, recent research in bottlenose dolphins on deep intrauterine inseminations with low doses (≤ 50 $x10^6$) of motile sexed spermatozoa is ongoing and already yielding positive results (2/4 pregnancies) [26].

Semen cryopreservation

Bottlenose dolphins produce high quality spermatozoa with motility, viability, normal acrosome and normal morphology reported to exceed 80% [6,13,19].

The first method used to cryopreserve semen of dolphins was pellets on dry ice using a canine cryodiluent comprised of egg yolk, lactose and glycerol [27]. Methods were later developed for cryopreservation of semen in straws using a diluent that contained egg yolk, buffers (TES, Tris), glucose and gentamicin (Test Yolk buffer, TYB) supplemented with glycerol [13]. In that study, high levels of initial sperm characteristics were maintained (0 h post-thaw, 0.5 mL straws: total motility of 53.8 ± 4.8%; percent progressive motility: 89.2 ± 1.4%) during a 6 h post-thaw incubation period with the use of a TYB/glycerol cryodiluent, slow cooling rate from 21 °C to 4 °C, rapid freezing rate, and medium (for 0.5 mL straw: 35 °C for 60 sec) or fast (for 0.5 mL: 50 °C for 10 sec) thawing rate [13]. Using a new trend in reproductive technology, O'Brien and Robeck [6] demonstrated that directional solidification using specialized cryopreservation equipment improved dolphin in vitro sperm characteristics at 6 h post-thaw (progressive motility: 54.1 ± 4.0%; viability: 63.4 ± 7.9%) when compared with the straw method (progressive motility: 48.7± 7.5%; viability: 52.0 ± 9.0%).

The recently developed freezing method, directional solidification, provides controlled seeding, cooling rates and ice front propagation [28]. The use of this technology allows large volume samples (up to 9 mL) to move through a linear temperature gradient, facilitating fine control of nucleation and ice crystal morphology [28]. In contrast, without the seeding process, uncontrolled ice crystal formation during cryopreservation using the straw method is believed to physically damage sperm membranes thus reducing in vitro sperm quality. In the aforementioned dolphin study, the quality of sexed dolphin spermatozoa, based on in vitro parameters of progressive motility, kinetic rating, viability and acrosome integrity, was significantly improved when samples were frozen using directional solidification compared with the straw method [6]. Improvements in post-thaw motility of non-sexed samples were also found when directional solidification was compared to conventional freezing methods in horses [29,30], and white rhinoceroses (*Ceratotherium simum*) [31]. Cryopreservation of Asian elephant (*Elephas maximus*) semen was not possible using conventional freezing methods. Semen from this species was cryopreserved for the first time using a directional freezer and a post-thaw sperm motility of 30% to 40% was achieved [32].

In addition to the dolphin, the use of directional solidification has been recently extended to two other cetaceans, also improving post-thaw in vitro sperm

characteristics. For the Pacific white-sided dolphin, 94% of initial motility was maintained post-thaw, compared with 50% using the straw method [2]. In beluga, spermatozoa exhibited greater susceptibility to damage during freeze-thaw processes (52.6% of raw progressive motility maintained) and the ejaculate volumes are substantially lower (1.6 ± 0.9 mL) than those for the previously described cetaceans [22,33], thereby resulting in low numbers of progressively motile beluga spermatozoa using conventional straw methods. A successful semen cryopreservation method for the beluga was recently achieved with the use of directional freezing and a trehalose-based cryodiluent [33]. With this combination of methods, sperm samples maintained 52.6% of their raw progressive motility, 95.1% of their raw kinetic rating and 67.4% of their raw viability [22]. These studies demonstrate that directional solidification provides superior maintenance of in vitro parameters during the freeze-thaw process compared to conventional straw freezing. Consequently, the technology has been integrated into semen freezing protocols for the bottlenose dolphin, Pacific white-sided dolphin, beluga and killer whale, with offspring produced using such samples from all four species after AI [18].

Sex pre-selection

Sorting of spermatozoa into X and Y chromosome-bearing populations using flow cytometry is the most efficient technique for sex pre-selection. It relies on the difference in DNA content of X- and Y-sperm cells. The method is widely used in the bovine industry; however, in order to be used in wild animals, some modifications in the flow cytometer settings for the correct orientation of gametes are necessary [7]. The DNA content varies among species and even breeds; cattle 3.7% (Brahman) and 4.22% (Jersey); possum (*Trichosurus sp.*) 2.3%, chinchilla (*Chinchilla sp.*) 7.5% and bottlenose dolphins 4.1% X-Y difference in DNA content [34]. This difference has been analyzed in many non-domestic species using flow cytometry and it can predict with some success the potential for spermatozoa to be successfully sorted (Table 1). However, it cannot predict the species and male-specific susceptibility of spermatozoa to the sorting process.

Table 1. Percentage of difference in DNA content between X and Y chromosome-bearing sperm nuclei (from O'Brien et al, 2009 [7]).

Order	Species common name (scientific name)	X-Y Difference (%)
Artiodactyla	Dromedary camel (*Camelus dromedarius*)	3.3
	Bison (*Bos bison*)	3.6
	Yak (*Bos mutus grunniens*)	3.6
	Pig (*Sus scrofa*)	3.6
	Hippopotamus (*Hippopotamus amphibius)*	3.7
	Cattle (*Bos indicus*)	3.7
	Cattle (*Bos Taurus*)	3.8, 4.0-4.2
	Elk (*Cervus elaphus nelsoni*)	3.8
	Sheep (*Ovis aries*)	4.2
	Dorcas gazelle (*Gazella dorcas*)	4.3
	Giraffe (*Giraffa camelopardalis)*	4.4
	White-tailed deer (*Odocoileus virginianus*)	4.4
	Reeve's muntjac (*Muntiacus reevesi*)	6.3
Carnivora	Dog (*Canis lupis familiaris*)	3.9
	Tiger (*Panthera tigris tigris*)	4.1
	Cat (*Felis catus*)	4.2
Cetacea	Bottlenose dolphin (*Tursiops truncatus*)	4.0, 4.1
	Pacific white-sided dolphin (*Lagenorhynchus obliqudens*)	4.0, 4.1
Diprotodontia	Brushtail possum (*Trichosurus vulpecula)*	2.3
Perissodactyla	Domestic horse (*Equus caballas*)	3.7
	Zebra (*Equus zebra hartmannae*)	3.7
	White rhinoceros (*Ceratotherium simum simum)*	4.1
	Black rhinoceros (*Diceros bicornis)*	4.1
	Indian rhinoceros (*Rhinoceros unicornis)*	4.1
Primates	Western lowland gorilla (*Gorilla gorilla gorilla*)	2.7-2.8
	Human (*Homo sapiens*)	2.8
	Bornean orangutan (*Pongo pygmaeus pygmaeus)*	3.2
	Common chimpanzee (*Pan troglodytes*)	3.3
	Ring-tailed lemur (*Lemur catta)*	4.3
	Rhesus macaque (*Macaca mulatta)*	4.3
Proboscidea	Asian elephant (*Elephas maximus*)	3.4
	African elephant (*Loxodonta africana*)	3.9
Rodentia	Rabbit (*Oryctolagus Cuniculas*)	3.0
	Mouse (*Mus musculus*)	3.3
	Plains rat (*Pseudomys australis*)	4.2
	Chinchilla (*Chinchilla laniger*)	7.5

Sperm sexing technology has been developed for use in numerous livestock species [34,35,36] and is also used in humans to avoid sex-linked diseases [37,38].

For non-domestic species, only elk (*Cervus canadensis*), buffalo (*Bubalus bubalis*) and dolphin spermatozoa have been sorted and offspring of pre-determined sex produced [7]. As a result of the potential benefits toward management of captive populations that AI with sex-selected spermatozoa offered bottlenose dolphin managers, a sperm sorting and reproductive physiology research facility (SeaWorld and Busch Gardens Reproductive Research Center (SWBGRRC), San Diego, CA, USA) was created. Successful efforts at developing sperm sorting and subsequent cryopreservation techniques combined with previously developed AI, led to the first pre-sexed bottlenose dolphin born in 2005 at SeaWorld San Diego [6]. The insemination was performed using sexed, frozen-thawed spermatozoa derived from liquid-stored semen. To date, eleven dolphin calves of pre-determined sex have been produced using cryopreservation and AI technology [O'Brien JK, Robeck TR personal communication].

Important research efforts are being made to overcome the challenges that some species present at sorting. For example, western lowland gorillas (*Gorilla gorilla gorilla*) have low total numbers of viable spermatozoa while Asian elephants present very different sorting rates between males [7].

Sorting and recryopreservation of frozen-thawed spermatozoa

Shipment of semen samples using liquid storage technology to a sorting laboratory should take less than 12 to 18 h in order to maintain high quality in vitro sperm characteristics prior to undergoing sorting and cryopreservation processes [7]. In the case of a facility requiring a longer shipping time, the sorting and re-cryopreservation of frozen-thawed spermatozoa can be performed. With this method, semen is cryopreserved at the site of collection, transported to the sorting facility where it is thawed, then processed for sorting and re-cryopreserved. Thereafter, sexed samples can be transported to any location in the cryopreserved state, or banked for long term storage.

From the time of collection to the insemination of the female, spermatozoa undergo a series of processes that cause detrimental effects to in vitro sperm characteristics [7]. The first and second cryopreservation/ thawing steps combined with

the sorting process are the most harmful stressors to the cells since these steps increase the number of spermatozoa with damaged membranes [11]. The sorting and recryopreservation of frozen-thawed spermatozoa has resulted in the total number of usable, sorted spermatozoa of a particular sex decreasing to less than 5% of the original number of spermatozoa in the fresh ejaculate [7,11].

Post-thaw spermatozoa are typically of lower quality (lower motility, viability and acrosome integrity) than fresh samples. In addition, they are thawed within their cryoprotectant medium which often includes glycerol, egg yolk and other diluent components. These lower quality samples and the presence of the cryodiluent can adversely affect the staining and sorting of the samples by decreasing the correct orientation of spermatozoa and sperm sorting rate. Therefore, post-thaw samples must undergo density gradient centrifugation or washing prior to staining and sorting in order to select live spermatozoa and to remove the cryodiluent. Cryoprotectants can interact with Hoechst 33342 (H33342) stain and cause poor resolution at sorting [8,39]. O'Brien et al. [8] determined that sorting rates of frozen-thawed ram spermatozoa undergoing density gradient processing were greater than samples that did not receive processing or washing (75 to 80%, 25 to 35%; respectively). When western lowland gorilla spermatozoa were sorted, the proportion of morphologically normal spermatozoa in frozen-thawed samples was increased by 28.3 ± 2.5% after the DGC process [10]. In this process both glycerol and egg yolk are removed. However, egg yolk is known to serve as a cell membrane stabilizer [40,41] and its presence in sperm solutions prior to staining with H33342 could improve or maintain in vitro sperm characteristics. Conversely, there is evidence that egg yolk interferes with H33342 penetration [42]. While necessary, the density gradient centrifugation or washing process adds an additional step (not including the original freeze thaw process) required to prepare spermatozoa for sorting which results in older spermatozoa when compared to fresh sorted spermatozoa. During aging, sperm cells utilize their limited energy storage capacity which may result in reduced fertility [43]. Thus, spermatozoa must be thawed in multiple batches based on sorting facility capacity to minimize the processing and sorting time prior to recryopreservation.

Due to the previously mentioned features of increased processing time and multiple freeze thaw cycles required to produce frozen-thawed, sorted then re-frozen

(FSF) spermatozoa, and the significant loss of gametes, the technology is unlikely to be used commercially in AI programs, but it has been recently integrated into commercial sorting operations for use with cattle IVF/ET programs [XY Inc., J Moreno, personal communication]. Despite its limitations, the sorting and re-cryopreservation of previously frozen spermatozoa has great potential for application to wild animals [8,10], especially bottlenose dolphins [7] which have multiple small populations located around the world that do not have immediate access to sorting facilities. The ability to sort frozen-thawed spermatozoa could also allow the incorporation of sexed spermatozoa from post-mortem samples of valuable wild specimens into ex situ populations. This potential approach is supported by the findings in several non-human primates where frozen-thawed epididymal spermatozoa were sorted without a negative impact on in vitro sperm characteristics [8]. So far, frozen-thawed spermatozoa from seven different primate species, including humans, have been successfully sorted [7].

One bottlenose dolphin was produced using frozen-thawed, sorted, re-cryopreserved spermatozoa [7]. It was estimated that 35 h of sorting time of frozen-thawed spermatozoa were required to produce an AI dose of 200 $x10^6$ progressively motile spermatozoa using one flow cytometer [7]. This calculation does not include time to prepare samples for sorting, nor flow cytometer preparation and maintenance times (approximately 4 h for every sorting day) [O'Brien JK, personal communication]. However, with the recent acquisition of a dual flow cytometer, and the use of a lower AI dose (150 $x10^6$ progressively motile spermatozoa [6]), the SWBGRRC has improved the production rate of sexed dolphin spermatozoa and the new estimated sorting time for a FSF AI dose is approximately 13 to14 h.

For domestic species, very little has been published about FSF spermatozoa, but the method has been described for bulls [44,45] and rams [9,43,46]. In sheep, FSF spermatozoa were used to produce normal offspring using in vitro fertilization and embryo transfer [9]. After a 6 h incubation period post-thaw, FSF and control (FNSF: frozen-thawed, non-sorted then re-frozen) spermatozoa had similar proportions of motile spermatozoa. However, FSF spermatozoa presented fewer intact acrosomes than controls over the same period.

In an additional study, FSF was compared to fresh sorted then frozen (SF) and Control (FNSF) ram spermatozoa. Sorting efficiency was evaluated by comparing the

correct orientation of the cells in the flow cytometer (flat surface of the spermatozoa head facing the laser beam) [43]. Similar sorting efficiency between SF (46.0 ± 3.8%) and FSF (42.0 ± 1.5%) spermatozoa ($P > 0.05$) were observed. However, at sorting, the proportion of non-viable cells from FSF (35.0 ± 1.0%) was greater than that of SF (29.0 ± 4.2%) ($P < 0.05$). Results from the aforementioned study showed evidence of a decrease in longevity, motility, viability, acrosome integrity and mitochondrial activity of spermatozoa from FSF samples. Despite the overall decrease in sperm quality, flow cytometric sorting produced a highly selected population of spermatozoa with improved survival and longevity when compared to the Control spermatozoa that underwent two cryopreservations without being sorted [43]. Standard laparoscopic insemination techniques using 15 $x10^6$ motile spermatozoa that underwent two cycles of freezing/thawing and sorting resulted in normal births in sheep [46], demonstrating the fertility of frozen-thawed ram spermatozoa which have been sex-sorted before re-cryopreservation and subsequent intrauterine insemination.

Production of lambs using FSF spermatozoa combined with IVF/ET technology or laparoscopic AI techniques provides strong evidence that embryos and subsequent fetuses development normally in utero [9,46]. However, in sheep, sorted spermatozoa derived from fresh semen have a reduced life span after freezing and thawing compared with their non-sorted counterparts [47]. Sorted frozen-thawed spermatozoa quickly attached to oviduct epithelial cells but for a short period of time, even shorter than non-sorted spermatozoa [47]. De Graaf et al. [43] reported that FSF spermatozoa were less likely to be bound after 1 h of incubation. Binding to oviduct cells allow spermatozoa to undergo capacitation, and this result suggest that sorted frozen-thawed spermatozoa require a short period of attachment to oviduct cells to reach the fully capacitated state [47]. Although the reduced lifespan in vitro could indicate a similarly short lifespan in vivo, the ability of FSF spermatozoa to migrate to the site of fertilization was maintained [47].

Regardless of the species under investigation, characterization of the in vitro functional capacity of FSF spermatozoa, determination of the life span of FSF spermatozoa and quantification of sperm losses that occur during FSF sperm preparation, are all necessary to identify detrimental steps which, if improved, may increase the applicability of this sex-preselection technology to species management.

MATERIALS AND METHODS

Three pilot trials (Pilot trials 1 to 3) and two experiments (Experiment 1 and 2) were performed using frozen semen from bottlenose dolphins (*Tursiops truncatus*) of proven fertility. A multi-male frozen semen pool (comprised of one ejaculate from three males) was used for the pilot trials and Experiment 1, and semen from three males, frozen individually, was used for Experiment 2. Semen was frozen using a directional solidification freezing method as described in subsequent sections. For all trials and experiments, frozen semen was stored in liquid nitrogen tanks at the SWBGRRC.

All procedures described within were reviewed and approved by the SeaWorld Institutional Animal Care and Use Committee and were performed in accordance with the Animal Welfare Act for the care of Marine Mammals. All samples were collected by SeaWorld staff using routine husbandry training and on unrestrained animals. Training of husbandry behavior to facilitate biological sample collection is currently viewed as the standard of care for marine mammals.

Reagents and media

All chemicals were of analytical grade. Disposable plastic ware was manufactured by BD Biosciences (BD, Bedford, MA, USA). Unless otherwise stated, all media components were purchased from Sigma-Aldrich (Sigma, St Louis, MO, USA) and were prepared with tissue-grade water (Sigma or Millipore, Billerica, MA, USA). All freezing and sorting diluents containing egg yolk (free range eggs < 48 h old) were clarified prepared by centrifugation for 1.5 h at 10000 x *g* at 10 ºC. The supernatant was filtered (0.22 µm; Millipore) and frozen at -80 ºC for a maximum of 18 months.

Evaluation of semen and sperm characteristics

Four proven breeding male bottlenose dolphins (Males 1 to 4; aged 23, 21, 20 and 16 years (in 2009), weighting 364, 230, 224 and 226 kg, respectively) housed at SeaWorld San Diego (CA, USA) were used for semen collection.

The fresh semen was transferred from a sterile Whirlpak® collection bag (Nasco International Inc, Fort Atkinson, WI) to a 50 ml polystyrene tube (BD Falcon™, Becton Dickinson, Franklin Lakes, NJ) and an aliquot was removed for assessment: pH (pH indicator strips; EM Science, Gibbstown, NJ, USA), osmolality (freezing point depression osmometer, Advanced Instruments Inc., Norwood, MA), sperm concentration (haemocytomer, X 400; Olympus, Tokyo, Japan), and motility parameters (Computer Assisted Sperm Analysis [CASA, Hamilton–Thorne, HTM-IVOS Version 12.2]) [48]. Plasma membrane integrity (viability) was assessed using live-dead exclusion stains (light microscopy: eosin-nigrosin; IMV International Corp., Maple Grove, MN, USA; fluorescence microscopy: propidium iodide). Acrosome integrity and viability were evaluated using a dual stain method (propidium iodide and FITC-PNA) [49,50]. An aliquot was frozen at each evaluation step and retrospectively analyzed after thawing for susceptibility of spermatozoa to DNA denaturation using the sperm chromatin structure assay (SCSA) [51,52]. All evaluations were conducted at the SWBGRRC with the exception of the SCSA which was performed at the Texas A&M University (Department of Large Animal Clinical Sciences).

Sperm concentration

A hemocytometer (Hausser Scientific, Horsham, PA) was used to determine the concentration of samples. Samples were diluted with a count solution (6% saline solution) and vortexed before loading the hemocytometer chambers. The dilution factors varied from 1:20 to 1:200 to avoid inaccuracies in the method by over- or under-dilution of the sperm sample and thereby allow for an appropriate number of cells for counting (approximately 150 to 200 spermatozoa per chamber). Chambers were loaded with 10 µL in standard fashion using a pipette [53]. A new pipette tip was used

for placement of the sperm suspension in each chamber, and the outside of the pipette tip was wiped with lint-free tissue (Kimwipe®, Kimberly-Clark Corp., Roswell, GA, U.S.A.) to prevent any excess volume from being loaded into the chamber. Before evaluation, the hemocytometer was placed for 5 min on a wet surface inside a covered petri dish to avoid dehydration. A minimum of four chambers (two hemocytometers) were assessed per sample. All spermatozoa within five or 25 squares were counted, including those touching the lower and right sides of each grid of 16 squares. Chambers were cleaned between counts with soap solution, rinsed with water and wiped with absorbent paper towels. Chambers were allowed to dry before reloading. In the case of counting five squares, the sperm concentration (spermatozoa/mL) was obtained by multiplying the average of the count by five, multiplying the result by the dilution factor and multiplying again by 10000. In the case of counting all 25 squares, the multiplication of the average by five is not necessary, and the average can be multiplied by the dilution factor and by 10000.

Recovery rate

Sperm concentration as previously described was used to determine the total number of spermatozoa in the resuspended pellet (after DGC) by multiplying the sperm concentration of the pellet by the total volume of the pellet. The total number of spermatozoa in the pellet was then multiplied by 100 and divided by the total number of spermatozoa in the thawed sample placed on the DGC, resulting in the percentage of spermatozoa recovered from the original thawed sample. This method was used to determine the recovery rate in all pilot trials and experiments.

Computer-assisted sperm analysis (CASA)

Sperm motility parameters were objectively assessed using computer-assisted sperm analysis (CASA; HTM-IVOS Version 12.2; Hamilton-Thorne, Inc., Beverly, MA, USA) in a manner similar to that previously described for beluga [21,33]. Semen was

diluted with Androhep Enduraguard™ (AE; Minitube of America, Verona, WI, USA) to a concentration of 15 to 25 x 10^6 spermatozoa/mL and 9 µL was transferred to a glass slide and overlaid with a 22 x 22 mm glass coverslip for analysis at 35 °C. After two to three minutes, a total of five to ten microscopic fields representing a minimum of 200 spermatozoa were randomly selected and examined per sample for the calculation of the following motility parameters: average pathway velocity (VAP, µm/sec), straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness of sperm movement (STR (%, VSL/VAP), total motility (TM, %; VAP > 20 µm), and progressive motility (PM, %; VAP> 50 µm/sec and STR > 80%). Preset values for the instrument consisted of the following: 30 frames at a frame rate of 60 frames/sec, minimum contrast of 80, minimum cell size (pixels) of five. Three additional groups based on velocity of movement were also determined: rapid (RAP, VAP > 50 µm/sec, %), medium (MED, 20 µm/sec < VAP < 50 µm/sec, %) and slow (SLOW, VAP < 20 µm/sec, %).

Sperm chromatin structure assay (SCSA)

This assay measures the susceptibility of sperm DNA to denaturation and has been previously described [51,52,54]. Individual samples were thawed in a water bath at 38 °C for 15 to 30 sec. An aliquot of 5 µL to 20 µL of semen was mixed with 180 to 195 µL of a TRIS buffer (0.186 g disodium EDTA, 0.790 g Tris-HCl, 4.380 g NaCl in 500 mL deionized water, pH 7.4) to a final volume of 200 µL. Four hundred microliters of acid- detergent solution (2.19 g NaCl, 1.0 mL of 2N HCl, 0.25 mL Triton-X, qs. 250 mL deionized water) was immediately added and after 30 seconds the solution was quenched with 1.2 mL of an acridine orange solution (3.8869 g citric acid monohydrate, 8.9429 g Na2HPO4, 4.3850 g NaCl, 0.1700 g disodium EDTA, 4 µg/mL acridine orange stock solution (1mg/mL), qs. 500 mL water, pH 6). Samples were immediately placed into the flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) and allowed to equilibrate for 30 sec prior to data acquisition. Samples, tubes and reagents were kept on ice during processing. Sample volume varied to accommodate a flow rate of 100 to 200 cells/sec. Five thousand events were accumulated per sample. Flow

cytometer settings calibrated using spermatozoa from a known fertile dolphin as the control. Using this control sample, settings were adjusted so that mean green fluorescence was 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 Standard Deviation (SD$_{αt}$). 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 (Fig. 1).

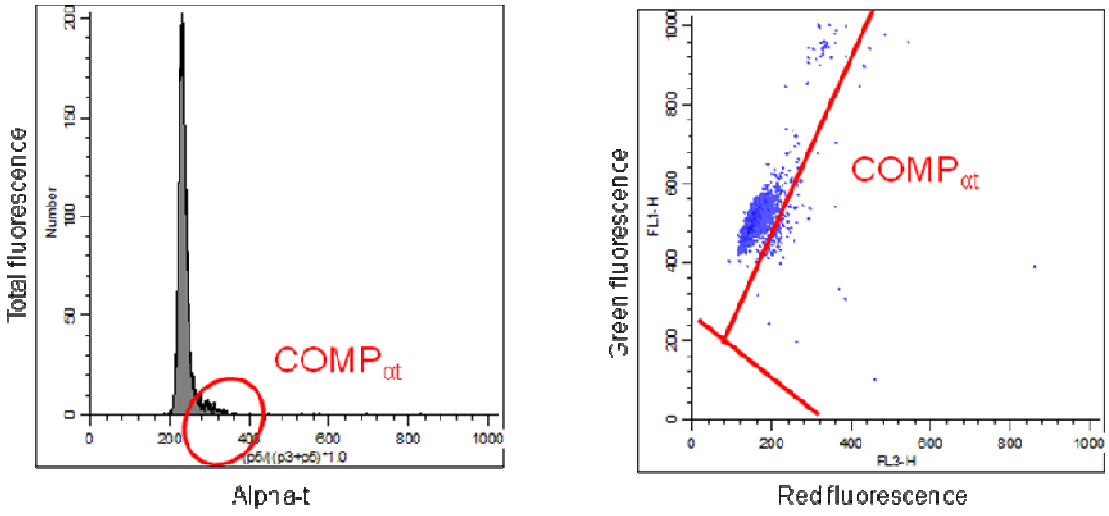

Fig. 1. Examples of data generated by the SCSA. Left: histogram of $α_t$ population, right: cytogram of green versus red fluorescence.

PI/FITC-PNA

Sperm plasma membrane integrity (viability) and acrosome integrity were measured simultaneously using the stains propidium iodide (PI, Sigma 287075) and fluorescein isothiocynate-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA, Sigma L-7381). The PI/FITC-PNA staining method used herein was a combination of different dual staining protocols [49,50,55]. 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 sec, 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 x 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 X 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 (viable cells with an intact acrosome), green staining in the acrosome region including the equatorial segment (viable cells with a damaged or reacted acrosome), red staining (non-viable cells with an intact acrosome), red and green staining (non-viable cells with a damaged or reacted acrosome). The dual staining method was validated for dolphin spermatozoa by observing 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 Androhep Enduraguard™ (AE; Minitube of America, Verona, WI, USA) without cryoprotectant, and >95% of cells were classified as non-viable and acrosome damaged/reacted after the second thawing.

Eosin-nigrosin stain

This assay has been extensively used to evaluate sperm viability [13,56]. In a glass slide, 10 µL of sample were mixed with 15 µL of eosin-nigrosin exclusion stain (IMV International Corp., Maple Grove, MN) for 30 sec. A smear was made and allowed to air-dry for immediate evaluation using bright field optics (1000 X). A minimum of 100 spermatozoa were examined and placed into one of two groups based on stain uptake by the sperm head and/or tail: live (no stain uptake) or dead (partial or complete stain uptake).

Semen processing and cryopreservation using directional solidification

Ejaculates were used for the multi-male sample in Pilot trials 1 to 3 (Males 1, 2 and 3) and in Experiments 1 and 2 (individual ejaculates from Males 2, 3 and 4) if the progressive sperm motility was greater than 85%, and the osmolality was within the normal range for bottlenose dolphins (330 ± 5 mOsm/kg).

For ejaculates of low sperm concentration (< 600 x 10^6 spermatozoa/mL), the tube of semen was centrifuged (600 x *g*, 10 min) and an appropriate volume of supernatant was removed to obtain a concentration of 600 x 10^6 spermatozoa/mL. For ejaculates of high sperm concentration, Part A extender (TYB; modified from Graham et al. 1972 [57]; 176 mM TES, 80 mM Trizma base (Tris), 9 mM fructose, 50 µg/mL gentamicin sulfate, 20% (v/v) egg yolk, 330 ± 5 mOsm/kg, pH 7.3 ± 0.1) [6] was added to raw, non-centrifuged semen in an appropriate volume to obtain the same final concentration of 600 x 10^6 spermatozoa/mL. Part A was added to semen (1:1, v/v) over a 2 to 3 min period at room temperature.

The sperm suspension was cooled to 5 °C over a 1.5 h to 2 h period (-0.2 °C/min) then diluted 2:1 (v/v) with Part B extender previously cooled to 5 °C (9% Glycerol glycerolated TYB, v/v) in a stepwise fashion over 30 min (three steps of 25%, 25% and 50% volume) to obtain a final concentration of 200 x 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). The hollow tubes moved through the first block of the machine (5 ºC) for 45 sec at a constant velocity (1 mm/sec) before reaching a 2 mm distance inside of the second block (second block maintained at -50 °C), where it was held for 60 sec for initiation of ice crystal formation (rapid induction of ice nucleation from the seeding point throughout the length of the glass tube) [6]. The tubes were then moved at the same velocity across the second block for 3 min before entering the collection chamber (-100 ºC to -110 ºC) followed by transfer to liquid nitrogen.

Pilot trials

Pilot trial 1: effect of frozen-thawed semen volume during density gradient centrifugation (DGC) on sperm recovery rate and in vitro sperm characteristics

Experimental design: a multi-male frozen semen pool was used to examine the effect of four semen volume treatments on sperm recovery rate using DGC. Three replicates were performed.

A large hollow glass tube containing 8.5 mL of frozen semen suspended at 200 x 10^6 spermatozoa/mL was thawed in air (21 °C) for 90 sec then placed at 35 ºC in a water bath for 50 sec. Four 15 mL polystyrene tubes were prepared with 2 mL of 45% density gradient medium (DGM) and 2.5 mL of 90% DGM. Density gradient medium was a Percoll-based (Percoll™ Plus, GE Healthcare Biosciences, Pittsburgh, PA) in-house preparation (320-330 mOsm/kg, pH 7.2-7.4). The 45% DGM was prepared by adding 45 mL of 100% DGM to 55 mL of staining medium, and the 90% DGM was prepared by adding 90 mL of 100% DGM to 10 mL of staining medium. Staining medium was a Tyrode's salt solution containing BSA (0.3%, v/v; Sigma A-4697), lactate, pyruvate and HEPES (HEPES-TALP staining medium; modified from Parrish et al. 1986 [58] supplemented with gentamycin (50 µg/mL); 330 ± 5 mOsm/kg and pH 7.3 ± 0.1; [6]). Semen was placed over the gradient at four volumes: 0.5 mL, 1.0 mL, 2.0 mL and 4.0 mL. The tubes were centrifuged at 800 x *g* for 20 min and the supernatant was removed using a disposable 3 mL transfer pipette. Using a calibrated pipettor

(Gilson Inc., Middleton, WI, USA) with a 1 mL pipette tip, the sperm pellets (1.6 mL) were aspirated and slowly combined in a new 15 mL polystyrene tube followed by resuspension with staining medium (1:3 v/v, sperm sample: staining medium). The sample was filtered (35 µm) and tube was then centrifuged at 800 x *g* for 10 min followed by removal of the supernatant (4.8 mL). The new pellet was then resuspended with the same volume (4.8 mL) of fresh staining medium that was removed post-centrifugation. Aliquots of each treatment were removed for assessments (comparison of motility parameters, viability and sperm recovery rate).

Pilot trial 2: effect of tube size during DGC on sperm recovery rate and in vitro sperm characteristics

Experimental design: a multi-male frozen semen pool was used to examine the effect of two centrifuge tube sizes on sperm recovery rate using DGC. Three replicates were performed.

Semen was thawed as described in Pilot trial 1. Treatments comprised DGC of semen in a 15 mL (2 mL of 45% DGM and 2.5 mL of 90% DGM) and 50 mL polystyrene centrifuge tube (2 mL of 45% DGM and 3 mL of 90% DGM). After DGC, the supernatant was removed using a 3 mL transfer pipette and the sperm pellet was aspirated and slowly combined in a new 15 mL polystyrene tube followed by resuspension with staining medium (1:3 v/v, sperm sample: staining medium). The samples were filtered (35 µm) then centrifuged at 800 x *g* for 10 min followed by removal of the supernatant and resuspension with the same volume of staining medium that was removed post-centrifugation. Aliquots of each treatment were removed for assessments (comparison of motility parameters, viability and sperm recovery rate).

Pilot trial 3: effect of a non-centrifugation (gravity) separation technique on sperm recovery rate and in vitro sperm characteristics

Experimental design: a multi-male frozen semen pool was used to examine the effect of a gravity separation technique after thawing. Three replicates were performed.

Semen was thawed as described previously and divided equally into two 15 mL polystyrene tubes. The first centrifugation of the DGC process was performed for both tubes as previously described in Pilot trials 1 and 2. One tube then underwent the second centrifugation as described previously for the DGC method. At the same time, the sperm solution in the second tube was resuspended with staining medium (1:3 v/v, sperm sample: staining medium) and centrifuged at 750 x *g* for 8 min. The supernatant was removed and the sperm pellet was resuspended with staining medium (to a final concentration of 600 x 10^6 spermatozoa/mL). After 30 min at room temperature, the supernatant was transferred to a new tube and aliquots were removed from both treatment groups for assessments (motility parameters, viability and sperm recovery rate).

Experiments

Experiment 1: effect of egg yolk as a component of HEPES-TALP medium for sperm washing procedures during DGC on sperm recovery and in vitro sperm characteristics

Experimental design: a multi-male frozen semen pool was used to examine the effect of two washing media (staining medium with and without 4% egg yolk) following DGC on sperm recovery rate and in vitro sperm characteristics. Nine replicates were performed.

A large hollow glass tube containing 8.5 mL of frozen semen from a multi male pool suspended at 200 x 10^6 spermatozoa/mL was thawed in air (21 °C) for 90 sec then placed at 35 ºC in a water bath for 50 sec. The optimum volume of frozen-thawed semen determined in Pilot trial 1 (4 mL) was placed in two polystyrene tubes of the

optimum tube size determined in Pilot trial 2 (15 mL), previously prepared with 2 mL of 45% DGM and 2.5 mL of 90% DGM. The tubes were centrifuged at 800 x *g* for 20 min and the supernatant was removed using a disposable 3 mL transfer pipette. Sperm pellets (1.6 mL) were aspirated using a pipettor and 1 mL pipette tip and transferred to new 15 mL polystyrene tubes. One tube was used to resuspend the sperm sample with staining medium (1:3, v/v, sperm sample: staining medium) and the other tube was used to resuspend the sperm sample with staining medium containing 4% egg yolk (1:3 v/v, sperm sample: staining medium plus egg yolk). The samples were filtered (35 µm) and placed in new 15 mL polystyrene tubes. Tubes were centrifuged at 800 x *g* for 10 min followed by removal of the supernatant and resuspension with the same volume of staining medium that was removed post-centrifugation. Aliquots of each treatment were removed for assessments (comparison of motility parameters, viability and sperm recovery rate).

Experiment 2: effect of conventional straw freezing and directional freezing technology on in vitro characteristics of non-sorted and sorted spermatozoa after recryopreservation

Experimental design: three cryopreserved ejaculates from three males were used to compare the effects of two cryopreservation methods (conventional straw freezing and directional freezing) on in vitro sperm characteristics of control (non-sorted) and sorted spermatozoa (2×2 factorial).

In vitro sperm characteristics were analyzed using aliquots of sperm suspensions from both refreezing methods and sperm types, non-sorted (control) and sorted spermatozoa, from both re-freezing methods at the following steps: post-DGC, post-staining with H33342 (post-stain), post- sorting and centrifugation (post-sort), post-cooling and cryodiluent equilibration (pre-freeze), and at fixed times post-thaw (0 h, 6 h, 12 h, 18 h and 24 h post-thaw).

Preparation and sorting of frozen-thawed spermatozoa

Frozen-thawed semen was processed for non-sorted (control) and sorted treatment groups as shown in Fig 2. Frozen-thawed spermatozoa destined for sorting were prepared using the optimum method devised from the three pilot trials which included: (i) the use of 4 mL of semen (or the equivalent volume containing 800 x 10^6 spermatozoa), (ii) 15 mL centrifuge tubes for DGC and washing steps, and (iii) re-suspension of the pellet after DGC in stain medium supplemented with 4% egg yolk. Sperm suspensions from each male were diluted to 200 x 10^6 spermatozoa/mL with staining medium and incubated with Hoechst H33342 (Sigma; 89 – 107 µM) for 45 min at 32.5 °C. The H33342 concentration was previously determined by a series of optimization experiments. Immediately before sorting, stained spermatozoa were diluted (1:1, v/v) with the staining medium containing 0.002% of food dye (FD&C #40, Warner Jenkinson Company Inc., St Louis, MO, USA) to a concentration of 100 x 10^6 spermatozoa/mL and filtered (35 µm). The food dye penetrates the membrane of the non-viable spermatozoa and reduces the intensity of the H33342 fluorescence. Consequently, only viable spermatozoa (with intact plasma membranes) were selected for sorting [59,60].

A high speed flow cytometer (SX MoFlo®, Dako Colorado Inc., Fort Collins, CO, USA) modified for sperm sorting [24,60,61] operated at 207 kPA (30 psi) was used to analyze and sort spermatozoa. The H33342 was excited with UV light from a diode-pumped solid state pulse laser (Vanguard 350 HMD-355; Spectra Physics, Mountain View, CA, USA) (333-363 nm multilines) operated at 175 mW. The sheath fluid contained staining medium without BSA.

Spermatozoa were sorted into 50 mL polystyrene tubes containing 3 mL of Part A (with 1% seminal plasma, v/v) for a maximum of two hours. Sort gates were placed on correctly 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 x 10^6 spermatozoa/mL. An aliquot of 0.1 x 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 [62].oriented spermatozoa (spermatozoa with the flat surface of their head oriented towards the laser beam) and sorting was

performed using Summit® software (Version 4.0, Dako Colorado Inc.). Sorting was performed by placing sort gates on the oriented, viable population to achieve purities of greater than 90% X- or Y-chromosome-bearing spermatozoa. Sexed spermatozoa were transferred to 15 mL polystyrene tubes and centrifuged at 850 x *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 x 10^6 spermatozoa/mL. An aliquot of 0.1 x 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 [62].

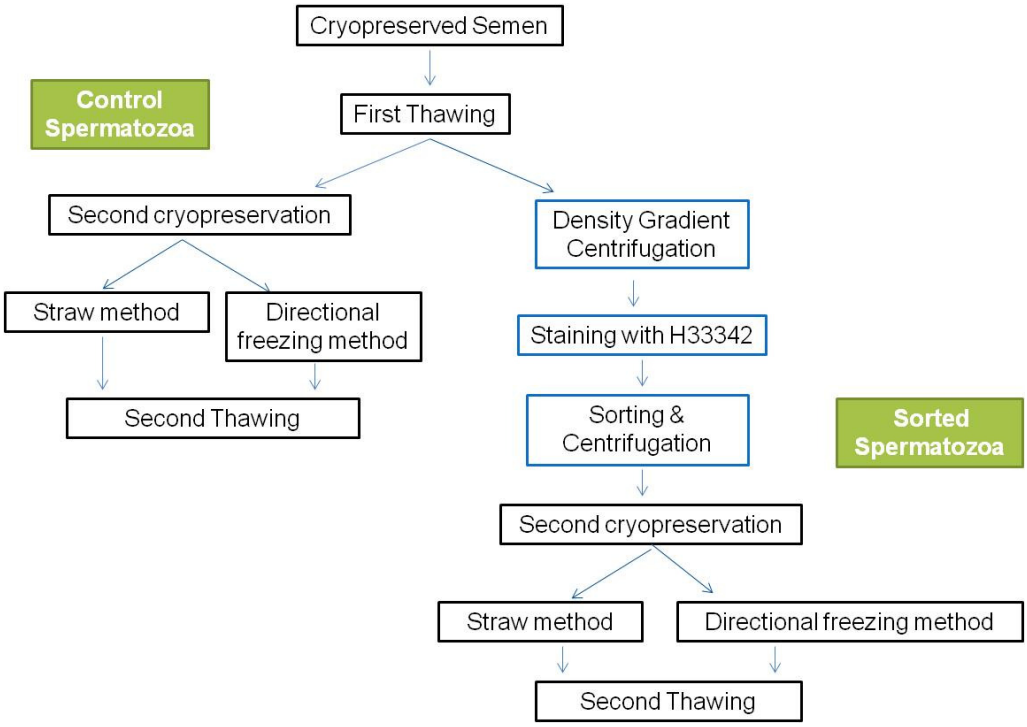

Fig. 2. Processing steps of non-sorted (Control) and sorted (FSF) spermatozoa of Experiment 2.

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

Non-sorted and sorted spermatozoa were frozen in straws as follows:

(i) Non-sorted spermatozoa: the sperm suspension was diluted with Part A extender at room temperature to obtain a final concentration of 100 x 10^6 spermatozoa/mL. Part A contained 1% (v/v) seminal plasma and 25% (v/v) sheath fluid (as used during sperm sorting).

(ii) Sorted spermatozoa: after sorting and processing as previously described; the sperm suspension contained 30 x 10^6 spermatozoa/mL (prior to cooling).

Non-sorted and sorted sperm suspensions were cooled to 5 °C over a 1.5 h to 2 h period (-0.2 °C/min) then diluted inside a cool room with Part B extender (with 3% glycerol, 1:1, v/v) in a stepwise fashion of 3 steps (25%, 25% and 50% of volume to be added) over 30 min (final concentration was 50 x 10^6 spermatozoa/mL with 1.5% glycerol) and equilibrated for 1 h.

Spermatozoa were loaded into 0.25 ml straws (Minitube of America, Verona, WI, USA) using a 1 mL syringe and a sterile 20-200 uL pipette tip attached to the syringe by an adaptor. An air space of 1 cm was left in the middle of the straw to allow for sample expansion during freezing. Straws were sealed with pre-cooled ball bearings (Minitube of America, Verona, WI, USA) and placed on a block of dry ice for 10 min and then transferred to liquid nitrogen.

Recryopreservation of non-sorted and sorted spermatozoa using a directional freezing method

Non-sorted and sorted spermatozoa were frozen in glass vials as follows:

(i) Non-sorted spermatozoa: the sperm suspension was cooled and diluted as previously described for the straw freezing method in order to obtain a final concentration of 50 x 10^6 spermatozoa/mL and 1.5% glycerol (v/v).

(ii) Sorted spermatozoa: the sperm suspension was cooled and diluted as previously described for the straw freezing method in order to obtain a final concentration of 15 x 10^6 spermatozoa/mL and 1.5% glycerol (v/v).

Sperm suspensions were transferred to 2 mL hollow glass tubes and cryopreserved using a directional freezing machine as previously described for non-sorted semen except that tubes were held for 30 sec (instead of 60 sec) to allow proper seeding. After entering the collection chamber, the tubes were transferred to liquid nitrogen.

Preparation of seminal plasma

Seminal plasma from the three males used in Experiment 2 was individually frozen after centrifugation of ejaculates displaying normal osmolality (10,000 x *g* for 1 h at 10 ºC). Seminal plasma was removed, snap frozen in liquid nitrogen (-196 ºC) and stored at -80 ºC until thawing at room temperature for use during the recryopreservation step.

Thawing of recryopreserved non-sorted and sorted spermatozoa

Straws were thawed in a water bath at 35 °C for 20 sec. Small hollow tubes were thawed in air for 45 sec then transferred to a 35 °C water bath equipped with

modifications to enable uniform sample thawing over 45 sec (Harmony CryoCare Activator™; IMT International).

Dilution of samples after second thawing

After thawing, samples were transferred to microcentrifuges tubes (Eppendorf North America) and diluted (1:0.1, v/v) with Androhep.

Statistics

Data for sperm motility parameters, viability, acrosome integrity, sperm recovery rate and sperm chromatin integrity across the different time points were analyzed using repeated measures analysis of variance (RMANOVA, SigmaStat®, Version 3.5, SSPS Inc., San Rafael, CA, USA). All pair wise multiple comparison procedures between means were conducted by Student-Newman-Keuls (SNK). $P < 0.05$ was considered significant. Data are presented as mean ± SD.

RESULTS

Pilot trials

Three replicates were performed for each pilot trial. Since a minimum of nine replicates is necessary for treatment comparisons, it must be acknowledged that the statistical analyses presented below are preliminary, as was the nature of the pilot trials.

Pilot trial 1: effect of frozen-thawed semen volume during DGC on sperm recovery rate and in vitro sperm characteristics

After DGC, no differences ($P > 0.05$) were found in the recovery rate, TM, PM, VAP, VSL and VCL when different volumes (0.5 mL, 1 mL, 2 mL and 4 mL) of thawed semen were placed on the density gradient (Table 2). The percentage of viable spermatozoa assessed using the eosin-nigrosin stain was higher ($P < 0.05$) for 4 mL than 0.5 mL or 1.0 mL treatment groups, but similar ($P > 0.05$) to the 2.0 mL group. Since motility parameters and recovery rates were similar across treatments, and viability was satisfactory for the 2 mL or 4 mL group, the greatest volume (4 mL) was used in all subsequent trials and experiments.

Table 2. Sperm characteristics and recovery rate of different semen volumes used during density gradient centrifugation in Pilot trial 1 (mean ± SD, n = 3).

Sperm Characteristics*	Volume of semen			
	0.5 mL	1.0 mL	2.0 mL	4.0 mL
TM (%)	80.7 ± 5.5	82.3 ± 1.5	83.3 ± 4.2	83.3 ± 5.8
PM (%)	66.7 ± 8.0	72.3 ± 5.1	63.7 ± 7.6	64.7 ± 11.2
VAP (µm/sec)	128.1 ± 24.5	143.9 ± 14.2	115.1 ± 7.0	138.2 ± 12.8
VSL (µm/sec)	117.4 ± 26.2	134.7 ± 16.4	102.2 ± 10.5	124.0 ± 17.5
VCL (µm/sec)	182.4 ± 13.0	187.1 ± 9.5	171.0 ± 5.2	188.4 ± 6.8
Viability (%)	82.0 ± 2.0[a]	82.7 ± 3.1[a,c]	89.3 ± 3.0[b,c]	90.5 ± 2.8[b]
Recovery rate (%)	31.8 ± 4.1	36.1 ± 4.9	31.5 ± 2.5	30.9 ± 2.2

***** TM = total motility (%); PM = progressive motility (%); VAP = average pathway velocity (µm/sec); VSL = straight-line velocity (µm/sec); VCL = curvilinear velocity (µm/sec). Viability was assessed using the eosin-nigrosin stain.
[a-c]Values with different superscripts within the same row are different based on preliminary statistics ($P < 0.05$).

Pilot trial 2: effect of tube size during DGC on sperm recovery rate and in vitro sperm characteristics

The recovery rate was greater ($P < 0.05$) for 50 mL tubes (32.8 ± 0.5%) than 15 mL tubes (28.1 ± 1.2%). Sperm samples from 15 mL tubes (n=3 per treatment) displayed higher ($P < 0.05$) VAP (148.5 ± 1.5 µm/sec) and VSL (133.1 ± 2.9 µm/sec) when compared to 50 mL tubes (VAP: 127.3 ± 6.0 µm/sec, VSL: 115.2 ± 8.1 µm/sec). However, TM, PM and VCL remained similar ($P > 0.05$) for 15 mL tubes (TM: 84.3 ± 4.9%, PM: 65.3 ± 2.1%, VCL: 199.2 ± 3.1 µm/sec) and 50 mL tubes (TM: 76.3 ± 6.7%, PM: 62.0 ± 1.7%, VCL: 178.5 ± 6.5). Due to the increased velocity parameters, 15 mL tubes were used for the remainder of the study.

Pilot trial 3: effect of a non-centrifugation (gravity) separation technique on sperm recovery rate and in vitro sperm characteristics

When compared to a gravity separation technique (n=3), DGC showed higher ($P < 0.05$) TM (88.3 ± 1.5% versus 52.0 ± 14.8%), PM (83.3 ± 2.5% versus 49.3 ± 12.7%) and sperm recovery rate (29.5 ± 1.4% versus 22.4 ± 9.9%). However, all velocities (VAP, VSL and VCL) remained similar ($P > 0.05$) for both the gravity separation technique (VAP: 180.0 ± 1.4 µm/sec, VSL: 173.7 ± 1.6 µm/sec, VCL: 211.4 ± 1.5 µm/sec) and DGC (VAP: 178.6 ± 4.8 µm/sec, VSL: 170.8 ± 5.5 µm/sec, VCL: 220.1 ± 6.4 µm/sec). Based on these results, DGC was used throughout subsequent experiments.

Experiments

Experiment 1: effect of egg yolk as a component of HEPES-TALP medium for sperm washing procedures during DGC on sperm recovery and in vitro sperm characteristics

Recovery rate and motility parameters

Egg yolk supplementation (4%, v/v) of the HEPES-TALP medium used to re-suspend the sperm pellet after the first centrifugation step during DGC did not influence ($P > 0.05$) the sperm recovery rate. However, sperm motility was enhanced ($P < 0.05$) for parameters of TM, PM, VAP, VSL, VCL, RAP (RAP) and SLOW velocity groups in the presence of egg yolk than in its absence. The remaining parameters BCF, STR and MED velocity group were similar ($P > 0.05$) between the two media, while ALH was higher ($P < 0.05$) for HEPES-TALP medium without egg yolk (Table 3).

Table 3. Effect of egg yolk in the HEPES-TALP medium during density gradient centrifugation on sperm recovery rate and in vitro sperm characteristics (mean ± SD, n=9).

Sperm motility characteristics*	HEPES-TALP medium	
	With egg yolk	Without egg yolk
Total Motility (%)	91.9 ± 3.4[a]	83.4 ± 4.7[b]
Progressive Motility (%)	82.6 ± 5.9[a]	72.3 ± 6.4[b]
VAP (µm/sec)	161.9 ± 19.6[a]	142.4 ± 16.3[b]
VSL (µm/sec)	150.7 ± 21.4[a]	130.3 ± 17.4[b]
VCL(µm/sec)	217.6 ± 13.2[a]	203.2 ± 9.5[b]
ALH (µm)	6.1 ± 1.2[a]	6.5 ± 0.9[b]
BCF (Hz)	37.2 ± 2.0	35.2 ± 2.7
STR (%)	92.2 ± 2.2	90.5 ± 2.1
RAP (%)	91.4 ± 3.7[a]	82.1 ± 4.7[b]
MED (%)	0.8 ± 0.8	1.4 ± 0.5
SLOW (%)	6.0 ± 2.3[a]	9.7 ± 4.3[b]
Recovery rate (%)	22.4 ± 7.0	22.6 ± 6.1
Viability (%)	93.7 ± 2.0[a]	89.5 ± 3.2[b]

*TM = total motility (%); PM = progressive motility (%); VAP = average pathway velocity (µm/sec); VSL = straight-line velocity (µm/sec); VCL = curvilinear velocity (µm/sec); ALH = amplitude of lateral head displacement (µm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%). Viability was assessed using the eosin-nigrosin stain.
[a,b]Values with different superscripts within the same row are significantly different ($P < 0.05$).

Viability

Viability assessment using the eosin-nigrosin stain showed that inclusion of egg yolk in the HEPES-TALP medium improved ($P < 0.05$) the number of viable spermatozoa following DGC (Table 3).

Experiment 2: effect of conventional straw freezing and directional freezing technology on in vitro characteristics of non-sorted and sorted spermatozoa after recryopreservation

Motility parameters

Overall, motility parameters were influenced by stage of processing (post-thaw, post-DGC, post-stain, post-sort, pre-freeze and post-second thaw) sperm type (non-sorted [Control] and sorted [FSF]) and freeze 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 4. Overall, compared with the non-sorted controls, the processing of frozen-thawed spermatozoa for sorting resulted in significantly improved motility parameters at the pre-freeze of recryopreservation (Table 4).

Motility parameters following the first thawing step (first post-thaw) and DGC (post-DGC)

Post-thaw samples from the first cryopreservation retained high proportions of their pre-freeze TM and PM (87.9 ± 7.3% and 92.2 ± 5.9%, respectively). The post-DGC sperm characteristics TM, PM, VAP, VSL, VCL, STR, RAP and MED were higher ($P < 0.05$, Table 4) than after the first thaw. SLOW and ALH were lower following DGC compared to post-thaw.

Table 4. Motility parameters of spermatozoa after the first post-thaw, during and after flow cytometric sorting, and prior to recryopreservation (mean ± SD, n=18).

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

*TM = total motility (%); PM = progressive motility (%); VAP = average pathway velocity (µm/sec); VSL = straight-line velocity (µm/sec); VCL = curvilinear velocity (µm/sec); ALH = amplitude of lateral head displacement (µm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).

[a-d]Values with different superscripts within the same row are significantly different ($P < 0.05$).

Motility parameters following staining and sorting (post-stain and post-sort)

With the exception of STR, the process of staining (incubation of spermatozoa at 35 °C with H33342) did not significantly affect motility parameters of spermatozoa selected by the DGC step (Table 4). Following the sorting process, PM, VCL, 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 ($P > 0.05$). As seen with samples after DGC, all post-sort motility parameters except ALH, were significantly improved compared to those observed at the first post-thaw step (Table 4).

Motility parameters prior to recryopreservation (Pre-freeze)

Pre-freeze characteristics of sorted spermatozoa remained similar to post-DGC, post-stain and post-sort values for TM, PM, VAP, VSL and MED ($P > 0.05$). Sorted spermatozoa VCL and RAP decreased ($P < 0.05$) and SLOW increased ($P < 0.05$) at the pre-freeze stage compared to the post-sort stage. Sorted 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. Sorted 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. Sorted 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. 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 sorted 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 4).

Motility parameters at 0 h after the second thawing step (0 h post-thaw)

The recryopreservation process had a negative impact ($P < 0.05$) on both Control and FSF spermatozoa for TM, PM, VAP, VSL, VCL and RAP. At 0 h post-thaw, within Control and FSF treatments, samples frozen using the straw and DF methods were similar ($P > 0.05$) in VAP, VSL, VCL, ALH and MED. For Control spermatozoa, TM, PM, BCF and RAP were also higher ($P < 0.05$) for DF than straw samples. For FSF spermatozoa, TM, PM and RAP were higher ($P < 0.05$) for samples frozen using DF than straws, and BCF was similar for both freeze methods ($P > 0.05$). When comparisons were made within each freezing method (DF or Straw), FSF spermatozoa exhibited higher TM, RAP and MED ($P < 0.05$), similar ($P > 0.05$) VAP, VSL, VCL, ALH and BCF, and lower ($P < 0.05$) SLOW than Control spermatozoa (Table 5; Fig. 3 to 5). However, compared to the straw method, DF samples of both sperm types displayed higher TM, PM, BCF and RAP, whereas VAP, VSL, VCL, ALH, STR and MED were similar across freezing methods, and SLOW was higher ($P < 0.05$) for the straw method (Table 5; Fig. 3 to 5).

The two combinations of freezing method and sperm treatment that achieved the highest TM and PM at 0 h post-thaw were FSF spermatozoa frozen with the DF method (TM: 38.1 ± 9.3% and PM: 34.3 ± 9.5%) and FSF spermatozoa frozen with the straw method (TM: 30.8 ± 7.5% and PM: 27.2 ± 6.8%).

Table 5. Sperm motility characteristics at 0 h post-thaw (mean ± SD, n=18).

Sperm motility characteristics*	Sperm type and freezing method			
	Control		Sort	
	Directional	Straw	Directional	Straw
ALH (µm)	4.2 ± 0.4	4.15 ± 0.4	4.3 ± 0.4	4.4 ± 0.5
BCF (Hz)	38.2 ± 2.2[a]	36.7 ± 2.3[b]	38.4 ± 2.3[a,b]	37.0 ± 2.5[a,b]
STR (%)	94.3 ± 1.7[a]	94.2 ± 1.8[a]	96.0 ± 1.2[b]	95.1 ± 1.0[b]
RAP (%)	24.2 ± 10.3[a]	21.1 ± 10.6[b]	35.1 ± 9.6[c]	28.3 ± 7.0[d]
MED (%)	1.7 ± 1.7[a]	1.5 ± 1.3[a]	2.9 ± 1.6[b]	2.6 ± 1.3[b]
SLOW (%)	19.0 ± 14.3[a]	17.9 ± 21.2[a]	11.0 ± 5.7[b]	12.3 ± 9.5[b]

*ALH = amplitude of lateral head displacement (µm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).
[a-d]Values with different superscripts within the same row are significantly different ($P < 0.05$).

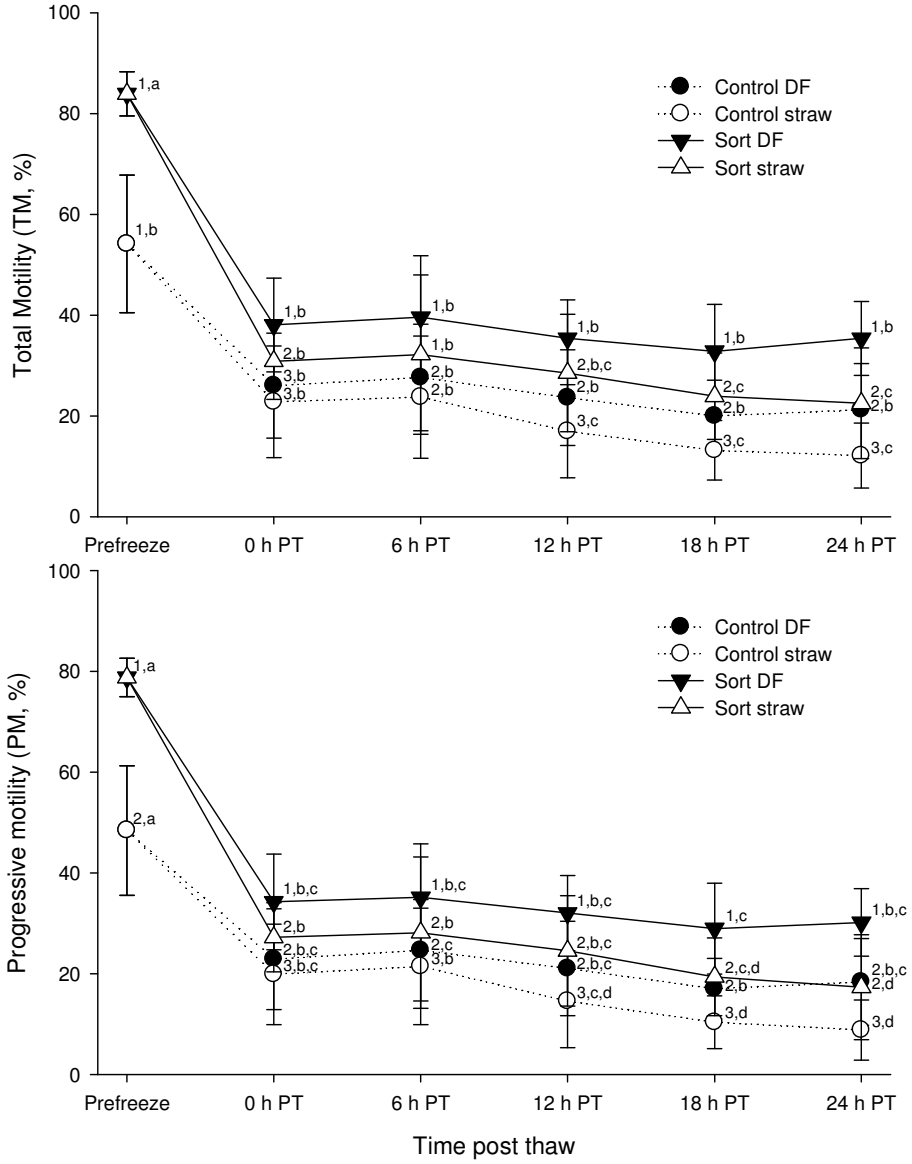

Fig. 3. Total motility (top graph) and progressive motility (bottom graph) of frozen-thawed, non-sorted, frozen-thawed (Control) spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, re-frozen-thawed (Sort) spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. [a-d,1-3]Values with different letters are significantly different ($P < 0.05$) across incubation time, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

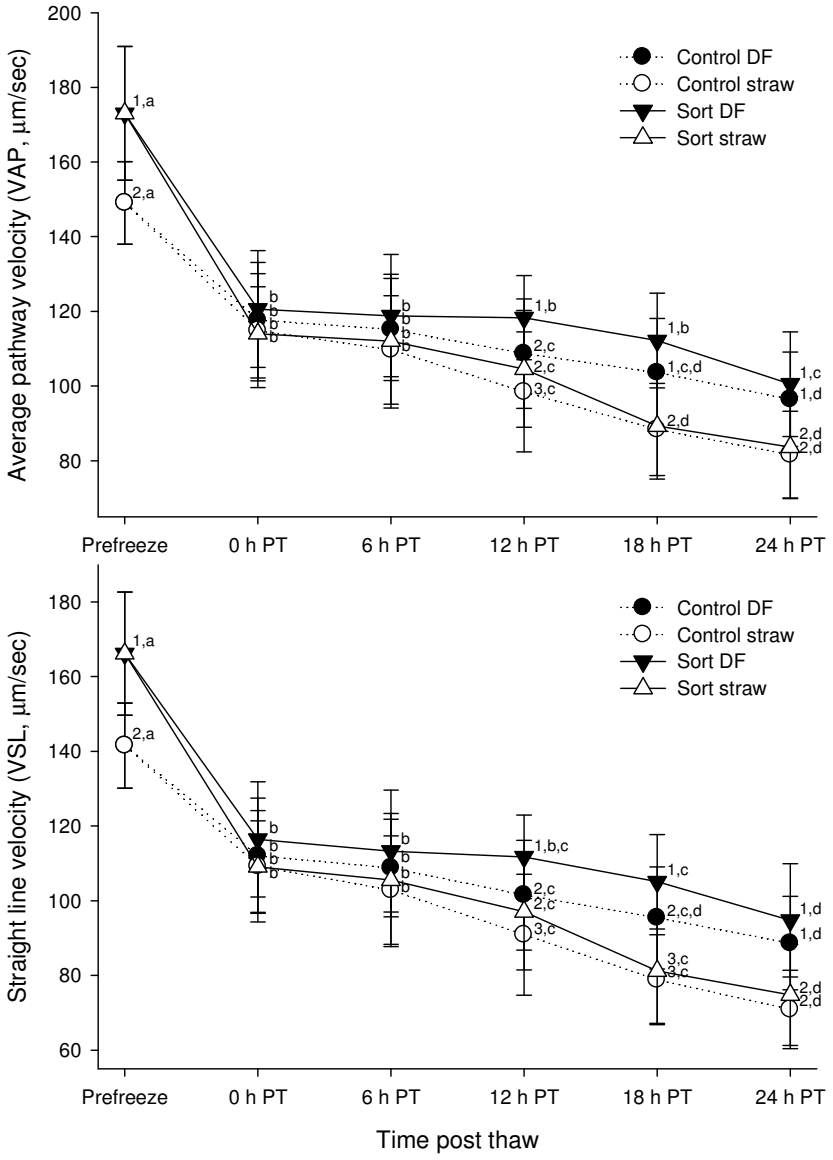

Fig. 4. Average pathway velocity (top graph) and straight line velocity (bottom graph) of frozen-thawed, non-sorted, frozen-thawed (Control) spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, re-frozen-thawed (Sort) spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. [a-d,1-3]Values with different letters are significantly different ($P < 0.05$) across incubation time, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

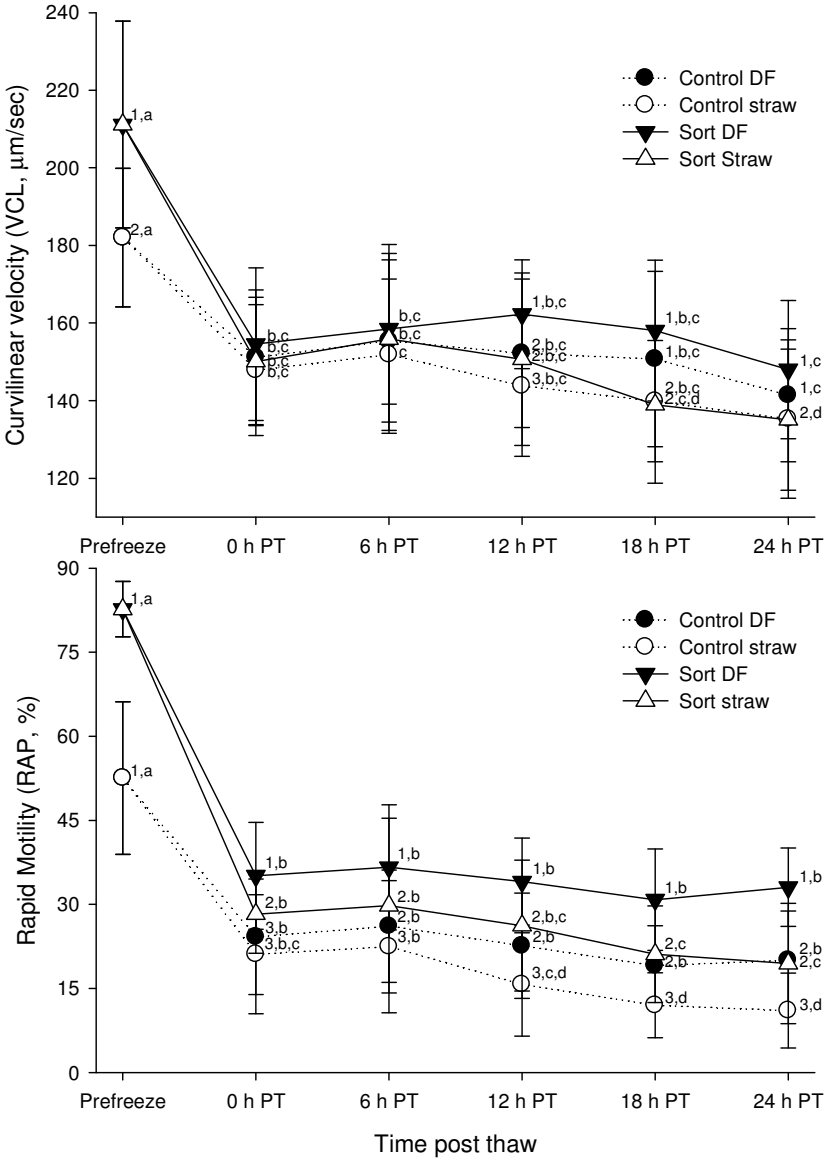

Fig. 5. Curvilinear velocity (top graph) and rapid velocity (bottom graph) of frozen-thawed, non-sorted, frozen-thawed (Control) spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, re-frozen-thawed (Sort) spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. [a-d,1-3]Values with different letters are significantly different ($P < 0.05$) across incubation time, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

Motility parameters at 6 h after the second thawing step (6 h post-thaw)

Within sperm type (Control or FSF), TM and PM were similar for DF and straw freezing methods ($P > 0.05$). Within freezing method, FSF spermatozoa presented higher ($P < 0.05$) TM, PM RAP and MED than Control spermatozoa, and similar VAP, VSL, VCL, ALH, BCF, STR and SLOW ($P > 0.05$). Regardless of the sperm type, DF samples displayed higher ($P < 0.05$) VAP, VSL and BCF than straw samples, and VCL, ALH, STR, RAP, MED and SLOW for both freeze methods were similar ($P > 0.05$) (Table 6; Fig. 3 to 5).

As observed at 0 h post-thaw, the two combinations of freezing method and sperm type that achieved the highest TM and PM at 6 h post-thaw were FSF spermatozoa frozen with the DF method (TM: 39.6 ± 12.2% and PM: 35.2 ± 10.6%) and FSF spermatozoa frozen with the straw method (TM: 32.2 ± 15.8% and PM: 28.1 ± 15.0%).

Table 6. Sperm motility characteristics at 6 h post-thaw (mean ± SD, n=18).

Sperm motility characteristics*	Sperm type and freezing method			
	Control		Sort	
	Directional	Straw	Directional	Straw
ALH (μm)	4.7 ± 0.6	5.0 ± 0.9	5.8 ± 0.5	5.1 ± 0.8
BCF (Hz)	35.3 ± 2.9[a]	34.1 ± 3.6[a]	36.9 ± 3.4[b]	33.3 ± 3.4[b]
STR (%)	93.7 ± 2.1	93.3 ± 2.1	94.5 ± 1.5	93.6 ± 2.1
RAP (%)	26.1 ± 10.0[a]	22.5 ± 11.8[a]	36.6 ± 11.2[b]	29.8 ± 15.6[a,b]
MED (%)	1.4 ± 1.3[a]	1.3 ± 1.2[a]	2.9 ± 2.3[b]	2.4 ± 1.9[b]
SLOW (%)	11.7 ± 1.3	14.1 ± 11.3	9.3 ± 6.8	9.7 ± 5.4

*ALH = amplitude of lateral head displacement (μm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).
[a,b] Values with different superscripts within the same row are significantly different ($P < 0.05$).

Motility parameters at 12 h after the second thawing step (12 h post-thaw)

Within sperm type (Control or FSF), all parameters (TM, PM, VAP, VSL, VCL, ALH, BCF, STR, RAP, MED and SLOW) were similar ($P > 0.05$) for DF and straw freezing methods. Within freeze method, Control and FSF spermatozoa presented similar ($P > 0.05$) values for all motility parameters: TM, PM, VAP, VSL, VCL, ALH, BCF, STR, RAP, MED and SLOW. Regardless of the freezing method, FSF spermatozoa presented higher ($P < 0.05$) TM, PM, VAP, VSL, VCL, BCF, STR and RAP than Control spermatozoa, and similar ALH, MED and SLOW ($P > 0.05$). For both sperm types, DF samples displayed higher TM, PM, VAP, VSL, VCL, BCF, STR and RAP ($P < 0.05$) than straw samples, and MED and SLOW for both freeze methods were similar ($P > 0.05$). Control and sorted samples displayed higher ALH after freezing with the straw than the DF method ($P < 0.05$; Table 7, Fig. 3 to 5).

As observed at 0 h and 6 h post-thaw, the two combinations of freezing method and sperm type that achieved the highest TM and PM at 12 h post-thaw were FSF spermatozoa frozen with the DF method (TM: 35.4 ± 7.7% and PM: 32.1 ± 7.5%) and FSF spermatozoa frozen with the straw method (TM: 28.5 ± 11.6% and PM: 24.5 ± 10.9%).

Table 7. Sperm motility characteristics at 12 h post-thaw (mean ± SD, n=18).

Sperm motility characteristics*	Sperm type and freezing method			
	Control		Sort	
	Directional	Straw	Directional	Straw
ALH (μm)	5.1 ± 0.7[a]	5.3 ± 0.6[b]	4.5 ± 0.5[a]	5.4 ± 1.0[b]
BCF (Hz)	33.5 ± 3.1[a]	30.4 ± 3.7[b]	36.2 ± 2.5[c]	31.4 ± 3.9[d]
STR (%)	92.5 ± 1.9[a]	91.6 ± 2.6[b]	93.8 ± 1.5[c]	92.3 ± 2.7[a,d]
RAP (%)	22.6 ± 9.4[a]	15.7 ± 9.2[b]	34.1 ± 7.8[c]	26.2 ± 11.7[a,d]
MED (%)	1.1 ± 0.8	1.1 ± 0.9	1.4 ± 0.8	2.1 ± 2.5
SLOW (%)	9.4 ± 5.8	12.2 ± 18.0	9.9 ± 0.8	10.0 ± 9.8

*ALH = amplitude of lateral head displacement (μm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).
[a-e]Values with different superscripts within the same row are significantly different ($P < 0.05$).

Motility parameters at 18 h after the second thawing step (18 h post-thaw)

Within sperm type (Control or FSF), TM, PM, VAP, VSL, VCL, ALH, BCF, RAP, MED and SLOW were similar ($P > 0.05$) for DF and straw freezing methods, and STR was higher ($P < 0.05$) for DF than the straw method. Within freeze method, Control and FSF spermatozoa presented similar ($P > 0.05$) values for TM, PM, VAP, VSL, VCL, ALH, BCF, RAP, MED and SLOW. However, STR was higher for FSF than Control spermatozoa within freeze method. Regardless of the freezing method, FSF spermatozoa presented higher ($P < 0.05$) TM, PM, VAP, VSL, BCF, STR, RAP and MED than Control spermatozoa, and similar VCL, ALH and SLOW ($P > 0.05$). For both sperm types, DF samples displayed higher TM, PM, VAP, VSL, VCL, BCF, STR and RAP ($P < 0.05$) than straw samples, and MED and SLOW for both freeze methods were similar ($P > 0.05$). Control and sorted samples displayed higher ALH after freezing with the straw method than the DF method ($P < 0.05$; Table 8, Fig. 3 to 5).

As observed at 0 h, 6 h and 12 h post-thaw, the two combinations of freezing method and sperm type that achieved the highest TM and PM at 18 h post-thaw were FSF spermatozoa frozen with the DF method (TM: 32.8 ± 9.3% and PM: 28.9 ± 9.0%) and FSF spermatozoa frozen with the straw method (TM: 23.9 ± 8.6% and PM: 19.4 ± 7.7%).

Table 8. Sperm motility characteristics at 18 h post-thaw (mean ± SD, n=18).

Sperm motility characteristics*	Sperm type and freeze method			
	Control		Sort	
	Directional	Straw	Directional	Straw
ALH (µm)	5.23 ± 0.7[a]	5.8 ± 0.5[b]	5.2 ± 0.6[a]	5.7 ± 0.7[b]
BCF (Hz)	32.1 ± 2.8[a]	27.1 ± 3.0[b]	34.7 ± 3.5[c]	28.3 ± 3.6[b,d]
STR (%)	91.4 ± 2.0[a]	88.9 ± 2.2[b]	92.9 ± 1.5[c]	90.6 ± 2.4[d]
RAP (%)	19.1 ± 7.1[a]	12.0 ± 5.8[b]	30.8 ± 9.0[c]	21.1 ± 8.6[d]
MED (%)	1.0 ± 1.0[a]	1.3 ± 0.6[a]	2.1 ± 1.6[b]	2.8 ± 1.8[b]
SLOW (%)	17.1 ± 12.2	17.3 ± 20.9	11.8 ± 10.0	15.3 ± 17.8

*ALH = amplitude of lateral head displacement (µm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).
[a-d]Values with different superscripts within the same row are significantly different ($P < 0.05$).

Motility parameters at 24 h after the second freezing step (24 h post-thaw)

Within sperm type (Control or FSF), TM, VSL, VCL, BCF, STR, MED and SLOW were similar ($P > 0.05$) between DF and straw freezing methods, PM, VAP and RAP were higher ($P < 0.05$) for DF than the straw method. Also within sperm type, ALH was higher ($P < 0.05$) for straw than DF method within Control spermatozoa but within FSF spermatozoa, ALH was similar ($P > 0.05$) for DF and Straw methods. Within freeze method, Control and FSF spermatozoa presented similar ($P > 0.05$) values for TM, VAP, VSL, VCL, ALH, BCF, STR and SLOW. However, PM, RAP and MED were higher ($P < 0.05$) for FSF than Control spermatozoa within freeze method, but MED was higher only within the straw method. Regardless of the freezing method, FSF spermatozoa presented higher ($P < 0.05$) TM, PM, VSL, BCF, STR, RAP, MED and SLOW than Control spermatozoa, and similar VAP, VCL, and ALH ($P > 0.05$). Regardless of sperm type, DF samples displayed higher TM, PM, VAP, VSL, VCL, BCF, STR and RAP ($P < 0.05$) than straw samples, MED and SLOW for both freeze methods were similar ($P > 0.05$), and ALH was higher for the straw method than the DF method ($P < 0.05$); Table 9; Fig. 3 to 5).

As observed as 0 h, 6 h, 12 h and 18 h post-thaw, the two combinations of freezing method and sperm type that achieved the highest TM and PM at 24 h were FSF spermatozoa frozen with the DF method (TM: 35.4 ± 7.3% and PM: 30.2 ± 6.7%) and FSF spermatozoa frozen with the straw method (TM: 22.5 ± 11.0% and PM: 17.3 ± 10.4%).

Table 9. Sperm motility characteristics at 24 h post-thaw (mean ± SD, n=18).

Sperm motility characteristics*	Sperm type and freezing method			
	Control		Sort	
	Directional	Straw	Directional	Straw
ALH (μm)	5. 2 ± 0.8[a]	5.6 ± 0.8[b]	5.3 ± 0.6[a]	5.7 ± 0.8[b]
BCF (Hz)	29.7 ± 3.4[a]	24.6 ± 2.9[b]	32.1 ± 4.0[a]	26.6 ± 3.9[b]
STR (%)	91.2 ± 1.3[a]	86.7 ± 3.4[b]	92.3 ± 1.9[c]	88.9 ± 2.8[d]
RAP (%)	20.1 ± 8.8[a]	11.1 ± 6.7[b]	33.0 ± 7.0[c]	19.4 ± 10.7[d]
MED (%)	1.1 ± 1.1[a]	1.1 ± 1.1[a]	3.1 ± 2.6[b]	3.1 ± 1.8[b]
SLOW (%)	8.7 ± 6.9[a]	9.3 ± 7.4[a]	13.9 ± 9.6[b]	18.1 ± 16.3[b]

*ALH = amplitude of lateral head displacement (μm); BCF = beat cross frequency (Hz); STR = straightness of sperm movement (%); RAP = rapid velocity group (%); MED= medium velocity group (%); SLOW = slow velocity group (%).
[a-d]Values with different superscripts within the same row are significantly different ($P < 0.05$).

Longevity of sperm motility after the second thawing within each treatment method

During the 24 h post-thaw incubation at room temperature, TM remained unchanged ($P > 0.05$) 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 TM after 12 h of incubation. The FSF spermatozoa frozen with the straw method also showed a decrease ($P < 0.05$) in TM, however, the rate of decrease was slower.

A constant ($P > 0.05$) PM was observed for FSF spermatozoa frozen with the DF method during the 24 h incubation (Fig. 3). For Control and FSF spermatozoa frozen with the straw method, PM remained unchanged ($P > 0.05$) during first 6 h post-thaw and the first decrease ($P < 0.05$) was observed at 12 h post-thaw followed by a constant decrease ($P < 0.05$) until 24 h post-thaw. For Control spermatozoa frozen with the DF method, PM remained unchanged ($P > 0.05$) during the first 12 h, decreased ($P < 0.05$) at 18 h and increased ($P < 0.05$) at 24 h.

Average pathway velocity remained unchanged ($P > 0.05$) for FSF spermatozoa frozen with the DF method until 18 h post-thaw, presenting a decrease ($P < 0.05$) in VAP only at 24 h post-thaw (Fig. 4). For FSF spermatozoa frozen with the straw method, the constant ($P > 0.05$) decrease ($P < 0.05$) in VAP was first observed at 12 h post-thaw. Control spermatozoa frozen with both straw and DF methods showed a decrease ($P < 0.05$) in VAP at 12 h and another decrease at 18 h, which remained unchanged ($P > 0.05$) at 24 h.

Straight line velocity decreased after 18 h and again at 24 h of incubation for FSF spermatozoa frozen with the DF method (Fig. 4). 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.

Both sperm types (Control and FSF) frozen with the DF method showed similar ($P > 0.05$) VCL across incubation periods (Fig. 5). Both sperm types (Control and FSF) frozen with the straw method showed a decrease ($P < 0.05$) in VCL at 24 h post-thaw.

Rapid velocity was similar ($P > 0.05$) throughout the incubation period for Control and FSF spermatozoa frozen with the DF method (Fig. 5). Control spermatozoa frozen with the straw method showed a decrease ($P < 0.05$) in RAP at 12 h post-thaw, whereas the same sperm type frozen with the DF method showed a decrease ($P < 0.05$) in RAP at 18 h post-thaw.

Overall, FSF spermatozoa frozen with the DF method maintained higher ($P < 0.05$) motility parameters across the 24 h post-thaw period than Control spermatozoa frozen with

the DF method and both Control and FSF spermatozoa frozen with the straw method (Fig 1 to 3).

Male effect on total and progressive motility

While there were no differences in pre-freeze TM and PM among males, significant differences were observed during incubation period post-thaw (Table 10). Overall, Male 4 displayed superior TM and PM than other males throughout the 24 h incubation period post-thaw ($P < 0.05$).

Table 10. Male effect on motility parameters during the 24 h incubation period after the second thawing step (mean ± SD, n=18).

Time post-thaw	Motility parameter	Male		
		2	3	4
0 h	TM (%)	27.2 ± 9.6[a]	26.5 ± 13.2[a]	34.6 ± 8.4[b]
	PM (%)	23.8 ± 8.7[a]	23.1 ± 12.3[a]	31.3 ± 8.4[b]
6 h	TM (%)	28.2 ± 12.2[a]	26.3 ± 12.2[a]	38.5 ± 14.2[b]
	PM (%)	25.5 ± 10.8[a]	22.3 ± 10.2[a]	35.2. ± 13.6[b]
12 h	TM (%)	25.0 ± 8.7[a]	22.0 ± 11.9[a]	31.3 ± 12.3[b]
	PM (%)	22.3 ± 8.1[a]	18.5 ± 11.4[a]	28.4 ± 11.6[b]
18 h	TM (%)	21.4 ± 10.1[a]	18.5 ± 8.2[a]	27.5 ± 11.2[b]
	PM (%)	18.5 ± 9.4[a]	15.0 ± 7.5[a]	23.3 ± 10.4[b]
24 h	TM (%)	23.1 ± 11.0[a,b]	19.8 ± 12.9[a]	25.5 ± 11.5[b]
	PM (%)	19.7 ± 10.2[a,b]	15.3 ± 11.4[a]	21.1 ± 11.0[b]

*TM = total motility (%); PM = progressive motility (%)

[a,b]Values with different superscripts within the same column are significantly different ($P < 0.05$).

Viability and acrosome integrity

Overall, viability and acrosome integrity were influenced by sperm type and stage of processing ($P < 0.05$, Table 11). The effect of sperm treatment on viability and acrosome integrity at various stages of processing following the first cryopreservation is displayed in Table 11. The effects of sperm treatment and freeze method on viability and acrosome integrity during incubation following the second thawing are displayed in Fig. 6.

Table 11. Viability and acrosome integrity of spermatozoa after the first post-thaw, during and after flow cytometric sorting, and prior to recryopreservation (mean ± SD, n = 18).

Sperm characteristics	Stage of processing					
	First post-thaw	Post-DGC	Post-stain	Post-sort	Pre-freeze Sort	Pre-freeze Control
Eosin-nigrosin						
Viability (%)	63.4 ± 8.4[a]	81.2 ± 7.7[b,c]	79.4 ± 7.5[b,c]	92.7 ± 3.2[d]	84.0 ± 4.9[b]	54.8 ± 10.7[e]
PI/FITC-PNA						
Viable, intact acrosome (%)	46.3 ± 9.1[a]	87.3 ± 6.2[b]	86.3 ± 5.8[b]	88.3 ± 5.9[b]	80.2 ± 5.2[b]	43.2 ± 8.7[a]
Viable, damaged/reacted acrosome (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
Non-viable, intact acrosome (%)	50.1 ± 9.2[a]	11.0 ± 6.3[b]	13.2 ± 6.1[b,c]	10.0 ± 5.4[b]	18.9 ± 4.8[c]	51.4 ± 7.6[a]
Non-viable, damaged/reacted acrosome (%)	3.7 ± 1.6	1.5 ± 0.5	0.4 ± 0.7	1.3 ± 0.9	1.5 ± 1.3	5.0 ± 2.9

[a-e] Values with different superscripts within the same row are significantly different ($P < 0.05$).

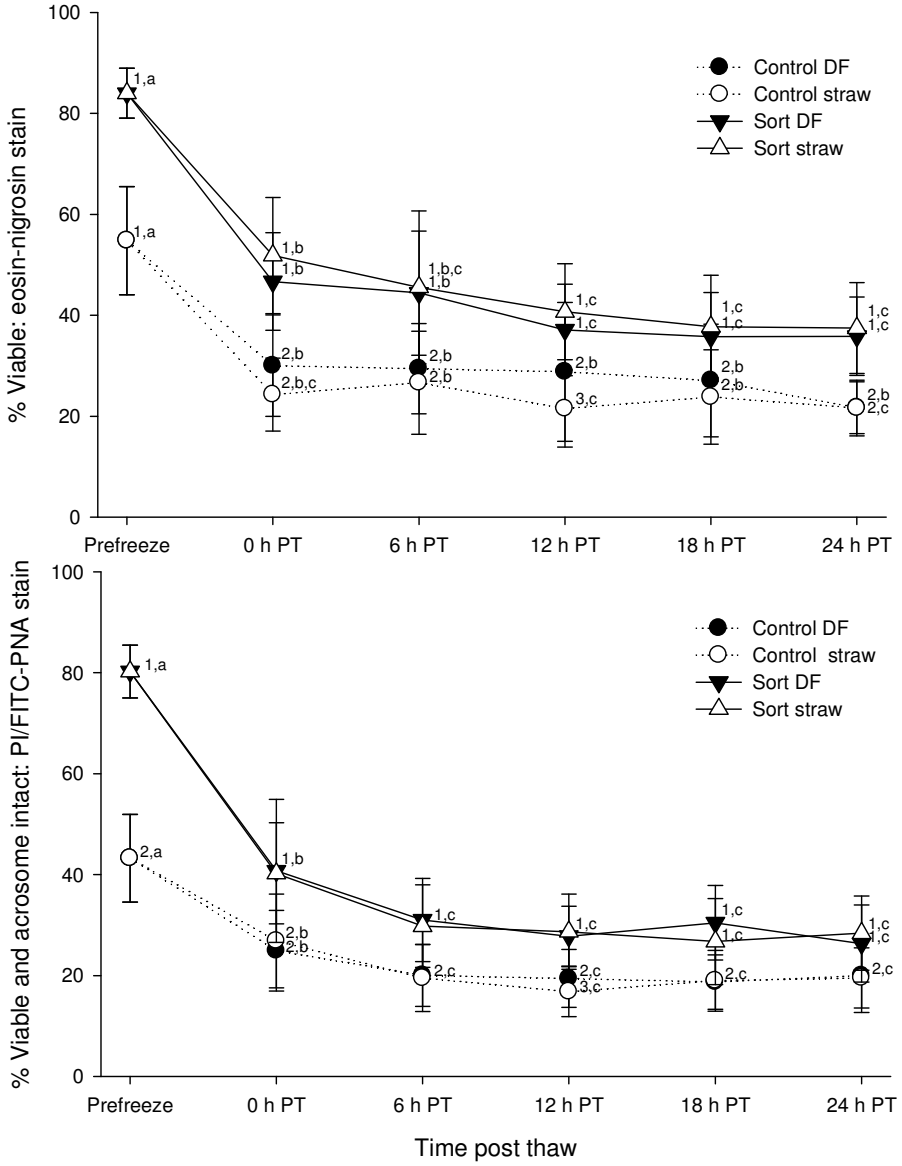

Fig. 6. Top graph: viable spermatozoa assessed with eosin-nigrosin stain, bottom graph: viable spermatozoa with intact acrosome stained with PI/FITC-PNA fluorescent stain before recryopreservation and during post-thaw incubation for 24 h at room temperature. [a-c]Values with different numbers in superscript row are significantly different ($P < 0.05$) across incubation time, and values with different letters in superscript are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

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 PI (in the PI/FITC-PNA staining method). Photomicrographs of spermatozoa after PI/FITC-PNA staining are displayed in Fig. 7.

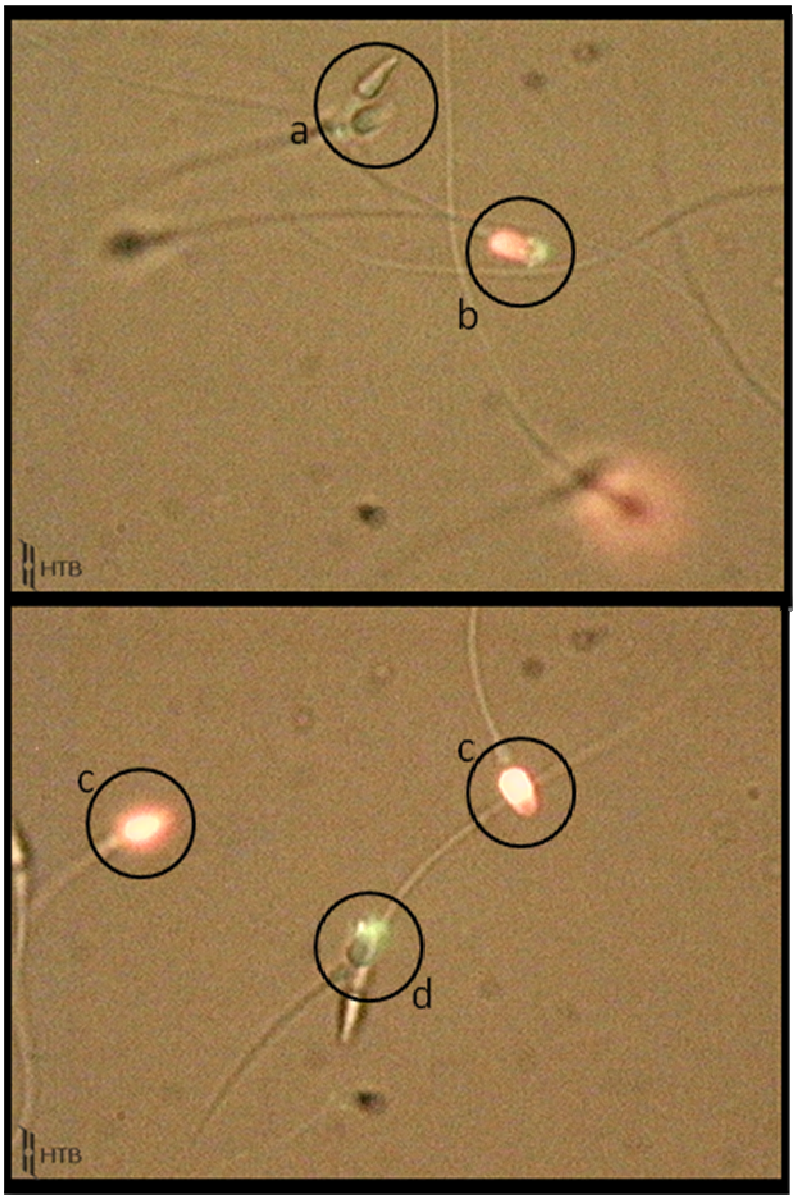

Fig. 7. Photomicrographs of spermatozoa after staining with PI/FITC-PNA (X 400 original magnification). Top picture: (a) two viable spermatozoa with an intact acrosome (no fluorescence) and (b) one non-viable spermatozoon with a damage/reacted acrosome (red and green fluorescence), bottom picture: (c) two non-viable spermatozoa with an intact acrosome (red fluorescence), one viable spermatozoon with a damaged/reacted acrosome (green fluorescence).

Viability and acrosome integrity following the first thawing step and density gradient centrifugation

The percentage of viable spermatozoa using eosin-nigrosin (percent viable) and PI/FITC-PNA (percent viable and acrosome intact) was higher ($P < 0.05$) after DGC than after the first post-thaw. The percentage of non-viable spermatozoa with an intact acrosome was higher ($P < 0.05$) at the first post-thaw than at the post-DGC step. The proportions of viable and non-viable spermatozoa with a reacted/damaged acrosome were similar at the post-thaw and post-DGC steps (Table 11).

Viability parameters and acrosome integrity following staining and sorting

Sperm viability and acrosome integrity remained unchanged ($P < 0.05$) between the DGC and staining steps. Flow cytometric sorting increased the proportion of viable spermatozoa ($P < 0.05$) when samples were evaluated with eosin-nigrosin. However, when PI/FITC-PNA was used, the post-sort viability and acrosome integrity results were similar to those observed at the post-stain step ($P > 0.05$, Table 11).

Viability and acrosome integrity prior to recryopreservation

Before recryopreservation, Control spermatozoa presented a decrease ($P < 0.05$) in viability (assessed with eosin-nigrosin) when compared to the post-sort step. When assessed using PI/FITC-PNA, Control spermatozoa showed unchanged ($P > 0.05$) non-viable and viable spermatozoa with or without damaged/reacted acrosome between the post-sort and pre-freeze steps.

Viability evaluated using eosin-nigrosin was higher for FSF than Control pre-freeze ($P < 0.05$). The percentage of viable spermatozoa with an intact acrosomes assessed by Pi/FITC-PNA was higher ($P < 0.05$) for FSF than Control spermatozoa prior to re-cryopreservation. Non-viable spermatozoa with an intact acrosome were higher ($P < 0.05$) for Control than FSF pre-freeze (Table 11).

Longevity of sperm viability and acrosome integrity after the second thawing

A detrimental effect ($P < 0.05$) of the second freeze-thaw process on sperm viability and acrosome integrity was observed after evaluation using eosin-nigrosin and PI/FITC-PNA staining, for Control and FSF spermatozoa. The reduction ($P < 0.05$) in viability between the pre-freeze and 0 h post-thaw step was observed for both sperm types (Control and FSF) and freezing methods (straw and DF). FSF spermatozoa showed a constantly higher ($P < 0.05$) viability than Control spermatozoa across incubation period. The percentage of live spermatozoa with a damaged or reacted acrosome was less than 2.1 % across all treatments.

Male effect on viability and acrosome integrity

The percentage of viable, acrosome intact spermatozoa was higher ($P < 0.05$) for Male 4 than Males 2 and 3 at all post-thaw time points (data not shown).

Sperm DNA denaturation

Sperm DNA denaturation following the first thawing step and density gradient centrifugation

$Mean_{\alpha t}$ and $COMP_{\alpha t}$ of sperm samples were similar ($P > 0.05$) between first post-thaw and post-DGC, whereas $SD_{\alpha t}$ was lower for spermatozoa at post-DGC than at first post-thaw (Fig. 8).

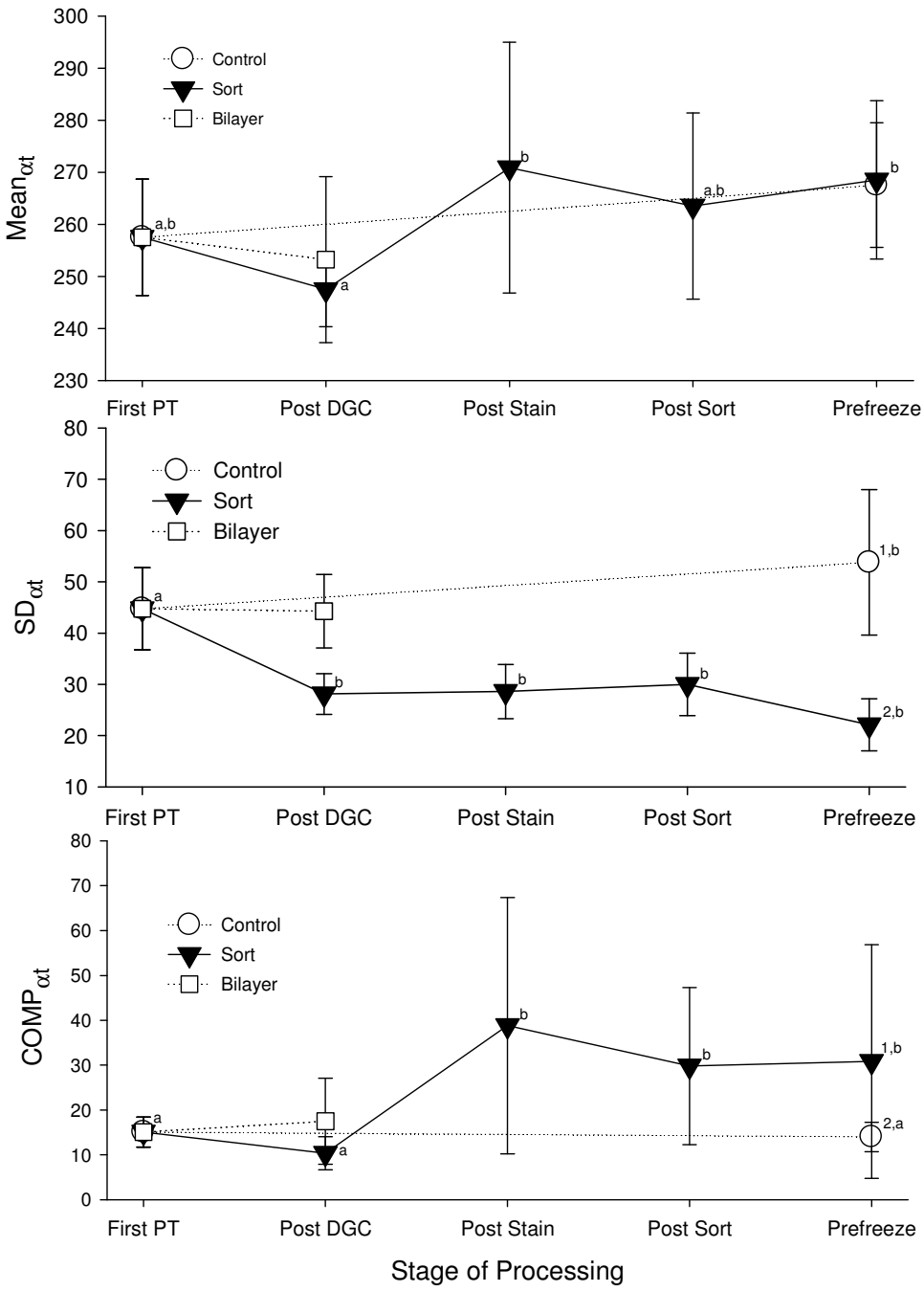

Fig. 8. Top graph: Mean$_{\alpha t}$, middle graph: SD$_{\alpha t}$ and bottom graph: COMP$_{\alpha t}$ of sperm samples at first thawing, during sorting process and prior to recryopreservation of sorted and control spermatozoa. Spermatozoa from bilayer samples are displayed at the post-DGC time point. ^{a,b,1,2}Values with different letters are significantly different ($P < 0.05$) across incubation time, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

Sperm DNA denaturation of bilayer spermatozoa

Spermatozoa from the Bilayer samples (n = 5) showed similar ($P > 0.05$) $Mean_{\alpha t}$ (Power = 0.069) and $COMP_{\alpha t}$ (Power = 0.163) to first post-thaw and post-DGC spermatozoa. Spermatozoa from the bilayer presented higher ($P < 0.05$) $SD_{\alpha t}$ (Power = 1.000) than post-DGC spermatozoa and similar ($P > 0.05$) to first post-thaw spermatozoa.

Sperm DNA denaturation following staining and sorting

Sperm samples presented an increase ($P < 0.05$) in $Mean_{\alpha t}$ and $COMP_{\alpha t}$ for post-stain compared to post-DGC, whereas $SD_{\alpha t}$ remained similar ($P > 0.05$, Fig. 8). Post-sort sperm samples exhibited higher ($P < 0.05$) $COMP_{\alpha t}$ and similar ($P > 0.05$) $Mean_{\alpha t}$ and $SD_{\alpha t}$ compared to post-stain sperm samples (Fig. 8).

Sperm DNA denaturation prior to recryopreservation

$Mean_{\alpha t}$ was similar ($P > 0.05$) for both sorted and Control spermatozoa and $SD_{\alpha t}$ was higher ($P < 0.05$) for Control than sorted samples (Fig. 8). Sorted spermatozoa exhibited higher ($P < 0.05$) $COMP_{\alpha t}$ than Control spermatozoa (Fig. 8).

Sperm DNA denaturation at 0 h post-thaw

Second cryopreservation decreased ($P < 0.05$) $Mean_{\alpha t}$ at 0 h post-thaw compared to pre-freeze stage for FSF spermatozoa frozen with straw and DF methods and Control spermatozoa frozen by the straw method, while Control spermatozoa frozen with the DF method presented similar ($P > 0.05$) $Mean_{\alpha t}$ than Control spermatozoa at the pre-freeze stage (Fig. 9). Regardless of the freezing method, $COMP_{\alpha t}$ of FSF and Control spermatozoa was lower compared to the pre-freeze stage

(Fig. 9). Control spermatozoa frozen with the straw and DF methods at 0 h post-thaw presented lower ($P < 0.05$) $SD_{\alpha t}$ compared to Control spermatozoa at the pre-freeze stage, while FSF spermatozoa frozen by the straw and DF methods exhibited similar ($P > 0.95$) $SD_{\alpha t}$ for pre-freeze and 0 h post-thaw (Fig 9).

$Mean_{\alpha t}$ was similar ($P > 0.05$) for both sperm type (Control and FSF) frozen with both freezing methods (DF and straw). Control spermatozoa frozen with the DF and straw method exhibited higher ($P < 0.05$) $SD_{\alpha t}$ than FSF frozen with the DF and straw method. Within sperm type, $COMP_{\alpha t}$ was similar ($P > 0.05$) for DF and straw freezing methods. Regardless of the freezing method, $COMP_{\alpha t}$ was higher ($P < 0.05$) for FSF spermatozoa than Control spermatozoa.

Sperm DNA denaturation at 6 h post-thaw

Within sperm type (Control and FSF), $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ were similar ($P > 0.05$) for both freezing methods, and FSF spermatozoa showed higher ($P < 0.05$) $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ than Control spermatozoa (Fig. 9).

Sperm DNA denaturation at 12 h post-thaw

As observed at 6 h post-thaw, within sperm type, $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ were similar ($P > 0.05$) for both freezing methods, and FSF spermatozoa showed higher ($P < 0.05$) $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ than Control spermatozoa (Fig. 9).

Sperm DNA denaturation at 18 h post-thaw

As observed at 6 h and 12 h post-thaw, within sperm type, $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ were similar ($P > 0.05$) for both freezing methods and FSF spermatozoa showed higher ($P < 0.05$) $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ than Control spermatozoa (Fig. 9).

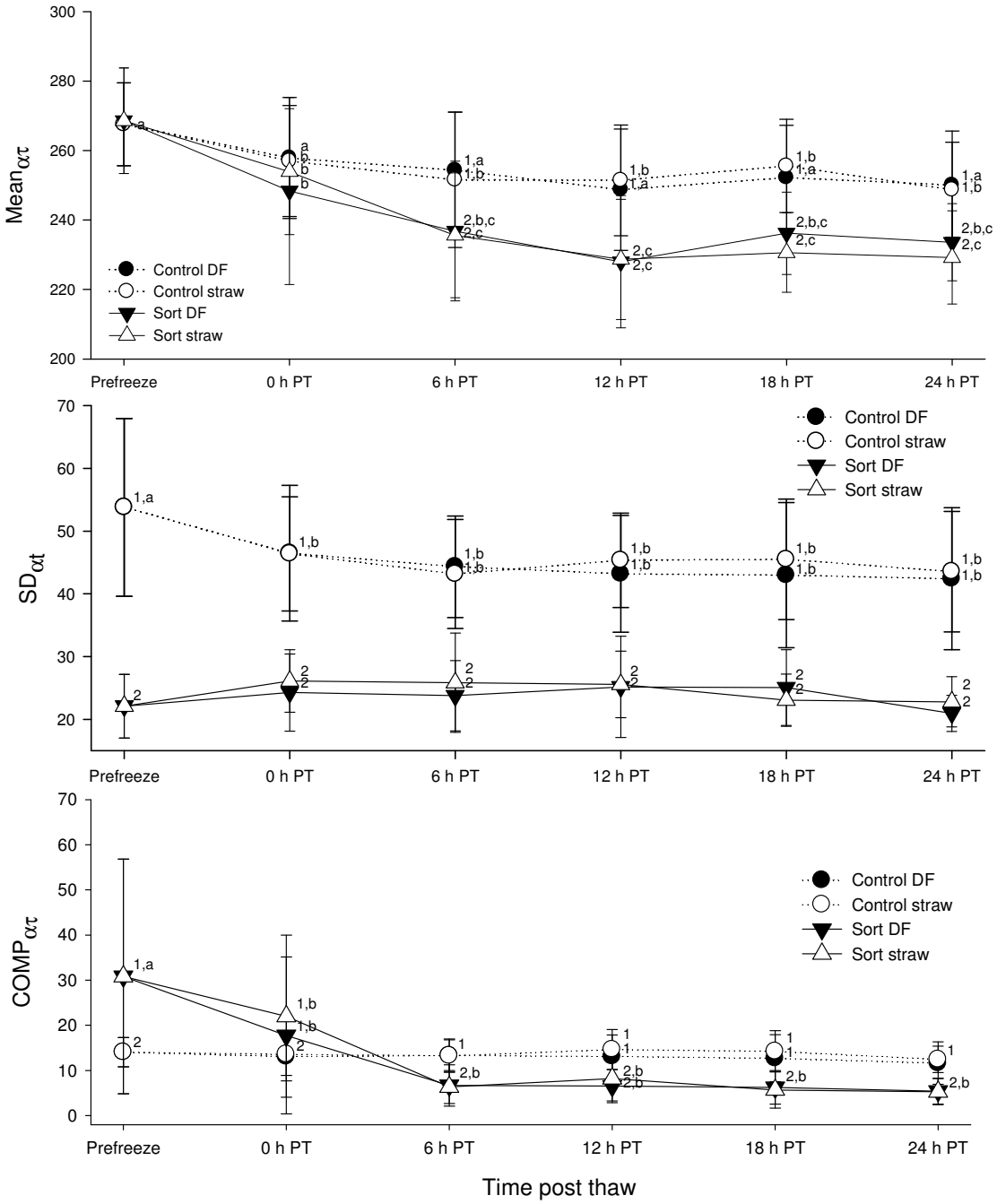

Fig.9. Mean$_{\alpha t}$ (top graph), SD$_{\alpha t}$ (middle graph), and COMP$_{\alpha t}$ (bottom graph) of frozen-thawed, non-sorted, frozen-thawed (Control) spermatozoa using straws and directional freezing (DF), and frozen-thawed, sorted, re-frozen-thawed (Sort) spermatozoa using straws and DF, before recryopreservation (pre-freeze) and during post-thaw incubation for 24 h at room temperature. ^{a,b,1,2}Values with different letters are significantly different ($P < 0.05$) across incubation time, and values with different numbers are significantly different ($P < 0.05$) within the same time point. Data are means ± SD.

Sperm DNA denaturation at 24 h post-thaw

As observed at 6 h, 12 h and 18 h post-thaw, within sperm type, $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ were similar ($P > 0.05$) for both freezing methods, and FSF spermatozoa showed higher ($P < 0.05$) $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ than Control spermatozoa (Fig. 9).

Overall changes in sperm DNA denaturation after the second thawing

$Mean_{\alpha t}$ was similar ($P > 0.05$) at 0 h post-thaw for Control and FSF spermatozoa frozen with DF and straw methods. However after 6 h of incubation, $Mean_{\alpha t}$ remained similar ($P > 0.05$) for Control spermatozoa across all time points (Fig. 9).

Within FSF spermatozoa, $Mean_{\alpha t}$ decreased ($P < 0.05$) between 0 h and 6 h post-thaw for the straw method and remained similar ($P > 0.05$) from 6 h until the end of the 24 h incubation period. $Mean_{\alpha t}$ was similar ($P > 0.05$) for FSF frozen with the DF method between 0 h and 6 h post-thaw. There was a decrease ($P < 0.05$) in $Mean_{\alpha t}$ at 12 h post-thaw and thereafter remained similar ($P > 0.05$) across the remaining incubation period (Fig. 9).

Overall, during the 24 h post-thaw incubation, FSF spermatozoa frozen with the DF and straw methods displayed consistently lower ($P < 0.05$) $SD_{\alpha t}$ and $COMP_{\alpha t}$ than Control spermatozoa frozen with DF and straw methods (Fig. 9).

Male effect on sperm DNA denaturation

No differences in $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ ($P > 0.05$) were observed among males during processing of Control and FSF samples prior to re-cryopreservation (data not shown). After the second thawing, no differences in $Mean_{\alpha t}$, $SD_{\alpha t}$ and $COMP_{\alpha t}$ ($P > 0.05$) were observed among males at 0 h post-thaw. However at 6 h, 12 h and 24 h post thaw, $COMP_{\alpha t}$ was higher ($P < 0.05$) for male 4 than males 2 and 3, whereas $Mean_{\alpha t}$ and $SD_{\alpha t}$ remained similar ($P > 0.05$). At 18 h post-thaw, male 4 showed lower $Mean_{\alpha t}$

than male 2 and similar $Mean_{\alpha t}$ to male 3, also male 4 showed higher $COMP_{\alpha t}$ than males 2 and 3 (data not shown).

DISCUSSION AND SUMMARY

Sex pre-determination has been established in breeding programs of captive populations of bottlenose dolphins using fresh sperm sorting, sperm cryopreservation and AI technologies [6,19]. 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 more than 12 h of transportation time away from a semen sorting facility [7]. The present study provides new information on the in vitro quality of sorted frozen-thawed bottlenose dolphin spermatozoa undergoing a second cryopreservation step (FSF), as well as the efficiency of the entire procedure. An optimized sperm recovery methodology using density gradient centrifugation was developed herein, and was combined with directional solidification. The results demonstrated that it is feasible to recryopreserve and thaw previously frozen-thawed then sorted bottlenose dolphin spermatozoa to obtain good in vitro sperm quality for up to 24 h post-second thaw.

The sorting of cryopreserved spermatozoa can only effectively be performed once the frozen-thawed sample is processed by DGC for removal of cryodiluent components and non-viable spermatozoa. This procedure relies on gradient medium properties, like the one used in this study, Percoll™ Plus (colloidal silica covalently coated with silane, iso-osmotic, pH-neutral) which enables the construction of varying layers of density dependent gradients [63]. These density bands will resist penetration by organic matter or cells that are less dense [64,65]. Thus, by formation of multiple density layers, cells and organic particles can be isolated based on their physical properties. For frozen-thawed semen, a 45% band is layered over a 90% density gradient medium forming two distinct concentration bands. As the frozen-thawed sperm suspension is layered over the 45% band and centrifuged, four different layers result. Glycerol, egg yolk and seminal fluid remain in the original sample layer while the dead and non-viable spermatozoa form a band between the 45% and 90% density layers [63,66,67]. Cryopreservation causes alteration in the sperm membrane stability, and it has been hypothesized that non-viable spermatozoa manifest a less dense state than viable spermatozoa caused by the contraction and swelling associated with addition

and removal of cryoprotectants [68,69]. Therefore, only viable and motile spermatozoa with a dense and homogenous nucleus [64] penetrate the 90% density gradient medium, forming a pellet on the bottom of the tube.

The volume of frozen-thawed semen placed on two-step discontinuous density gradients is dependent on the species (sperm head morphology), sperm concentration of the sample and the application of recovered spermatozoa. Cheng and Bongso [70] placed 3 mL of fresh human semen over a three layer Puresperm® (Nidacon, Göteborg, Sweden) column and increased the sperm concentration, total sperm motility, percentage viability and percentage normal sperm morphology of the final sperm solution compared to the placement of 0.75 mL of semen. Although there is more probability of rafting due to excessive numbers of debris and spermatozoa when a larger volume of semen is placed over a density gradient medium [71], pilot trial 1 provided similar results to those observed by Cheng and Bongso [70]. In the present study, it was determined that the most efficient separation technique involved using the largest volume of 4 mL frozen-thawed bottlenose dolphin semen (containing 800 x 10^6 spermatozoa) on 4.5 mL of density gradient medium (2 mL of 45% and 2.5 mL of 90%). Due to differences in sperm morphology and gradient volumes it is difficult to make direct comparisons to results from other species, but the observed recovery rate in this study is comparable to the recovery rate observed after DGC of 2 mL of frozen-thawed bull semen [72].

The directional solidification freezing method can freeze up to five glass tubes, each holding approximately 2 mL or 8.5 mL of semen, in one freezing cycle. The ability of the directional freezer to freeze large volumes of bottlenose dolphin semen [2,6] makes it the perfect freezing method for processing 4 mL of semen with the optimized DGC method. When compared to the DF method, cryopreserving dolphin semen with 0.25 mL or 0.5 mL straws would add increased labor required for loading and thawing of individual straws. In addition to the increase in labor required for the use of straws with the optimized DGC method, the decrease in quality of thawed spermatozoa using this freezing method [6] may change the dynamics of the DGC system. Thus, if DGC using straws was attempted, optimization trials would have to be conducted to determine the effect of the decreased semen quality on the efficiency of this system.

Although the recovery rate of spermatozoa at the DGC step was significantly greater for 50 mL tubes than 15 mL tubes, additional replicates of this trial (Pilot trial 2) were not performed because manipulation of 50 mL tubes was found to be inappropriate for the methodologies of this study. This was due to the sperm pellet at the bottom of the density gradient layers being presented with a larger surface area from which spermatozoa could swim out of the initial pellet. The recovery of a larger pellet volume was therefore required to ensure an equal or greater recovery rate to that achieved using the 15 mL tubes. The increased pellet volume using 50 mL tubes would subsequently translate into an insufficient pre-stain sperm concentration, and staining with H33342 could not be accomplished at the minimum concentration for optimum staining for sorting of 200 x 10^6 spermatozoa/mL [6]. Thus, in an effort to maintain adequate sperm concentration for H33342 staining, 15 mL tubes were used throughout this study.

For pilot trial 3 of this study, a post-thaw sperm processing technique that was originally designed as a swim-up method was examined. The swim-up method typically includes a centrifugation step for the removal of cryodiluent components followed by incubation (> 30 °C) of the sperm pellet beneath a layer of medium. Motile spermatozoa are then isolated from the upper layers after the incubation period [65]. In contrast, dolphin spermatozoa herein were washed, resuspended in staining medium then held at room temperature to determine the effectiveness of a gravity separation step. It was theorized that non-viable spermatozoa would remain in the bottom part of the tube and motile spermatozoa would be present in the upper layer. As in the swim up technique, spermatozoa from the upper layer would be selected for transfer to a new tube. However in the modification used in the present study, the selection would be without increasing the temperature of the sperm sample before the staining process. The results from this trial showed that higher motility of spermatozoa from the upper layer was not observed. Swim-up techniques have been proven to provide high quality sperm samples in humans [64] and non-human primates [8]. Direct comparisons of the DGC and swim-up methods demonstrated that DGC delivers higher numbers of motile spermatozoa than the swim-up technique in different species (humans [65], cattle [67] and sheep [9,69]. Results from this trial with bottlenose dolphin spermatozoa were similar to previous work with bull [67,11] and ram [9] spermatozoa, where DGC

separation resulted in significantly higher sperm recovery rates compared to swim-up separation techniques. These results ratify the use of DGC as an optimum technique to select high quality dolphin spermatozoa for further staining and sorting.

Egg yolk contains lipoproteins, phospholipids, and cholesterol, among other less abundant components [73] that provide protection against sperm loss induced by the cooling stage prior to cryopreservation and thawing [40]. However, high concentrations of egg yolk cause non-uniform H33342 staining and contribute to poor X-Y population resolution and reduced sorting rates [8,42]. Thus, it is important to remove the egg yolk and glycerol from the post-thaw sperm suspension using DGC prior to staining. However, this removal of egg yolk may decrease the in vitro survivability of spermatozoa through the post DGC washing step, and the results of this study demonstrated that the addition of a low concentration of egg yolk (4% v/v) to the HEPES-TALP washing medium, significantly improved sperm motility parameters and viability post-centrifugation. The positive effects that supplementation of egg yolk provided to the sperm cells at this step may have been a result of additional protection provided to the sperm membranes prior to the centrifugation. The low-density lipoproteins found in egg yolk have been postulated to increase sperm membrane fluidity [74] and thus may make spermatozoa more resilient to physical forces encountered during this step. The addition of 4% egg yolk after staining is commonly used for cattle [9,75,76] and ram [43,46] as well as the addition of egg yolk to the sheath fluid in the collection tube after sorting, where sorted spermatozoa have to adapt to a more concentrated egg yolk medium and maintain their viability [60].

In the second experiment, FSF spermatozoa frozen with the DF method showed significant superior PM across the 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 differ from those using chilled dolphin spermatozoa [6] where PM was greater for Control than sorted spermatozoa frozen with the DF method during 6 h of incubation post-thaw. This difference could be due to damage from the two cryopreservation processes that Control spermatozoa underwent in the present study, combined with the absence of a DGC procedure and therefore lack of selection for a higher quality sperm population prior to recryopreservation.

The improvements in sperm motility parameter (TM, PM, VAP, VSL, VCL, STR, RAP, MED and SLOW) during sorting processing of frozen-thawed bottlenose dolphin semen were observed at post-DGC and maintained at post-sort stages, as observed in studies with frozen-thawed cattle [11] and ram [8] semen. Maxwell et al. [72] reported a similar increase in TM, VAP, VSL, VCL and ALH in bull sperm samples from the first post-thaw to post-DGC using Puresperm®. The DGC and sorting process selected a motile, viable sperm population able to maintain in vitro characteristics after recryopreservation. Spermatozoa selected by DGC in the present study presented similar characteristics to those seen in fresh-chilled bottlenose dolphin semen undergoing sorting and cryopreservation [6].

The significant increase in PM post-sorting compared to post-staining in the present study was also observed in sorted ram spermatozoa from frozen-thawed semen [43], but not in sorted spermatozoa from fresh-chilled bottlenose dolphin semen [6] or from frozen-thawed bull semen [11].

Although PM of FSF spermatozoa at the pre-freeze stage (78.8 ± 3.8%) was similar to sorted spermatozoa of fresh-chilled bottlenose dolphin semen (76.8 ± 5.3%) [6], FSF spermatozoa presented lower PM (34.3 ± 9.5%) than sorted spermatozoa from fresh-chilled semen (46.2 ± 6.9%) at 0 h of incubation period when sorted spermatozoa were frozen with the DF method in both studies. For sorted spermatozoa frozen with the straw method, a lower PM was also observed for FSF spermatozoa (27.2 ± 6.0%) than sorted spermatozoa from fresh-chilled semen (38.9 ± 6.0%) at 0 h of incubation period. However, at 6 h of incubation period, PM of FSF spermatozoa (35.2 ± 10.6%) was similar to PM of sorted spermatozoa from fresh-chilled semen (36.7 ± 9.4%) within DF method. Similarly, within straw method at 6 h of incubation period, PM of FSF spermatozoa was 28.2 ± 15.0% and PM of sorted spermatozoa from fresh-chilled semen was 21.4 ± 11.2%. Although PM was lower at 0 h of incubation period in the present study than in the previous study using fresh-chilled semen, PM of FSF spermatozoa remained unchanged within freezing methods from 6 h of the incubation period until the end of it.

Even though TM and PM are clearly indispensable for in vitro and in vivo fertilization, semen analysis using CASA equipment has been shown to provide increased details and improve the accuracy of information collected on sperm motility

characteristics. In cattle, specific aspects of sperm movement such as velocities (VAP, VSL and VCL) have been positively associated with fertility rates [77,78]. In the present research, there was a tendency for FSF spermatozoa frozen with the DF method to present higher velocities (VAP, VSL, VCL) across the 24 h post-thaw incubation than Control spermatozoa frozen with the straw or DF methods. However, ALH was similar among freezing treatments until 12 h post-thaw and significantly higher for the straw freezing method from 12 h until 24 h of incubation. The lateral head displacement (ALH) is characterized by a side-to-side movement of the sperm head and although it does not directly measure the flagellar bend, it is associated with sperm hyperactivation, observed as an asymmetrical beat pattern often seen as a circular pathway [79].

In vivo, hyperactivation is a consequence of sperm capacitation in the female tract [80], whereas in vitro, hyperactivation of sperm motility is seen when the spermatozoon is separated from seminal plasma [65]. Mortimer [65] hypothesized that hyperactivated motility serves to benefit the spermatozoon as it decreases the probability of the spermatozoon to be trapped in the folds and crypts of the oviduct, increases the probability of contact with the oocyte, provides a constant supply of nutrients from the environment by the tail movement, allows the spermatozoon to enter the cumulus oophorus, and finally, provides enough force required for the spermatozoon to penetrate the zona pellucida. Hyperactivation of spermatozoa has been seen in all analyzed eutherian spermatozoa [81], including the bottlenose dolphin [82]. However, there are differences between the hyperactive pattern of sperm movement among species. When compared to fresh semen, relatively high VCL and ALH and low LIN of the trajectories are common features exhibited by hyperactivated spermatozoa after an incubation period [81]. For example, hyperactivated motility of human spermatozoa is defined as VCL ≥ 150 µm/sec and linearity (LIN) ≤ 50% and ALH ≥ 7.0 [65]. Further research on sperm motility using CASA of spermatozoa incubated at 35 °C is warranted to examine sperm hyperactivation in the bottlenose dolphin.

Within spermatozoa frozen with the DF method, BCF was similar for FSF to Control spermatozoa at 0 h post-thaw but maintained significantly higher for FSF from 6 h to 18 h and decreased at 24 h, being similar to Control at this point. A relationship between beat frequency and sperm progressive velocity has been described [83] and

the constant value of BCF presented by FSF samples indicates that there were no gross changes in the flagellar beat pattern [65].

Both post-DGC and post-sort steps selected a sperm population with higher viability than their previous step. The improvement in viability parameters following DGC has been discussed. The addition of food-dye to the sperm solution after staining, safely quenches the fluorescence of H33342 of dead or damaged spermatozoa to be sorted, allowing those cells to be discarded by the flow cytometer [60,84,85]. Control samples were not exposed to the DGC or sorting process, and at the pre-freeze stage, such samples exhibited lower viability than sorted spermatozoa. Plasma membrane integrity is an indirect indicator of sperm viability and can be evaluated by conventional live-dead stains such as eosin-nigrosin, or fluorescent stains using fluorescent microscopy or flow cytometry [86,87]. Propidium iodide is a permeable stain that crosses the damaged sperm membrane and binds to the chromatin, resulting in red fluorescence from the sperm head [88]. Comparable results of plasma membrane integrity were achieved using eosin-nigrosin and PI stains [49]. In the present study, it was also found that the percentage of viable spermatozoa detected using eosin-nigrosin positively correlated ($r^2 = 0.79$) with PI (in the PI/FITC-PNA method) using fluorescent microscopy.

Sperm capacitation occurs in the female tract and involves hyperactivated motility (mentioned previously) and biochemical and structural changes of protein and lipid membrane organization. These changes lead to sperm binding the zona pellucida and an immediate acrosome reaction, an irreversible event involving the formation of multiple vesicles between the sperm plasma membrane and the outer acrosome membrane. The rupture in the sperm membranes causes the release of acrosomal enzymes that enables the spermatozoon to penetrate the zona pellucida [89,90,91]. In vitro, sperm capacitation can be induced by the addition of small molecules such as caffeine, adenosine, or adrenaline to mention a few (reviewed in [92]). However, sorting and cryopreservation processes also can induce sperm capacitation and acrosome reaction in vitro [47]. Staining with fluorescein-conjugated lectins as *Arachis hypogea* (peanut) agglutinin (PNA), *Pisum sativum* agglutinin (PSA) or concanavalin A are the most frequently used methods to evaluate acrosome integrity. While FITC-PSA binds to acrosomal glycoprotein contents, the FITC-PNA binding site is located at the

outer acrosome membrane, therefore reacted/damaged spermatozoa display green fluorescence at the acrosomal region and acrosome intact spermatozoa display no fluorescence [49,55,93]. The combination of PI and FITC-PNA generates four categories of spermatozoa: viable or non-viable combined with damaged/reacted or intact acrosome [55].

Spermatozoa are exposed to different sources of stress during sex-sorting, including staining, exposure to the laser beam, and electrical charge [34]. However, passage through the pressurized flow cytometer is the main source of stress for the sperm population [34,94]. Lower pressure (40 psi instead of 50 psi) has been used in an effort to decrease the damage caused to the bovine sperm membrane in the passage through the flow cytometer [95,96]. In this study, a pressure of 30 psi was used to sex sort bottlenose dolphin spermatozoa, as opposed to 40 psi used previously [6]. The results of the present study indicated that sorted spermatozoa assessed with PI/FITC-PNA had significantly higher viability prior to the second cryopreservation than Control spermatozoa, and although the difference was not significant when assessed using eosin-nigrosin, sorted spermatozoa also displayed higher viability than Control spermatozoa. The significant difference observed in viability with PI/FITC-PNA was consistent across incubation time and suggests that the selection of a more viable sperm population by DGC and sex-sorting processes can overcome the damage caused by the factors related to the sorting process mentioned previously.

The employment of flow cytometry to determine sperm viability and membrane integrity using fluorescent stains would improve the accuracy of the method performed in the present study (fluorescent microscopy). A few advantages of flow cytometry are: thousands of cells per sample can be analyzed using the flow cytometer, samples require minimum preparation and the analysis is done in a short period of time [49]. However, if a flow cytometer is used, interference from the binding of FITC-PNA to the small vesicles from acrosome reaction can occur and overestimate the sperm damaged/reacted acrosome population [97]. To overcome this event, in bulls, a triple-stain using SYBR-14/PE-PNA/PI was developed and proved to give more accurate results than PI/FITC-PNA using flow cytometer since binding of triple-stains occurs exclusively to sperm DNA [97]. However, flow cytometric assessment of sperm viability utilizing this stain combination has yet to be tested in the bottlenose dolphin.

No correlation was established between the PI viability method and in vivo fertility using frozen-thawed spermatozoa in horses [87] or pigs [98]. However, it is known that damaged/deteriorated spermatozoa are not able to fertilize an oocyte in vivo, which can be overcome by using the ICSI method in a laboratory. Therefore, if the spermatozoa are going to be used for a conventional IVF or AI, integrity of the sperm membrane is essential. Although sorting and cryopreservation can cause spermatozoa to undergo acrosome damage or an acrosome reaction, in this study less than 5 % of spermatozoa, across all treatments, presented a damaged/reacted acrosome.

In the present study, the DNA quality was evaluated by the sperm chromatin structure assay (SCSA) [51,52]. Though the integrity of the DNA is not critical for fertilization of the oocyte, chromatin abnormalities can interfere with further embryo development [99]. In addition to this, severe DNA alterations are related to male infertility in humans [52], cattle [100], pigs [101] and horses [51]. In a study with rams; high DNA fragmentation index (DFI) was related to positive breeding potential [102]. Sperm chromatin is highly condensed, coated with protamines and presents a toroid structure with inter- and intramolecular disulfide cross-links between the cysteine-rich protamines that protect the DNA against mechanical and environmental factors [99,103]. Each toroid is connected to each other by damage-sensitive chromatin while the DNA that is compacted within the toroid is not accessible to damage [103]. Sperm DNA compaction reduces the cell volume allowing efficient transportation through the female tract, minimization of damage by exogenous agents and inactivation of genome transcription [104]. Damage to sperm DNA is reported to 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 [104,105,106]. External factors that result in sperm DNA damage include heat [107], chemotherapeutics [108], radiation [109,110], pollution [111] and age [104] as well as in vitro semen processing like type of extender, prolonged incubation time [112] and cryopreservation [113]. It was speculated that sex-sorting of bull spermatozoa could also cause damage to the sperm DNA. Mechanical stress and exposure to H33342 combined with 150 mW laser exposure were proved to be safe steps in the process, since the damage to the sperm DNA was increased by less than 3% when compared to

control spermatozoa [34]. The effect of sex-sorting on sperm DNA quality has not previously been examined in bottlenose dolphins.

Damage to sperm DNA can be detected by several fluorescent assays. Defective packaging of chromatin can be detected by the Comet assay, where deprotaminated DNA is identified by a fluorescent dye in agarose gel under an electric field [99]. This assay is commonly used to evaluate DNA damage of human spermatozoa handled in fertility clinics. An alkaline Comet assay has been used to detect DNA damage of stallion spermatozoa [114] and boar spermatozoa [115]. The TUNEL assay (terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling) is commonly used to determine DNA breaks in human spermatozoa, detecting both damaged single stranded (ss) and intact double stranded (ds) breaks mainly in the toroid linker regions. The enzyme (TdT) adds uridine residues to the 3' OH ends of nicked and broken DNA [103]. Instead of using an enzyme as for the TUNEL assay, SCSA samples are treated with mild acid to denature DNA that has nicks and the acridine orange stains ss (damaged) DNA red and ds (intact) DNA green [103]. The resulting ratio of red fluorescence/red + green fluorescence determined the DNA fragmentation index (DFI), also indicated as $COMP_{\alpha t}$ (Cells outside the main population). The total population evaluated is indicated by alpha-t [α_t] and following analysis of at least 5000 spermatozoa per sample, is presented as $Mean_{\alpha t}$) [51]. The standard deviation of α_t ($SD_{\alpha t}$) is associated with the extent of sperm DNA denaturation in humans [116] while the denatured DNA is indicated by the $COMP_{\alpha t}$. The TUNEL assay and the SCSA require a flow cytometer to evaluate the florescence of the samples [103].

Selection of a sperm population displaying less susceptibility to DNA denaturation has been observed after density gradient (Percoll®) centrifugation of human spermatozoa [117]. In the present study, density gradient centrifugation did not select for spermatozoa with higher DNA quality than frozen-thawed spermatozoa, but $SD_{\alpha t}$ was lower for spermatozoa at post-DGC than at first post-thaw, indicating that after DGC, DNA quality of the sperm population were more homogenous than that observed at the first post-thaw. A sample of the "bilayer" was removed from the layer formed between the 45% and 90% gradient media and although statistic analyses could not be performed due to unequal sampling across treatments, results indicated that

spermatozoa from the bilayer presented higher $COMP_{\alpha t}$, $Mean_{\alpha t}$, and $SD_{\alpha t}$ than spermatozoa recovered from the pellet of post-DGC samples. Morrel et al. [118] observed an improvement in the proportion of spermatozoa with high DNA quality following colloidal centrifugation of fresh stallion spermatozoa. However, morphologically abnormal stallion spermatozoa in that study were in a higher proportion (63-74%) than what has been reported for fresh bottlenose dolphin spermatozoa where abnormal forms represent less than 10% of ejaculated cells [13]. Although sperm morphology results were not determined in the present study, the morphology status of fresh spermatozoa was within the normal range for the bottlenose dolphin (data not shown).

In the present study, Control spermatozoa did not demonstrate any change in DNA quality from the first post-thaw until after the 24 h post second thaw incubation. This finding differs from a previous report with human sperm that demonstrated increased DNA damage after each of three freeze/thaw cycles [119]. However, in that study, spermatozoa that did not undergo processing (DGC, washing, and resuspending) demonstrated lower DNA damage than spermatozoa selected by DGC and resuspended with new cryoprotectant media [119]. Thus, the results observed herein suggest that Control spermatozoa were able to survive a second cycle of cryopreservation/thawing without altering sperm DNA quality because recryopreservation was performed with minimal post-thaw processing.

At the post-stain step in the current study, samples presented a significant increase in $COMP_{\alpha t}$ and $Mean_{\alpha t}$, whereas $SD_{\alpha t}$ remained the same compared to the post-DGC step. The proportion of spermatozoa exhibiting potential DNA damage as determined by the SCSA did not change between staining, sorting and pre-freeze steps. Garner [34] did not observe a significant increase in bull sperm potential DNA damage after evaluating sorting of stained spermatozoa with SCSA. De Ambrogi et al. [94] evaluated sex-sorted boar spermatozoa with Sperm-*Sus*-Halomax® kit (ChromaCell SL, Madrid, Spain) and did not observe a significant reduction in DNA integrity during the sorting process, while in the present study, $COMP_{\alpha t}$ values after staining and sorting were more than double to that observed in the previous step (post-DGC).

For FSF dolphin spermatozoa, a unique pattern in SCSA parameters was observed throughout pre- and post-sorting stages. Although there was a clear increase

in $COMP_{\alpha t}$ and $Mean_{\alpha t}$ during staining of spermatozoa for sorting, the values underwent a decrease between the pre-freeze and 0 h post-thaw steps. Both $COMP_{\alpha t}$ and $Mean_{\alpha t}$ continued to decline from 0 h 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 [120,121]. 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 then decrease, suggests that processing of the sperm samples for sex-sorting introduced an artifact. It is hypothesized that this artifact was caused by an interaction of AO with another factor(s) present in sperm chromatin, leading to differences in $COMP_{\alpha t}$ between FSF and Control spermatozoa.

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 [122]. 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 [123]. In the present study, the amount of AO bound to ds DNA may have been artificially decreased due to H33342 interference but the extent of AO intercalation between base pairs of ss DNA was not affected, because H33342 only binds to ds DNA. Therefore, it suggests that $Mean_{\alpha t}$ (determined as [red/red+green]), for each sorted spermatozoon presented lower green fluorescence which thereby artificially increased $COMP_{\alpha t}$. The hypothesized interference of AO staining lasts as long as the H33342 is present in the sperm nucleus, and it potentially explains the gradual “normalization” of $COMP_{\alpha t}$ values as the H33342 diffused out of the cells during the pre-freeze and post-thaw dilution and incubation periods.

Sorted bull spermatozoa are commonly frozen at a concentration of 10×10^6 spermatozoa/mL [34] as opposed to 15×10^6 spermatozoa/mL in the present study with dolphin spermatozoa. The longer post-sorting time at high dilution, and overall higher dilution rate that bull spermatozoa undergo from sorting to freezing would purportedly lead to diffusion of H33342 from the cell at a greater rate than that of dolphin spermatozoa under the sorting protocol used in this study. Though the concentration of H33342 in dolphin spermatozoa after sorting is unknown, due to the higher dilution

effect experienced by bull spermatozoa, H33342 concentration in dolphin spermatozoa is likely higher than that of bull spermatozoa after sorting.

The dramatic difference of $SD_{\alpha t}$ between Control and FSF spermatozoa before recryopreservation and during the 24 h post-thaw incubation indicates that DNA quality of sperm populations selected during the sorting process (DGC and flow cytometry) were more homogenous than those of Control spermatozoa.

Control spermatozoa were frozen approximately 2 h after first thawing, while the counterpart spermatozoa was being prepared for sorting and resulted in sorted samples being frozen approximately 4 h after Control samples. A previous study in horses demonstrated that temperature at storage can affect sperm DNA quality (ie. $COMP_{\alpha t}$) [124]. However, in the aforementioned study, a rise in SCSA values of sperm samples stored at 20°C were detected by 7 h of incubation. In this study, Control samples underwent less handling than FSF samples and FSF spermatozoa presented lower $COMP_{\alpha t}$ than Control spermatozoa after 6 h of post-thaw incubation. Further studies in the bottlenose dolphin are needed to evaluate the effects of sperm handling procedures (storage temperature and storage time), on sperm DNA quality.

In humans, the SCSA-derived DFI > 30% was associated with low fertility and DFI < 15% corresponded to high fertility status [52]. For pigs, most fresh boar ejaculates present DFI values < 5% when evaluated by the SCSA [101] and the Sperm-*Sus*-Halomax® kit [94]. In bottlenose dolphins, in 2006 the SCSA was used to compare fresh ejaculates of healthy males to an animal with orchitis, which displayed a higher DFI (33%) than the healthy males (4%) (O'Brien JK, Robeck TR, Evenson DP, unpublished results).

Although further research is necessary to understand the reasons for the possible artifact found herein, 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. 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.

In conclusion, the present study demonstrated that characteristics of bottlenose dolphin spermatozoa undergoing cryopreservation, sorting and recryopreservation steps were well maintained in vitro, and that such samples are of suitable quality for AI. In addition to this, the large volume of bottlenose dolphin semen that can be frozen with

the directional solidification method is a convenient and effective method to bank semen for future sorting. This method also resulted in the highest recovery of frozen-thawed bottlenose dolphin semen and should be considered the primary methodology to be used with sorted spermatozoa.

The successful birth of a bottlenose dolphin calf using sorted, frozen-thawed spermatozoa from previously cryopreserved semen [7], and the improvements to the sperm processing procedures for sex-sorting described herein, provide the potential for widespread application of sorting of cryopreserved semen from bottlenose dolphins located around the globe.

REFERENCES

[1] CITES – [website] Convention on International Trade in Endangered Species of Wild Fauna and Flora, consulted on July 15th, 2010. Available on http://www.cites.org/esp/resources/species.html

[2] Robeck TR, Steinman KJ, Greenwell M, Ramirez K, Van Bonn W, Yoshioka M, Katsumata E, Dalton L, Osborn S, O´Brien JK. Seasonality, estrous cycle characterization, estrus synchronization, semen cryopreservation, and artificial insemination in the Pacific white sided-dolphin (*Lagenorhynchus obliquidens*). Reproduction 2009;138:391-405.

[3] Robeck TR, Curry BE, Mcbain JF, Kraemer DC. Reproductive biology of the bottlenose dolphin (*Tursiops truncatus*) and the potential application of advanced reproductive technologies. J Zoo Wild Med 1994;25(3):321-336.

[4] Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 1989;41(2):199-203.

[5] Wells R, 2000. Reproduction in wild bottlenose dolphins: overview of patterns observed during a long-term study. In: The Bottlenose Dolphin Breeding Workshop, Duffield D, Robeck TR (EDs.) Marine Mammal Taxon Advisory Group, American Zoo and Aquarium Association (AZA): Silver Springs, MD; 2000. p. 57-73.

[6] O'Brien JK, Robeck TR. Development of sperm sexing and associated assisted reproductive technology for sex preselection of captive bottlenose dolphins (*Tursiops truncatus*). Reprod Fert Dev 2006;18:319-29.

[7] O'Brien JK, Steinman KJ, Robeck TR. Application of sperm sorting and associated reproductive technology for wildlife management and conservation. Theriogenology 2009;71:98-107.

[8] O'Brien JK, Hollinshead FK, Evans KM, Evans G, Maxwell WMC. Flow Cytometric sorting of frozen-thawed spermatozoa in sheep and non-human primates. Reprod Fertil Dev 2003;15:367-75.

[9] Hollinshead FK, Evans G, Evans KM, Catt SL, Maxwell WMC, O'Brien JK. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. Reproduction 2004;127:557-68.

[10] O'Brien JK, Stojanov T, Crichton EG, Evans KM, Leigh D, Maxwell WMC, Evans G, Loskutoff NM. Flow cytometric sorting of fresh and frozen-thawed spermatozoa in the western lowland gorilla (*Gorilla gorilla gorilla*). Am J Primatol 2005;66:297-315.

[11] Hollinshead FK, O'Brien JK, Maxwell WM, Evans G. Assessment of in vitro sperm characteristics after flow cytometric sorting of frozen-thawed bull spermatozoa. Theriogenology 2004;5:958-68.

[12] Rommel SA, Lowenstine LJ. Gross and microscopic anatomy. In: CRC Handbook of Marine Mammal Medicine (2nd Edition), Dierauf LA, Gulland MD (Eds.), CRC Press LLC, New York, New York; 2001 pp.129-64.

[13] Robeck TR, O´Brien JK. Effect of cryopreservation methods and pre-cryopreservation storage on bottlenose dolphin (*Tursiops truncatus*) spermatozoa. Biol Reprod 2004;70:1340-8.

[14] Robeck TR, Atkinson SKC, Brook F. Reproduction. In: CRC Handbook of Marine Mammal Medicine (2nd Edition), Dierauf LA, Gulland MD (Ed), CRC Press LLC, New York, New York; 2001. pp. 193-236.

[15] Dudley, M. Bottlenose dolphin North American Studbook. Marine Mammal Taxon Advisory Group, American Zoo and Aquarium Association (AZA): Silver Spring, MD. 2008.

[16] Brook F. The use of diagnostic ultrasound in assessment of the reproductive status of the bottlenose dolphin, *Tursiops aduncas*, in captivity and applications in management of a controlled breeding programme, Ph.D. thesis, Hong Kong Polytechnic University, Kowloon, Hong Kong, 1997 pp. 339.

[17] Schroeder JP, Keller KV. Seasonality of serum testosterone levels and sperm density in *Tursiops truncatus*. J Exp Zool 1989,249:316-21.

[18] O'Brien JK, Robeck TR. The value of ex situ cetacean populations in understanding assisted reproductive technology for ex situ and in situ species

management and conservation efforts. International J Comp Psychol 2010; in press.

[19] Robeck TR, Steinman KJ, Yoshioka M, Jensen E, O´Brien JK, Katsumata E, Gili C, McBain JF, Sweeney J, Monfort SL. Estrous cycle characterisation and artificial insemination using frozen-thawed spermatozoa in the bottlenose dolphin (*Tursiops truncatus*). Reproduction 2005;129:659-74.

[20] Robeck Tr, Steinman KJ, Gearhart S, Reidarson TR, McBain JF, Monfort SL, 2004, Reproductive physiology and development of artificial insemination technology in killer whales (*Orcinus orca*). Biol Reprod 2004;71:650-60.

[21] O'Brien JK, Steinman KJ, Schmitt T, Robeck TR. Semen collection, characterization and artificial insemination in the beluga (*Delphinapterus leucas*) using liquid-stored spermatozoa. Reprod Fert Dev 2008;20:770-83.

[22] Robeck TR, Steinman KJ, Montano GA, Katsumata E, Osborn S, Dalton L, Dunn JL, Schmitt T, Reidarson T, O'Brien JK. Deep intra-uterine artificial inseminations using cryopreserved spermatozoa in beluga (*Delphinapterus leucas*). Theriogenology 2010, Epub ahead of print.

[23] Brook FM, Kinoshita R, Brown B, Metreweli C. Ultrasonographic imaging of the testis and epididymis of the bottlenose dolphin, *Tursiops truncates aduncas*. J Reprod Fertil 2000;119:233-240.

[24] Sharpe JC, Evans KM. Advances in flow cytometry for sperm sexing. Theriogenology 2009;71:4-10.

[25] Buchanan BR, Seidel GE, McCue PM Jr, Schenk JL, Herickhoff LA, Squires EL. Insemination of mares with low number of either unsexed or sexed spermatozoa. Theriogenology 2000;53:1333-44.

[26] Robeck TR, Steinman KJ, Montano GA, Morrisseau M, Sweeney J, Osborn S, O'Brien JK. Low dose, deep intra-uterine artificial insemination using cryopreserved sexed spermatozoa in the bottlenose dolphin (*Tursiops truncatus*): preliminary findings. In: Proceedings of the international Association of Aquatic Animal Medicine 2010. pp.193-4.

[27] Searger S, Gilmartin W, Moore L, Platz C, Kirby V. Semen collection (electroejaculation), evaluation, and freezing in the Atlantic bottlenosed dolphin

(Tursiops truncatus). In: Proceedings of the American Association of Zoo Veterinarians 1981. pp 136.

[28] Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H. New trends in gamete's cryopreservation. Mol Cell Endocrinol 2002;187:77-81.

[29] Zirkler H, Gerbes K, Klug E, Sieme H. Cryopreservation of stallion semen collected from good and poor freezers using a directional freezing device (Harmony CryoCare – Multi Thermal Gradient 516). Anim Reprod Sci 2005;89:291-4.

[30] Saragusty J, Gacitua H, Pettit MT, Arav A. Directional freezing of equine semen in large volumes. Reprod Domest Anim 2007;42:610-5.

[31] Reid CE, Hermes R, Blottner S, Goeritz F, Wibbelt G, Walzer C, Bryant BR, Portas TJ, Streich WJ, Hildebrandt TB. Split-sample comparison of the directional and liquid nitrogen vapour freezing method on post-thaw semen quality in white rhinoceroses (*Ceratotherium simum simum* and *Ceratotherium simum cottoni*). Theriogenology 2009;71:275-91.

[32] Hermes R, Arav A, Saragusty J, Goeritz F, Pettit M, Blottner S, Flach E, Eshkar G, Boardman W, Hildebrand TB. Cryopreservation of spermatozoa from asian elephant (*Elephas maximus*) using directional freezing. In: Proceedings American Association of Zoo Veterinarians 2003. pp 264.

[33] O'Brien JK, Robeck TR. Preservation of beluga (Delphinapterus leucas) spermatozoa using a trehalose-based cryodiluyent and directional freezing technology. Reprod Fertil Dev 2010;22:653-63.

[34] Garner DL. Flow cytometric sexing of mammalian sperm. Theriogenology 2006;65: 943–57.

[35] Johnson LA. Sexing mammalian sperm for production of offspring: the state-of-the-art. Anim Reprod Sci 2000;60–61:93–107.

[36] Maxwell WMC, Evans G, Hollinshead FK, Bathgate R, de Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK. Integration of sperm sexing technology into the ART toolbox. Anim Reprod Sci 2004;82–3.

[37] Levinson G, Keyvanfar K, Wu JC, Fugger EF, Fields RA, Harton GL, Palmer FT, Sisson ME, Starr KM, Dennison-Lagos L, Calvo L, Sherins RJ, Bick D, Schulman JD, Black SH. DNA-based X-enriched sperm separation as an

adjunct to preimplantation genetic testing for the prevention of X-linked disease. Hum Reprod 1995;10:979-82.

[38] Fugger EF, Black SH, Keyvanfar K, Schulman JD. Births of normal daughters after MicroSort sperm separation and intrauterine insemination, in vitro fertilization, or intracytoplasmic sperm injection. Hum Reprod 1998;13:2267-70.

[39] Murdoch RN, Jones RC. The effects of glycerol on the metabolism and ultrastructure of boar spermatozoa. J Reprod Fert 1978;54:419-22.

[40] Holt WV, North RD. Partially irreversible cold-induced lipid phase transitions in mammalian sperm plasma membrane domains: freeze-fracture study. J Exp Zool 1984;230:473– 83.

[41] Gamzu R, Yogev L, Botchan A, Amit A, Lessing JB, Lichtenberg D, et al. Effect of sperm preparation with TEST yolk buffer on sperm-binding capacity under hemizona assay conditions. Andrologia 1997;29:17–21.

[42] Johnson LA, Welch GR. Sex preselection: high-speed flow cytometry sorting of X and Y sperm for masimum efficiency. Theriogenology 1999;52:1323-41.

[43] De Graaf SP, Evans G, Maxwell WMC, O'Brien JK. In vitro characteristics of fresh and frozen-thawed ram spermatozoa after sex sorting and re-freezing. Reprod Fertil Dev 2006;18:867-74.

[44] Stap J, Hoebe RA, Merton JS, Haring RM, Bakker PJM, Aten JA. Improving the resolution of cryopreserved X- and Y-sperm during DNA flow cytometric analysis with the addition of percoll to quench the fluorescence of dead sperm. J Anim Sci 1998;76:1896-1902.

[45] Lu KH, Cran DG, Seidel GE Jr. In vitro fertilization with flow-cytometrically-sorted bovine sperm. Theriogenology 1999;52:1393-1405.

[46] De Graaf SP, Evans G, WMC Maxwell, Cran DG, O'Brien JK. Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. Theriogenology 2007;67:391-8.

[47] Hollinshead FK, Gillan L, O'Brien JK, Evans G, Maxwell WMC. In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. Reprod Fertil Dev 2003;15:351-9.

[48] Holt WV, O'Brien JK, Abaigar T. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reprod Fertil Dev 2007;19:709-18.

[49] Graham JR, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow citometry. Biol Reprod 1990;43:55-64.

[50] Pena AI, Johannisson A, Linde-Forsberg C. Validation of flow cytometry for assessment of viability and acrosomal integrity of dog spermatozoa and for evaluation of different methods of cryopreservation. J Reprod Fertil Suppl 2001;57:371-76.

[51] Love CC, Kenney RM. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenology 1998;52:955-972.

[52] Evenson DP, Kjersten LL, Jost LK. Sperm chromatin Structure Assay: its clinical use for detectin sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25-43.

[53] Seaman EK, Goluboff E, BarChama N, Fisch H. Accuracy of semen counting chambers as determined by the use of latex beads. Fertil Steril 1996;66:662-5.

[54] Evenson DP, Jost LK. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 2000;22:169-189.

[55] Szasz F, Sirivaidyapong S, Cheng FP, Voorhout WF, Marks A, Colenbrander B, Solti AL, Gadella BM. Detection of calcium ionophore induced membrane changes in dog sperm as a simple method to predict the cryopreservability of dog semen. Mol Reprod Dev 2000;55:289–98.

[56] Dott HM, Foster GC. A technique for studing the morphology of mammalian spermatozoa which are eosinophilic in a differential 'live/dead' stain. J Reprod Fert 1972;(29):443-5.

[57] Graham EF, Crabo BG, Brown KI. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa I. Bull. J Dairy Sci 1972;55(3):372-78.

[58] Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986;25:591-600.

[59] Johnson LA, Welch GR, Rens W. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci 1999;77(2):213-220.

[60] Johnson LA, Welch GR. Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 1999;52:1323-41.

[61] Rens W, Welch GR, Johnson LA. A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X and Y chromosome-bearing sperm. Cytometry 1998;33:476-481.

[62] Welch GR, Johnson LA. Sex preselection: laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA. Theriogenology 1999;52:1343-52.

[63] Pertoft H. Fractionation of cells and subcellular particles with Percoll. J Biochem Biophys Methods 2000;(44):1-30.

[64] Le Lannou DL, Blanchard Y. Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure. J Reprod Fert 1988;84:551-6.

[65] Mortimer D. Sperm preparation methods. J Androl 2000;21:357-66.

[66] Parks JE, Graham JK. Effects of cryopreservation procedure on sperm membranes. Theriogenology 1992;38:209-22.

[67] Somfai T, Bodó S, Nagy S, Papp AB, IváncsicsJ, Baranyai B, Gócza E, Kovács A. Effect of swim up and percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. Reprod Dom Anim 2002;37:285-90.

[68] Schweisguth DC, Hammerstedt RH. Evaluation of plasma membrane stability by detergent-induced rupture of osmotically swollen sperm. J Biochem Biophys Methods 1992;24:81-94.

[69] Cesari A, Kaiser GG, Mucci N, Mutto A, Vincenti A, Fornés MW, Alberio RH. Integrated morphophysiological assessment of two methods for sperm selection in bovine embryo production in vitro. Theriogenology 2006;66:1185-93.

[70] Cheng MJ, Bongso A. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum Reprod 1999;14:759-64.

[71] Mortimer D, Mortimer ST. Laboratory investigation of the infertile male. In: The text book of in vitro fertilization and assisted reproduction (2nd Edition), Brinsden P (Ed), The Parthenon Publishing Book, New York, USA, 1999. pp 53-82.

[72] Maxwell WMC, Parrila I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D. Retained functional integrity of bull spermatozoa after double freezing and thawing using puresperm® density gradient centrifugation. Reprod Dom Anim 2007;42:489-94.

[73] Kuksis A. Yolk lipids. Biochim Biophys Acta 1992;1124(3):205-22.

[74] Barbas JP, Mascarenhas RD. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 2009;10:49-62.

[75] Underwood SL, Bathgate R, Maxwell WMC, Evans G. Birth of offspring after artificial insemination of heifers with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. Anim Reprod Sci 2010;118:171-5.

[76] Underwood SL, Bathgate R, Ebsworth M, Maxwell WMC, Evans G. Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. Anim Reprod Sci 2010;118:7-12.

[77] Cseh S, Polichronopoulos T, Solti L. Prediction of bull fertility by computer assisted semen analysis. Reprod Fertil Devel 2004;16:128-9 (Abstr.).

[78] Gillan L, Kroetsch T, Maxwell WMC, Evans G. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 2008,103:201-14.

[79] Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update 2008;14:647-57.

[80] Luconi M, Baldi E. How do sperm swim? Molecular mechanisms underlying sperm motility. Cell Mol Biol 2003;49:357-69.

[81] Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update 1997,3:403-39.

[82] Fleming AD, Yanagimachi R, Yanagimachi H. Spermatozoa of the Atlantic bottlenosed dolphin, *Tursiops truncatus*. J Reprod Fert 1981;63:509-14.

[83] Serres C, Feneux D, Jouannet P, David G. Influence of the flagellar wave development and propagation on the human sperm movement in seminal plasma. Gamete Res 1984;9:183-95.

[84] Garner DL. Sex-sorting mammalian sperm: concept to application in animals. J Androl 2001;22:519-26.

[85] Seidel GE Jr. Overview of sexing sperm. Theriogenology 2007;68:443-6.

[86] Magistrini M, Guitton E, Levern Y, Nicolle JC, Vidament M, Kerboeuf D, Palmer E. New staining methods for sperm evaluation estimated by microscopy and flow cytometry. Theriogenology 1997;48:1229-35.

[87] Katila T. In vitro evaluation of frozen-thawed stallion semen: a review. Acta Vet Scand 2001;42:199-217.

[88] Garner DL, Johnson LA, Yue ST, Roth BL. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 1994;15:620-9.

[89] Neild DN, Gadella BM, Agüero A, Stout TAE, Colenbrander B. Capacitation, acrosome function and chromatin structure in stallion sperm. Anim Reprod Sci 2005;89:47-56.

[90] Gadella BM, Tsai PS, Boerke A, Brewis IA. Sperm head membrane reorganization during capacitation. Int J Dev Biol 2008;52:473-80.

[91] Sigman M, Zini A. Semen analysis and sperm function assays: what do they mean? Semin Reprod Med 2009;27:115-23.

[92] Fraser LR. The role of small molecules in sperm capacitation. Theriogenology 2008;70:1356-9.

[93] Flesch FM, Voorhout WF, Colenbrander B, van Golde LMG, Gadella BM. Use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. Biol Reprod 1998;59:1530-9.

[94] De Ambrogi M, Spinaci M, Galeati G, Tamanini C. Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology 2006;66:1994-2000.

[95] Suh TK, Schenk JL. Pressure during flow sorting of bull sperm affects post-thaw motility characteristics. Theriogenology 2003;59:516 (Abstr.).

[96] Campos-Chillon LF, de la Torre JF. Effect of concentration of sexed bovine sperm sorted at 40 and 50 psi on developmental capacity of in vitro produced embryos. Theriogenology 2003;59:506 (Abstr.).

[97] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68:1828-35.

[98] Guthrie HD, Welch GR. Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenology 2005;63:396-410.

[99] Silva PFN, Gadella BM. Detection of damage in mammalian sperm cells. Theriogenology 2006;65:958-78.

[100] Ballachey BE, Evenson DP, Saacke RG. The sperm chromatin structure assay. Relationship with alternate tests of semen quality and heterospermic performance of bulls. J Androl 1988,9:109-15.

[101] Evenson DP, Thompson L, Jost L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 1994;41:637-51.

[102] Kasimanickam R, Pelzer KD, Kasimanickam V, Swecker WS, Thatcher CD. Association of classical semen parameters,sperm DNA fragmentation indez, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. Theriogenology 2006;65:1407-21.

[103] Shaman JA, Ward WS. Sperm chromatin stability and susceptibility to damage in relation to its structure. In: The sperm cell – Production, maturation, fertilization, regeneration. (1st Edition), De Jonge CJ, Barratt CLR (Ed), Cambridge University Press, New York, 2006. pp 31-48.

[104] Singh NP, Muller CH, Berger R. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 2003;80:1420-30.

[105] Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM. Isola tion of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem 1990;265:20662–6.

[106] Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. Reprod Biomed Online 2003;7:428–32.

[107] Paul C, Murray AA, Spears N, Saunders PTK. A single, mild, transient scrotal heat stress causes DNS damage, subfertility and impairs formation of blastocyts in mice. Reproduction 2008;135:73-84.

[108] Delbes G, Hales BF, Robaire B. Effects of the chemotherapy cocktail used to tread testicular cancer on sperm chromatin integrity. J Androl 2007;28(2):241-9.

[109] Sailer BL, Jost LK, Erickson KR, Tajiran MA, Evenson DP. Effects of X-irradiation on mouse testicular cells and sperm chromatin structure. Environ Mol Mutagen 1995;25:23-30.

[110] Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM, King BV. Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. Int J Androl 2005;28:171-9.

[111] Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, Perreault SD. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod 20,2776-83.

[112] Krzyzosiak J, Evenson D, Pitt C, Jost L, Molan P, Vishwanath R. Changes in susceptibility of bovine sperm to in situ DNA denaturation during prolonged incubation at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor. Reprod Fertil Dev 2000;12:251-61.

[113] Hammadeh ME, Dehn CH, Hippach M, Zeginiadou T, Stieber M, Georg T, Rosenbaum P, Schmidt W. Comparison between computerized slow-stage and static liquid nitrogen vapour freezing methods with respect to the deleterious effect on chromatin and morphology of spermatozoa from fertile and subfertile men. Int J Androl 2001;24:66-72.

[114] Linfor JJ, Meyers SA. Detection of DNA damage in esponse to cooling injury in equine spermatozoa using single-cell gel electrophoresis. J Androl 2002;23:107-113.

[115] Boe-Hansen GB, Ersbøll AK, Greve T, Christensen P. Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology 2005,15:2006-19.

[116] Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 2006;65:979-91.

[117] O'Connell M, McClure N, Powell LA, Steele EK, Lewis SEM. Differences in mitochondrial and nuclear DNA status of high-density and low density sperm fractions after density centrifugation preparation. Fertil Steril 2003,79:754-62.

[118] Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez H. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod Dom Anim 2009;44:512-7.

[119] Thomson LK, Fleming SD, Barone K, Zieschang, Clark AM. The effect of repeated freezing and thawing on human sperm DNA fragmentation. Fertil Steril 2010;93:1147-56.

[120] Genesca A, Caballin MR, Miró R, Benet J, Germà JR, Egozcue. Repais of human chromosome aberrations in the hamster egg. Hum Genet 1992;89:181-6.

[121] Daudoune JP. Expression of mammalian spermatozoa nucleoproteins. Microsc Res Tech 2003;61:56-75.

[122] Kapuscinski J. Interactions of nucleic acids with fluorescent dyes: spectral properties of condensed complexes. J Histochem Cytochem 1990;38:1323-9.

[123] Garner DL. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 2009;71:11-21.

[124] Love CC, Thompson JA, Lowry VK, Varner DD. Effect of storage time and temperature on stallion sperm DNA and fertility. Theriogenology 2002;57:1135-42.

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