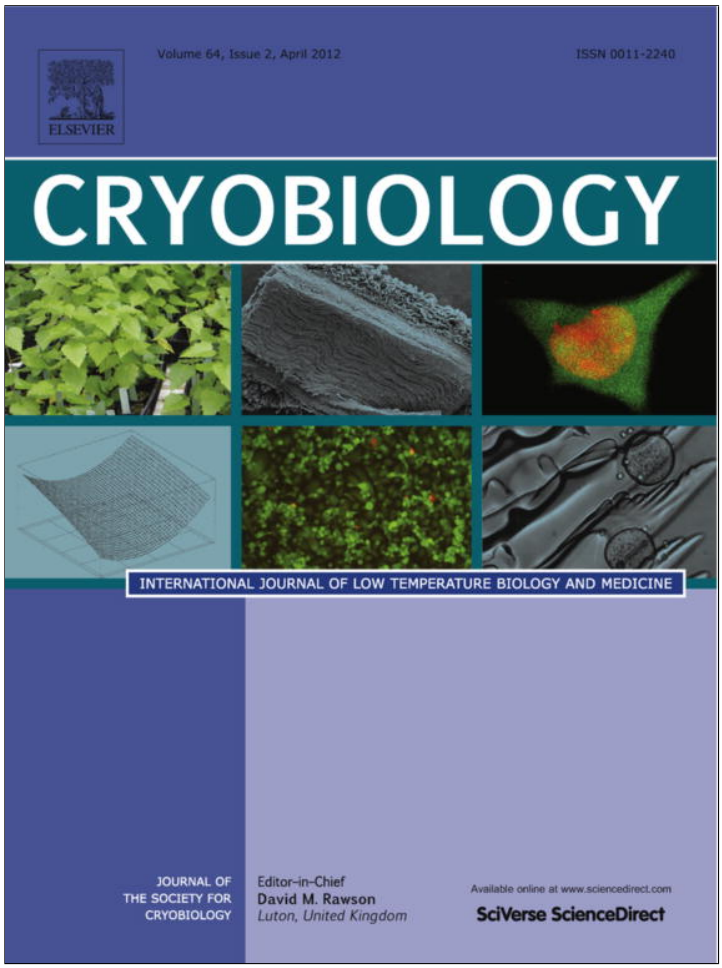

Cryobiology 64 (2012) 110–117

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Different patterns of metabolic cryo-damage in domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) spermatozoa ☆

Kimberly A. Terrell [a,b,*], David E. Wildt [a], Nicola M. Anthony [b], Barry D. Bavister [c], S.P. Leibo [b,d], Linda M. Penfold [e], Laurie L. Marker [f], Adrienne E. Crosier [a]

[a] Center for Species Survival, Smithsonian Conservation Biology Institute, 1500 Remount Road, Front Royal, VA 22630, USA
[b] Department of Biological Sciences, University of New Orleans, 2000 Lakeshore Dr, New Orleans, LA 70148, USA
[c] University of Puerto Rico, Medical Sciences Campus, P.O. Box 365067, San Juan, PR 00936-5067, USA
[d] Audubon Center for Research of Endangered Species, 14001 River Road, New Orleans, LA 70131, USA
[e] White Oak Conservation Center, 581705 White Oak Rd, Yulee, FL 32097, USA
[f] Cheetah Conservation Fund, P.O. Box 1755, Otjiwarongo, Namibia

ARTICLE INFO

Article history:
Received 4 June 2011
Accepted 22 December 2011
Available online 29 December 2011

Keywords:
Sperm
Cryopreservation
Metabolism
Swim-up
Accudenz
Density gradient
Mitochondria
Lactate
Felid
Teratospermia

ABSTRACT

Felid spermatozoa are sensitive to cryopreservation-induced damage, but functional losses can be mitigated by post-thaw swim-up or density gradient processing methods that selectively recover motile or structurally-normal spermatozoa, respectively. Despite the importance of sperm energy production to achieving fertilization, there is little knowledge about the influence of cryopreservation or post-thaw processing on felid sperm metabolism. We conducted a comparative study of domestic cat and cheetah sperm metabolism after cryopreservation and post-thaw processing. We hypothesized that freezing/thawing impairs sperm metabolism and that swim-up, but not density gradient centrifugation, recovers metabolically-normal spermatozoa. Ejaculates were cryopreserved, thawed, and processed by swim-up, Accudenz gradient centrifugation, or conventional washing (representing the 'control'). Sperm glucose and pyruvate uptake, lactate production, motility, and acrosomal integrity were assessed. Mitochondrial membrane potential (MMP) was measured in cat spermatozoa. In both species, lactate production, motility, and acrosomal integrity were reduced in post-thaw, washed samples compared to freshly-collected ejaculates. Glucose uptake was minimal pre- and post-cryopreservation, whereas pyruvate uptake was similar between treatments due to high coefficients of variation. In the cat, swim-up, but not Accudenz processing, recovered spermatozoa with increased lactate production, pyruvate uptake, and motility compared to controls. Although confounded by differences in non-specific fluorescence among processing methods, MMP values within treatments were positively correlated to sperm motility and acrosomal integrity. Cheetah spermatozoa isolated by either selection method exhibited improved motility and/or acrosomal integrity, but remained metabolically compromised. Collectively, findings revealed a metabolically-robust subpopulation of cryopreserved cat, but not cheetah, spermatozoa, recovered by selecting for motility rather than morphology.

Published by Elsevier Inc.

Introduction

Despite extensive research to optimize sperm cryopreservation protocols for the domestic cat [1] and various wild felids [2], freezing/thawing causes significant damage to these cells [3–6]. The degree of cryo-injury is particularly severe in the cheetah and other species that produce teratospermic ejaculates containing high proportions of sperm pleiomorphisms [7–12]. In such cases, thawed spermatozoa consistently exhibit reduced motility and decreased proportions of cells with intact acrosomes [3–12]. This cellular damage is linked to disruption of sperm membranes from osmotic stress during freezing, thawing, and/or cryoprotectant removal [9,13–15]. It is possible that the physiological stress experienced by spermatozoa during cryopreservation also disrupts cellular metabolism. For example, one recent investigation of epididymal spermatozoa from the domestic cat determined that mitochondrial membrane potential (MMP) declined rapidly after cryopreservation and post-thaw washing [16]. Sperm MMP also is impaired in frozen-thawed ejaculates from the human [17], boar

☆ *Statement of funding:* Supported by the National Science Foundation Graduate Research Fellowship Program, Louisiana State University and Audubon Center for Research of Endangered Species Grant Program, the Ohrstrom Family Foundation, and the William H. Donner Foundation, Inc.

* Corresponding author at: Center for Species Survival, Smithsonian Conservation Biology Institute, 1500 Remount Road, Front Royal, VA 22630, USA. Fax: +1 540 635 6506.
E-mail address: terrellk@si.edu (K.A. Terrell).

0011-2240/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.cryobiol.2011.12.006

[18], ram [19], stallion [20], bull [21], and elephant [22]. Such published observations reinforce the need to explore the impact of cryopreservation on metabolic function of felid spermatozoa. Our recent studies of the domestic cat and cheetah have provided an understanding of baseline sperm metabolism (i.e., metabolism of freshly-collected ejaculates) for these felids and have revealed intriguing species differences in metabolic function [23–25]. In both species, sperm glucose metabolism was minimal, while rates of pyruvate uptake and lactate production were correlated positively to cellular function (i.e., sperm motility, acrosomal integrity, and/or normal morphology) [24]. Sperm MMP was essential for lactate production [25], and these metabolic indicators were substantially reduced in cheetah compared to domestic cat ejaculates [24,25].

Although being a potential source of osmotic stress, post-thaw removal of sperm cryoprotectant [26] provides an opportunity to isolate functionally-normal spermatozoa. For example, sperm 'swim-up' into fresh medium increases the proportions of motile, structurally-intact cells in cat ejaculates [27,28] and improves sperm morphology in cryopreserved cheetah samples [29]. Evidence in other mammals indicates that, in addition to improved motility and/or morphology, spermatozoa isolated by selective processing also exhibit high metabolic rates. In the ram, density gradient processing of thawed ejaculates improves sperm MMP [19], whereas swim-up processing has the same influence on bull spermatozoa [20] and increases oxygen consumption by ~20-fold in freshly-ejaculated human semen [30]. While either approach could enhance metabolic function in thawed felid ejaculates, swim-up processing (targeting vigorous, motile cells with high rates of ATP production) [31–33] might be expected to recover the most metabolically-robust spermatozoa. By contrast, density gradient processing (targeting cells with normal morphology) may be less effective, because it is known that metabolism can be impaired in structurally-normal cheetah spermatozoa [24].

Our general aim was to determine the impact of cryopreservation and post-thaw processing on the metabolic function of felid spermatozoa. This novel approach compared two species (domestic cat and cheetah) for which baseline patterns of sperm metabolism are well-characterized [23,24]. Because felid sperm metabolism is influenced both by teratospermia [24] and species physiology [23–25], domestic cats previously known to produce low proportions of structurally-normal spermatozoa [24] were included for comparison with the teratospermic cheetah. To assess the influence of cellular function versus morphology, metabolism was assessed in thawed spermatozoa selected on the basis of motility (swim-up) or normal morphology (density gradient). We hypothesized that the metabolism of cat and cheetah spermatozoa, specifically rates of pyruvate and lactate utilization, would be impaired by cryopreservation. Given the known differences in cryosensitivity and baseline metabolism in spermatozoa from these species, we expected sperm metabolic cryo-damage to be more severe in the cheetah compared to the domestic cat. We also predicted that a subpopulation of thawed spermatozoa in each species would exhibit robust metabolic function (similar to baseline values) and would be recovered more effectively by isolating motile rather than normal-appearing cells.

Materials and methods

Animals

All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee (ACUC) and similar committees of the White Oak Conservation Center (WOCC) and the San Diego Zoo Safari Park (SDZSP). Electroejaculates were collected from domestic cats and cheetahs using methods described below. For the former, a total of 16 semen samples were recovered from five adult males (ages, 1.5–8 yr) that were known to consistently produce either normospermic or teratospermic ejaculates [23–25]. Teratospermia has been described previously in felids and is defined as the ejaculation of <40% structurally-normal spermatozoa [34]. Three normospermic cats were used to produce five ejaculates (1–2/individual) and two teratospermic males produced five samples (2–3/individual). Three additional ejaculates were collected from one male in each group for the optimization of Accudenz density gradients (described below). The management protocol for domestic cats maintained at the Smithsonian Conservation Biology Institute (SCBI, Front Royal, VA) has been described in detail [24].

Electroejaculates (1/individual) were collected from 11 adult cheetahs (ages, 2.5–10 yr). These animals were managed at the Cheetah Conservation Fund (CCF, Otjiwarongo, Namibia, $n = 9$), WOCC (Yulee, FL, $n = 1$), or the SDZSP (Escondido, CA, $n = 1$). Males at CCF and the WOCC were wild-caught and captive-born, respectively, and maintained under previously described protocols [24,29]. The male at SDZSP was captive-born and managed with two male siblings off-exhibit in a 1300 m^2 outdoor enclosure and fed a commercial carnivore diet (Natural Balance Pet Foods Inc., Pacoima, CA).

Semen collection

A surgical plane of anesthesia was induced in domestic cats and cheetahs according to protocols determined by institutional veterinarians for these species [29,35]. Semen was collected using a rectal probe of 1 cm (domestic cat) or 1.9 cm (cheetah) in diameter and an electrostimulator (P.T. Electronics, Boring, OR) as described previously [24,36]. A sample of raw semen containing $\sim 2 \times 10^5$ spermatozoa was fixed in 0.3% glutaraldehyde in phosphate-buffered saline for assessment of sperm morphology according to previous descriptions for our laboratory [9,24,36].

Sperm processing and metabolic assessments

Each ejaculate was diluted immediately with an equal volume of a chemically-defined, protein-free, modified mouse tubal fluid medium (cMTF) [37] supplemented with 2% polyvinyl alcohol (PVA) [38]. The cMTF medium was prepared as described previously [24] and contained 98.4 Mm NaCl, 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 1 mM glucose, 1 mM Na-pyruvate, 25 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and 0.02 mg/mL phenol red. Sperm concentration was determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) [39]. Osmolality of the final cMTF medium (300–345 mOsm) was determined using a vapor pressure osmometer (Wescor, Inc., Logan, UT) and was within 10% of the physiological value of domestic cat semen (323 mOsm).

A sample containing $\sim 3 \times 10^6$ motile spermatozoa was removed from each diluted ejaculate, washed by centrifugation for 8 min (300*g* for domestic cat, 100*g* for cheetah), resuspended in fresh cMTF, and then incubated (37 °C) in the dark in a microcentrifuge tube (3×10^6 motile sperm/ml) under oil to prevent evaporation, as described previously [24]. Assessments of sperm percentage motility (% M), forward progression (FPS), and acrosomal integrity were made at 0, 1, 3, 5, and 7 h of incubation. However, the low percentages of spermatozoa recovered in samples processed by swim-up or gradient centrifugation (see below) precluded sampling at more than three time points. Therefore, data obtained at 1 and 5 h were omitted from analysis. Motility was assessed visually (200×), and FPS was rated on a 0–5 scale, with a rating of five equivalent to most rapid, linear progress [36]. A

sperm motility index was calculated using the formula (% M + (FPS × 20) ÷ 2) [28]. Spermatozoa (~2 × 10^5 cells) were fixed in 4% paraformaldehyde and stained with Coomassie Blue G-250 (Fisher Biotech, Springfield, NJ) to evaluate acrosomal integrity, as described previously [7,40].

Glucose and pyruvate uptake and lactate production also were assessed at each time point, and metabolic rates were calculated from substrate concentrations at 0, 3, and 7 h of incubation. To determine metabolic substrate concentrations, medium samples (110 μl) were centrifuged for 8 min (1000*g*) through a CoStar Spin-X 0.22-μm nylon filter tube (Corning Incorporated, Corning, NY) and stored at −80 °C until analysis using a HK/G6PDH (glucose), or LDH (pyruvate and lactate) fluorescence assay, as described previously [24,41]. Rates of substrate utilization were calculated as the change in medium substrate concentration over time divided by sperm concentration.

Sperm cryopreservation

After removing a sperm sample for metabolic assessment, the remainder of each diluted ejaculate was cryopreserved in 4% glycerol as described previously [7,29]. Briefly, each diluted ejaculate was centrifuged for 8 min (300*g* domestic cat, 100*g* cheetah), resuspended in TEST-yolk buffer (TYB) refrigeration medium without glycerol (Irvine Scientific, Santa Ana, CA), and cooled slowly (~3.5 h) in a water bath from ambient temperature (19–22 °C) to 4 °C. After cooling, an equal volume of TYB containing 8% glycerol (prepared as a 1:2 mixture of refrigeration medium and TYB with 12% glycerol, Irvine Scientific) was added in a step-wise manner over 30 min (¼ volume at 0 min, ¼ volume at 15 min, and ½ volume at 30 min). Samples were loaded into 0.25-ml plastic straws (Veterinary Concepts, Spring Valley, WI) with a final sperm concentration of ~60 × 10^6 motile spermatozoa/ml, frozen in liquid nitrogen vapor (1 min at ~7.5 cm and 1 min at ~2.5 cm above liquid nitrogen), and then plunged directly into liquid nitrogen. Samples were stored in liquid nitrogen until thawing (2 days to 30 months).

Comparison of post-thaw processing methods

Cryopreserved straws were thawed individually for 10 s in air (19–22 °C) followed by immersion in a 37 °C water bath for 30 s [7,29]. Each straw was dried, and its contents emptied into a sterile microcentrifuge tube. After thorough mixing, sperm motility, acrosomal integrity, and morphology were assessed as described above. Each sample was divided among three processing treatments: (1) wash, (2) swim-up, and (3) Accudenz density gradient. For wash and swim-up treatments, 100 μl of the thawed sample was diluted to 1 ml by the slow, drop-wise addition of cMTF medium [29]. Each washed sample was centrifuged for 8 min (100*g*), the supernatant removed, and the pellet resuspended in 500 μl of cMTF medium. Each swim-up sample was centrifuged as above, the supernatant removed, and the sperm pellet gently overlaid with 100 μl of cMTF medium. These samples were maintained at ambient temperature for 45 min in the dark to allow motile spermatozoa to enter the supernatant. The top 90 μl of supernatant was removed and diluted to 500 μl in fresh cMTF.

For the Accudenz treatment, 100 μl of thawed sperm solution was gently layered on top of a 4–10% (wt/vol) discontinuous density gradient in cMTF (except for domestic cat samples, described below) [29]. The Accudenz gradient was created by layering 100 μl of 10% solution underneath 500 μl of 4% solution in a microcentrifuge tube. After adding the sperm sample, each gradient was centrifuged for 8 min (100*g*). The entire suspension formed three distinct layers after centrifugation: (1) the top layer (predominantly composed of TYB), (2) the interphase layer containing motile spermatozoa (except for domestic cat samples, described below), and (3) the bottom layer containing non-motile cells. The interphase (~80 μl) was removed and diluted to 500 μl. To recover sufficient Accudenz and swim-up processed spermatozoa for metabolic assessments, treatments were replicated in separate microcentrifuge tubes (3–5 replicates/treatment for each thawed ejaculate, depending on the ejaculate volume available) and combined before determining sperm concentration. For each processing treatment, the combined sample was diluted to a standard concentration (3 × 10^6 motile spermatozoa/ml), and final volumes ranged from 0.5 to 1.4 ml. Processed samples were incubated (37 °C) and motility, forward progression, acrosomal integrity, and metabolic rates were evaluated at 0, 3, and 7 h, as described above. A volume of sample (containing ~2 × 10^5 cells) was removed at the start of incubation for assessment of sperm morphology, as described above.

Sperm mitochondrial membrane potential (MMP) of domestic cat samples was evaluated immediately post-thaw and after processing at 0, 3, and 7 h of incubation. Cheetah sperm MMP was not assessed because too few spermatozoa were recovered from swim-up and Accudenz treatments. Additionally, a recent study from our laboratory demonstrated that MMP values of fresh cheetah ejaculate are extremely low [24], suggesting that it would be impossible to detect differences among post-thaw treatments for this species. At each time point, 48 μl of domestic cat sperm suspension were removed, combined with 2 μl of 12.5 nM MitoTracker® Red CMXRos (Molecular Probes, Inc., Eugene, OR) in cMTF (0.5 nM final concentration), and incubated (1 h at ambient temperature) in the dark. To quantify MMP, 100 spermatozoa per ejaculate were individually analyzed (400×) using a BX40 fluorescence microscope (Olympus America Inc., Center Valley, PA, 555 nm excitation) and a Sensicam qe high performance camera (Cooke Corp., Romulus, MI) and IP Lab v4.04 software (BD Biosciences, Rockville, MD). Spermatozoa with MMP values below the background fluorescence threshold were considered to have non-functional mitochondria. The presence of sperm structural abnormalities in MMP samples was recorded as described previously [25], with each spermatozoon classified as being one of the following five morphotypes: structurally-normal, midpiece droplet, flagellar droplet, spermatid, and 'other.' The 'other' category included less common abnormalities (head deformities, malformation of the midpiece or flagellum) that collectively represented ⩽10% of spermatozoa in domestic cat ejaculates.

Accudenz gradient optimization for domestic cat spermatozoa

Initial attempts to process domestic cat samples by Accudenz density gradient centrifugation resulted in very low sperm recovery (<5%), compared to percentages of cheetah spermatozoa recovered by the same method in this study and previously (~25%) [29]. This double layer gradient (4–10%) centrifugation for 8 min (300*g*) method resulted in a high concentration of motile spermatozoa in the pellet, suggesting that the density of the medium was below that needed to localize these cells in the target interphase layer. Therefore, we tested the efficacy of a triple layer gradient (4–10–30%) and reduced centrifugation speed (100*g*). This higher concentration was chosen because a 12–30% discontinuous Accudenz gradient has been successfully used to recover motile spermatozoa in the chicken, with the cells isolated from the interface between the two layers [42,43]. Cryopreserved sperm samples from two domestic cats (one normospermic, the other teratospermic, $n = 3$ ejaculates per male) were thawed as above. Each thawed sample was mixed thoroughly, and the total volume (90–150 μl) was divided equally among three centrifugation treatments: (1) double gradient at 300*g*, (2) triple gradient at 300*g*, (3) triple gradient at 100*g*. Double gradients were prepared as described above.

Triple gradients were prepared using 200 μl each of 4%, 10%, and 30% (wt/vol) Accudenz in cMTF. The solutions were added to the microcentrifuge tube in order of increasing density, with each new layer added underneath the previous. After centrifugation, each layer was transferred to a new tube (in the order of interphase, top, and bottom layer) and assessed for sperm motility index and percent recovery. Sperm morphology and acrosomal integrity also were evaluated in the interphase layer. The interphase was considered to be the volume at the interface of the 4% and 10% solutions for the double gradient, or the 10% and 30% solutions for the triple gradient.

Chemicals

All reagents were purchased from Sigma Aldrich (St. Louis, MO) except enzymes (LDH, GPT, HK, and G6PDH) that were obtained from Roche Applied Science (Indianapolis, IN) and Accudenz, which was from Accurate Chemical and Scientific Corporation (Westbury, NY).

Statistical analyses

Data were analyzed with statistical analysis software (SAS) version 9.1 (SAS Institute, Cary, NC), and percentage data were arcsine-transformed before evaluation. Within species, the influence of post-thaw processing method was assessed at each time point using a General Linear Model (GLM) [44], with sperm motility index, acrosomal integrity, metabolic rates, percent normal morphology (0 h only), mean MMP (domestic cat only), and mean percent non-functional mitochondria (domestic cat only), included as response variables. Within domestic cats, the influence of teratospermia on sperm cryo-damage was investigated by testing for an interaction between treatment (freshly collected or post-thaw wash, swim up, or Accudenz) and group (normospermic or teratospermic) in a GLM that included all time points and response variables. Differences in MMP (0 h only) among sperm morphotypes were assessed using a GLM with data for all processing treatments combined. Accudenz gradient optimization data were analyzed using a separate GLM for each gradient layer (top, interphase, and bottom). Gradient type and male were considered class variables, and sperm percent recovery, motility index, acrosomal integrity (interphase only), and morphology (interphase only) were included as response variables. When treatment effects were significant, differences among means were evaluated using Duncan's Multiple Range Test. Pearson's correlation was used to evaluate the relationships among sperm motility index, acrosomal integrity, and metabolic metrics. Results were considered significant at $P < 0.05$ and are reported as least-squares (LS) means ± SEM.

Results

Accudenz density gradient optimization for domestic cat spermatozoa

Although motile spermatozoa were recovered from all layers of each gradient, most (⩾73%) were isolated from the pellet-containing bottom layer when centrifuged at 300*g* (Table 1). However when the centrifugation speed was reduced (100*g*), the percentage of spermatozoa recovered from the target interphase layer of the triple gradient increased ($P < 0.05$) ~3 to 20-fold (Table 1). Reduced centrifugation speed also was associated with a ~50% improvement ($P < 0.05$) in sperm motility index (SMI) compared to the double gradient (Table 1). Acrosomal integrity (% IA) and percentages of structurally-normal spermatozoa were not different ($P > 0.05$) among gradient treatments (Table 1). Primary head abnormalities (i.e., macro/microcephaly) were more prevalent ($P < 0.05$) in the double versus triple gradient (Table 1). Other malformations did not differ ($P < 0.05$) among gradient treatments (data not shown). There was no interaction ($P > 0.05$) between male and gradient treatment.

Table 1
Characteristics of domestic cat* post-thaw sperm samples recovered from Accudenz density gradients. Error bars represent means ± SEM.

Parameter	Density gradient		
	Double†	Triple†	Triple, low speed‡
Interphase (target layer)			
Sperm recovery (%)	4 ± 7[a]	24 ± 7[a]	70 ± 7[b]
Sperm motility index	32 ± 5[a]	40 ± 5[a,b]	48 ± 4[b]
Intact acrosomes (%)	34 ± 6	43 ± 6	43 ± 6
Normal sperm morphology (%)	54 ± 4	65 ± 4	52 ± 4
Macro/microcephaly (%)	12 ± 1[a]	4 ± 1[b]	3 ± 1[b]
Top layer			
Sperm recovery (%)	2 ± 4[a]	3 ± 4[a]	18 ± 4[b]
Sperm motility index	35 ± 7	25 ± 7	39 ± 7
Bottom layer and sperm pellet			
Sperm recovery (%)	94 ± 7[a]	73 ± 7[a]	12 ± 7[b]
Sperm motility index	53 ± 5	42 ± 5	44 ± 5

* $n = 2$ males, 3 ejaculates per male.
† 8 min at 300*g*.
‡ 8 min at 100*g*.
[a,b] Within rows, values with different superscripts differed ($P < 0.05$).

Comparison of post-thaw processing methods

Domestic cat

There was no interaction ($P > 0.05$) between treatment (freshly collected or washed, swim-up, or Accudenz-processed) and domestic cat group (normospermic or teratospermic) for SMI, lactate production (ΔL), glucose uptake (ΔG), sperm MMP, or the percentage of cells with non-functional mitochondria (% NFM). A treatment/teratospermia interaction was observed ($P < 0.05$) for acrosomal integrity (data not shown), and this reflected the known sensitivity of teratospermic ejaculates to cryo-induced acrosomal damage [8]. A treatment/teratospermia interaction also was detected ($P < 0.01$) for pyruvate and was driven by higher rates of uptake in teratospermic compared to normospermic post-thaw, swim-up samples. However, coefficients of variation for pyruvate uptake of these samples were relatively high (17–140%), and this pattern was not observed with respect to lactate uptake, which was previously determined to be a more reliable indicator of sperm function due to the inherent qualities of the corresponding fluorescence assays [24]. Given the lack of a treatment/teratospermia interaction for SMI, ΔL, ΔG, MMP, and % NFM, data from normospermic and teratospermic cats were combined for subsequent analyses.

Cryopreservation and post-thaw washing of domestic cat spermatozoa resulted in decreased (~30–70%, $P < 0.05$) SMI, % IA, and ΔL compared to freshly-collected ejaculates (Fig. 1A–C). Although values of pyruvate uptake (ΔP) were lower in washed versus freshly-collected samples, this difference was non-significant ($P > 0.05$, Fig. 1D). Consistent with previous findings [24], sperm lactate production was correlated positively ($P < 0.05$) to SMI and % IA (Table 2), whereas ΔG was minimal and was not influenced ($P > 0.05$) by cryopreservation or post-thaw processing (Sup. Fig. 1A). Swim-up processing recovered metabolically-robust cells with SMI, ΔL, and ΔP values that were increased ($P < 0.05$) compared to washed samples and similar to ($P > 0.05$) or greater than ($P < 0.05$) corresponding values for freshly-collected ejaculates (Fig. 1A, C, and D). There was no change ($P > 0.05$) in ΔL and ΔP of swim-up samples after 3 h, indicating that normal metabolic function was maintained for an extended time period (Fig. 1C

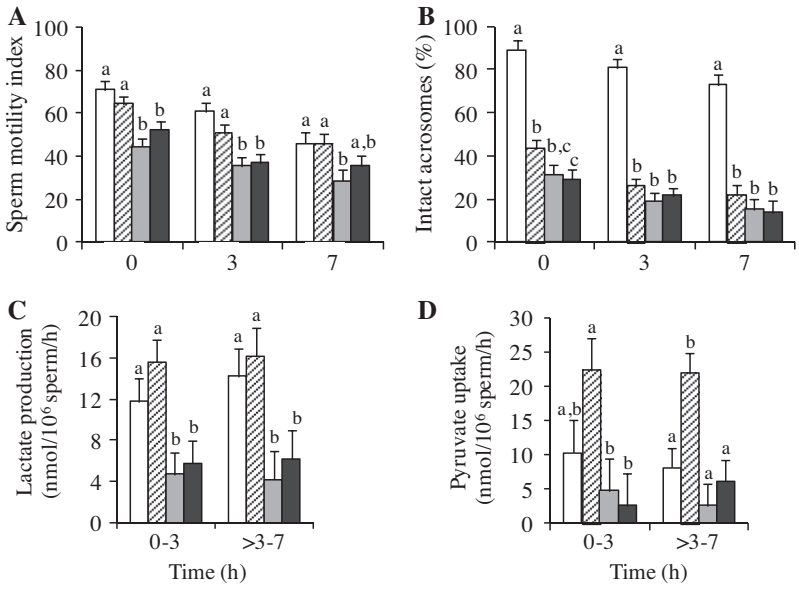

Fig. 1. Sperm motility index (A), acrosomal integrity (B), lactate production (C), and pyruvate uptake (D) in domestic cat ejaculates before cryopreservation (open bar) and after post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) methods. Within each time interval, bars with different superscripts differed ($P < 0.05$). Error bars represent means ± SEM.

Table 2
Correlation coefficient (r) values for metabolic indicators versus domestic cat[†] or cheetah[‡] sperm quality metrics after cryopreservation.

Metabolic indicator	Sperm motility index	Intact acrosomes (%)
Domestic cat		
Lactate production (nmol/10^{6}sperm/h)	0.54[c]	0.55[c]
Mitochondrial membrane potential	0.25[a]	0.28[b]
Non-functional mitochondria (%)	−0.33[b]	−0.27[b]
Cheetah		
Lactate production (nmol/10^{6}sperm/h)	0.41[c]	0.25[b]

[†] $n = 5$ males, 10 total ejaculates.
[‡] $n = 11$ males, 11 total ejaculates.
[a] $P = 0.07$.
[b] $P < 0.05$.
[c] $P < 0.0001$.

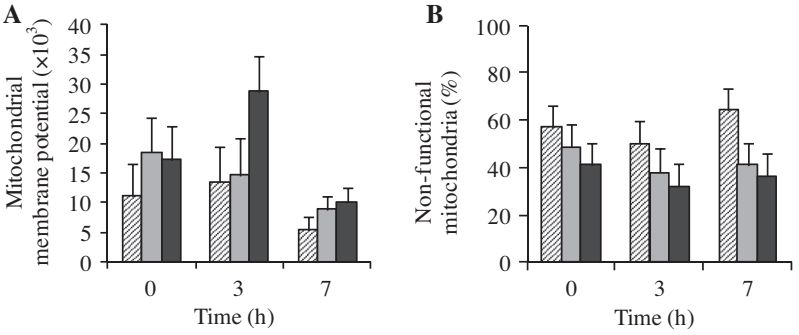

Fig. 2. Sperm mitochondrial membrane potential (A) and percentages of cells with non-functional mitochondria (B) in domestic cat ejaculates after cryopreservation and post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) methods. Error bars represent means ± SEM.

and D). Although swim-up processing increased ($P < 0.05$) % IA relative to washed samples, this improvement was not ($P > 0.05$) sustained after 0 h, and values remained reduced ($P < 0.05$) compared to fresh ejaculates (Fig. 1B). In contrast to spermatozoa isolated by swim-up, the subpopulation recovered by density gradient centrifugation experienced impaired cellular function (SMI, % IA, ΔL, and ΔP) similar ($P > 0.05$) to washed samples (Fig. 1A–D).

Post-thaw processing did not influence ($P > 0.05$) sperm MMP or % NFM (Fig. 2A and B). However, high levels of non-specific fluorescence were observed in spermatozoa from washed and Accudenz treated samples relative to swim-up counterparts (Sup. Fig. 2A–C), possibly confounding a treatment effect. We suspected that non-specific fluorescence was related to the amount of cryopreservation buffer (TYB) carryover, which likely was minimal in

Table 3
Correlation coefficient (r) values for mitochondrial function versus domestic cat[†] sperm quality.

Treatment	Mitochondrial membrane potential		Non-functional mitochondria (%)	
	SMI	% IA	SMI	% IA
Wash	NS	0.56[a]	NS	−0.60[a]
Swim-up	0.63[a]	0.48[a]	−0.63[a]	−0.47[a]
Accudenz	0.59[a]	0.44[b]	−0.51[a]	NS

SMI = sperm motility index, % IA = acrosomal integrity.
NS = not significant ($P > 0.05$).
[†] $n = 5$ males, 10 total ejaculates.
[a] $P < 0.05$.
[b] $P = 0.07$.

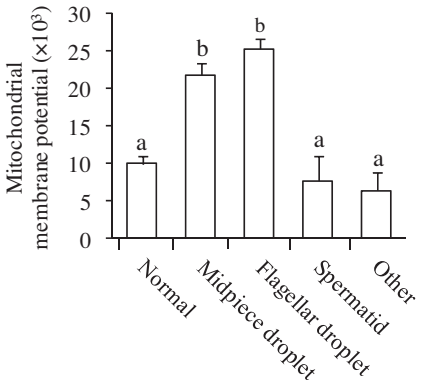

Fig. 3. Sperm mitochondrial membrane potential in relation to cellular morphotype for all domestic cat post-thaw processing treatments combined. Among sperm morphotypes, bars with different superscripts differed ($P < 0.05$).

swim-up samples. We also observed high percentages (85–100%) of spermatozoa with non-fluorescent mitochondria immediately post-thaw (data not shown), suggesting that high concentrations of TYB inhibited MitoTracker® staining.

When data from all sperm treatments were combined, both MMP and % NFM were correlated (positively and negatively, respectively, $P < 0.05$) to sperm motility and acrosomal integrity, although the relationship between MMP and motility represented a trend ($P = 0.07$, Table 2). Compared to sperm lactate production, MMP and % NFM were less accurate predictors of motility and acrosomal integrity (Table 2), likely due to the confounding influence of non-specific fluorescence after MitoTracker® staining. Sperm MMP and % NFM were better correlated to motility and acrosomal integrity when treatment groups were analyzed individually (Table 3), although certain relationships became non-significant due to the smaller sample sizes. Finally, consistent with previous findings in freshly-collected cat and cheetah ejaculates [25], MMP was greater ($P < 0.05$) in spermatozoa with a retained cytoplasmic droplet at the midpiece or flagellum compared to other cellular morphotypes (Fig. 3).

Cheetah

The influence of cryopreservation and post-thaw washing on cheetah spermatozoa was consistent with results for the domestic cat. Sperm SMI, % IA, and ΔL in washed samples was markedly reduced (~30–70%, $P < 0.05$) compared to freshly-collected ejaculates (Fig. 4A–C), and ΔP also appeared to decrease, although non-significantly ($P > 0.05$, Fig. 4D). Similar to results for cat spermatozoa, cheetah sperm ΔG was minimal before and after cryopreservation (Sup. Fig. 1B). Selective processing by swim up and Accudenz methods increased ($P < 0.05$) SMI compared to washed samples, but values remained decreased ($P < 0.05$) relative to fresh ejaculates, and this improvement was not maintained after 3 h (Fig. 4A). Similarly, Accudenz centrifugation improved ($P < 0.05$) % IA compared to washed samples, but after 3 h there were no differences ($P > 0.05$) among processing treatments (Fig. 4B). Sperm ΔL and ΔP were similar ($P > 0.05$) among all three processing treatments (Fig. 4C and D). Finally, ΔL was correlated positively with SMI and % IA (Table 2).

Discussion

Our investigation of cellular metabolism in cryopreserved felid spermatozoa yielded four significant discoveries. First, conventional cryopreservation protocols for felid spermatozoa not only

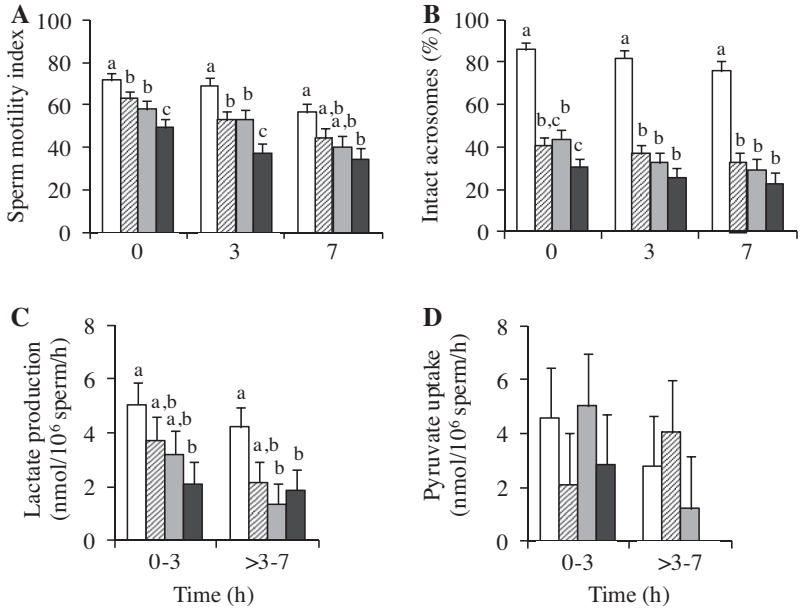

Fig. 4. Sperm motility index (A), acrosomal integrity (B), lactate production (C), and pyruvate uptake (D) in cheetah ejaculates before cryopreservation (open bar) and after post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) methods. Within each time interval, bars with different superscripts differed ($P < 0.05$). Error bars represent means ± SEM.

impaired sperm motility and membrane integrity (as determined previously [7,8]), but also disrupted pathways of cellular metabolism, as demonstrated by decreased rates of lactate production. Second, despite an overall reduction in metabolic function of post-thaw ejaculates, a subpopulation of domestic cat spermatozoa appeared to be unaffected by cryopreservation as indicated by high rates of pyruvate uptake and lactate production. Third, in contrast to the domestic cat, cheetah spermatozoa were unable to sustain baseline metabolic profiles after thawing (despite using an identical cryopreservation protocol), indicating species-specificity in metabolic cryo-sensitivity. Perhaps the mechanistic basis of this finding is related to the more overt morphological deficits of spermatozoa from this genetically impoverished species [45–47]. Finally, we determined that it was possible to isolate the most metabolically-robust domestic cat spermatozoa post-thaw by targeting motile, rather than structurally-normal cells. Given the link between sperm metabolism and cellular function [23–25], we predict that this subpopulation of thawed cells has the greatest chance of achieving fertilization.

Although the extent of injury was different between domestic cat and cheetah spermatozoa, our observations revealed that both species experienced sperm metabolic cryo-damage. Therefore, felids appear to be similar to other mammalian species, including in the human [17], boar [18], ram [19], stallion [20], bull [21], and elephant [22], that produce ejaculates exhibiting impaired post-thaw metabolism. The exact origin of this disruption remains to be elucidated. However, it was important to consider that cryopreservation-induced stress to plasma membranes could cause leakage of sperm glycolytic enzymes [48]. Therefore, we postulated that sperm metabolic rates (measured by changes in medium glucose, pyruvate, and lactate concentrations) might falsely appear to be *increased* post-thaw due to greater activity of enzymes released into the medium. Cytoplasmic droplets (a common abnormality in cat and cheetah spermatozoa [24,47]) are particularly susceptible to post-thaw LDH leakage [48], and this could confound the use of lactate production as an indicator of cellular quality in thawed ejaculate. Nonetheless, there was no evidence of glycolytic enzyme leakage from cat or cheetah spermatozoa. On the contrary, rates of pyruvate and lactate metabolism were reduced (non-significantly in the case of pyruvate), and glucose uptake remained minimal post-thaw. Furthermore, rates of lactate production were correlated positively to sperm motility, acrosomal integrity, and MMP. This finding provides strong support of our previous studies [23–25] and indicates that lactate production is a reliable metric of sperm function in both the domestic cat and cheetah.

Although rates of lactate production revealed metabolic cryo-damage in both the domestic cat and cheetah, we also identified an important species difference in the extent of cellular injury. Unlike the domestic cat, metabolic function in cheetah spermatozoa was uniformly impaired by cryopreservation and not improved by selective processing via swim-up or density gradient centrifugation. Therefore, future efforts to improve the post-thaw function of cheetah spermatozoa should focus on preventing damage to the freshly-collected cells, perhaps by testing alternative cryoprotectants (e.g., non-permeating sugars [49]) or preservation approaches (e.g., vitrification [50]). Regardless, the revealed differences re-emphasize the value of conducting cross-species comparisons, and, for felids, the importance of the domestic cat as a research model [8,23–25,28,35,51,52]. Besides determining what reproductive mechanisms are conserved, this comparative approach can identify unique traits for a given species that eventually may permit large-scale, systematic cryo-banking of germplasm. Because the present study demonstrated that a proportion of domestic cat spermatozoa survived cryopreservation with their metabolic machinery intact, this cellular subpopulation could be especially useful for future research studies designed to understand the key biological factors that contribute to sperm cryo-survival.

The relatively high rates of substrate utilization in cat ejaculates processed by swim-up versus Accudenz centrifugation confirmed our prediction that post-thaw sperm metabolic function was more closely linked to cellular motility than morphology. Although impaired in teratospermic felids, sperm lactate production also is compromised in freshly-collected cheetah ejaculates that contain high proportions of structurally-normal spermatozoa [24]. We have speculated [24] that disrupted metabolism could explain why even structurally-normal spermatozoa from teratospermic ejaculates often are incapable of fertilization [27]. Furthermore, we previously discovered important differences in sperm glycolytic [23] and oxidative metabolism [25] between the cat and cheetah that were related to species physiology rather than sperm morphology. For example, sperm MMP was ~95% lower in the cheetah compared to both normospermic and teratospermic domestic cats [25]. Based on these collective observations, one would expect metabolically-robust spermatozoa to be recovered post-thaw by isolating cells on the basis of motility rather than structural integrity. Furthermore, results of the present study suggest that structurally-normal cells are as susceptible to metabolic cryo-damage as their abnormal counterparts. However, because only structurally-intact spermatozoa are capable of achieving fertilization [27], cellular morphology remains an important consideration for optimizing post-thaw sperm fertilizing potential. Indeed, it now appears that the most effective cryopreservation and processing protocols should yield post-thaw samples containing high proportions of structurally-normal, motile, and metabolically-robust cells.

In conclusion, our findings confirmed that important differences exist in gamete physiology among felids and in the inherent ability of spermatozoa to retain metabolic function after cryopreservation. In this case, cheetah spermatozoa, well known for a high incidence of structural pleiomorphisms [45,47,53,54], was particularly susceptible to metabolic cryo-damage. In contrast, a proportion of domestic cat spermatozoa retained normal metabolism after thawing, and perhaps this cellular subpopulation can provide clues for improving sperm cryo-survival in other species or populations with less vigorous ejaculate quality. Finally, our results confirmed that sperm metabolism, specifically the rate of lactate production, is a reliable metric of cellular quality that can be applied to evaluate and enhance sperm cryo-survival.

Acknowledgments

The authors thank Drs. Budhan Pukazhenthi and Pierre Comizzoli for helpful advice, Drs. Luis Padilla, Katharine Hope, Carlos Sanchez for veterinary support, and Jenny Santiestevan and Marianne de Jonge for technical assistance. This study is dedicated to the memory of Dr. JoGayle Howard, whose pioneering research made our work possible.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cryobiol.2011.12.006.

References

[1] G.C. Luvoni, E. Kalchschmidt, S. Leoni, C. Ruggiero, Conservation of feline semen, Part I: Cooling and freezing protocols, J Feline Med Surg 5 (2003) 203–208.
[2] W.F. Swanson, Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids, ILAR J 44 (2003) 307–316.
[3] N. Cocchia, F. Ciani, R. El-Rass, M. Russo, G. Borzacchiello, V. Esposito, S. Montagnaro, L. Avallone, G. Tortora, R. Lorizio, Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction, Zygote 18 (2010) 1–8.

[4] J.M. Tebet, M.I.M. Martins, V.H. Chirinea, F.F. Souza, D. Campagnol, M.D. Lopes, Cryopreservation effects on domestic cat epididymal versus electroejaculated spermatozoa, Theriogenology 66 (2006) 1629–1632.

[5] J.R. Herrick, M. Campbell, G. Levens, T. Moore, K. Benson, J. D'Agostino, G. West, D.M. Okeson, R. Coke, S.C. Portacio, K. Leiske, C. Kreider, P.J. Polumbo, W.F. Swanson, In vitro fertilization and sperm cryopreservation in the black-footed cat (*Felis nigripes*) and sand cat (*Felis margarita*), Biol Reprod 82 (2010) 552–562.

[6] D.L.K. Baudi, K. Jewgenow, B.S. Pukazhenthi, K.M. Spercoski, A.S. Santos, A.L.S. Reghelin, M.V. Candido, M.L. Javorouski, G. Müller, R.N. Morais, Influence of cooling rate on the ability of frozen-thawed sperm to bind to heterologous zona pellucida, as assessed by competitive in vitro binding assays in the ocelot (*Leopardus pardalis*) and tigrina (*Leopardus tigrinus*), Theriogenology 69 (2008) 204–211.

[7] A.E. Crosier, B.S. Pukazhenthi, J.N. Henghali, J.G. Howard, A.J. Dickman, L. Marker, D.E. Wildt, Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival, Cryobiology 52 (2006) 169–181.

[8] B. Pukazhenthi, K. Pelican, D. Wildt, J.G. Howard, Sensitivity of domestic cat (*Felis catus*) sperm from normospermic versus teratospermic donors to cold-induced acrosome damage, Biol Reprod 61 (1999) 135–141.

[9] B.S. Pukazhenthi, E. Noiles, K. Pelican, A.M. Donoghue, D.E. Wildt, J.G. Howard, Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors, Cryobiology 40 (2000) 139–150.

[10] B. Pukazhenthi, D. Laroe, A.E. Crosier, L.M. Bush, R. Spindler, K.M. Pelican, M. Bush, J.G. Howard, D.E. Wildt, Challenges in cryopreservation of clouded leopard (*Neofelis nebulosa*) spermatozoa, Theriogenology 66 (2006) 1790–1796.

[11] N. Ganan, R. Gonzalez, J.J. Garde, F. Martinez, A. Vargas, M. Gomendio, E.R.S. Roldan, Assessment of semen quality, sperm cryopreservation and heterologous IVF in the critically endangered Iberian lynx (*Lynx pardinus*), Reprod Fertil Dev 21 (2009) 848–859.

[12] K. Thiangtum, W.F. Swanson, J. Howard, W. Tunwattana, D. Tongthainan, W. Wichasilpa, P. Patumrattanathan, T. Pinyopoommintr, Assessment of basic seminal characteristics, sperm cryopreservation and heterologous in vitro fertilization in the fishing cat (*Prionailurus viverrinus*), Reprod Fertil Dev 18 (2006) 373–382.

[13] B.S. Pukazhenthi, R.E. Spindler, D.E. Wildt, L.M. Bush, J.G. Howard, Osmotic properties of spermatozoa from felids producing different proportions of pleiomorphisms: Influence of adding and removing cryoprotectant, Cryobiology 44 (2002) 288–300.

[14] P. Mazur, S.P. Leibo, G.E. Seidel Jr., Cryopreservation of the germplasm of animals used in biological and medical research: Importance, impact, status, and future directions, Biol Reprod. 78 (2008) 2–12.

[15] S.P. Leibo, L. Bradley, Comparative cryobiology of mammalian spermatozoa, in: C. Gagnon (Ed.), The Male Gamete: From Basic Science to Clinical Application, Cache River Press, Vienna, IL, 1999, pp. 502–516.

[16] P. Thuwanut, K. Chatdarong, A. Johannisson, A.S. Bergqvist, L. Soderquist, E. Axner, Cryopreservation of epididymal cat spermatozoa: Effects of in vitro antioxidative enzymes supplementation and lipid peroxidation induction, Theriogenology 73 (2010) 1076–1087.

[17] M. O'Connell, N. McClure, S.E.M. Lewis, The effects of cryopreservation on sperm morphology, motility and mitochondrial function, Hum Reprod 17 (2002) 704–709.

[18] E. Flores, J.M. Fernandez-Novell, A. Pena, T. Rigau, J.E. Rodriguez-Gil, Cryopreservation-induced alterations in boar spermatozoa mitochondrial function are related to changes in the expression and location of midpiece mitofusin-2 and actin network, Theriogenology 74 (2010) 354–363.

[19] O. Garcia-Alvarez, A. Maroto-Morales, M. Ramon, E. del Olmo, V. Montoro, A.E. Dominguez-Rebolledo, A. Bisbal, P. Jimenez-Rabadan, M.D. Perez-Guzman, A.J. Soler, Analysis of selected sperm by density gradient centrifugation might aid in the estimation of in vivo fertility of thawed ram spermatozoa, Theriogenology 74 (2010) 979–988.

[20] D. Schober, C. Aurich, H. Nohl, L. Gille, Influence of cryopreservation on mitochondrial functions in equine spermatozoa, Theriogenology 68 (2007) 745–754.

[21] T. Hallap, S. Nagy, Ü. Jaakma, A. Johannisson, H. Rodriguez-Martinez, Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633, Theriogenology 63 (2005) 2311–2322.

[22] M. Sa-Ardrit, J. Saikhun, N. Thongtip, M. Damyang, S. Mahasawangkul, T. Angkawanish, S. Jansittiwate, T. Faisaikarm, Y. Kitiyanant, K. Pavasuthipaisit, A. Pinyopummin, Ultrastructural alterations of frozen-thawed Asian elephant (*Elephas maximus*) spermatozoa, Int J Androl 29 (2006) 346–352.

[23] K.A. Terrell, D.E. Wildt, N.M. Anthony, B.D. Bavister, S.P. Leibo, L.M. Penfold, L.L. Marker, A.E. Crosier, Glycolytic enzyme activity is essential for domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) sperm motility and viability in a sugar-free medium, Biol Reprod 84 (2011) 1198–1206.

[24] K.A. Terrell, D.E. Wildt, N.M. Anthony, B.D. Bavister, S.P. Leibo, L.M. Penfold, L.L. Marker, A.E. Crosier, Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*), Biol Reprod 83 (2010) 833–841.

[25] K.A. Terrell, D.E. Wildt, N.M. Anthony, B.D. Bavister, S.P. Leibo, L.P. Penfold, A.E. Crosier, Oxidative phosphorylation is essential for felid sperm function, but is substantially lower in cheetah (*Acinonyx jubatus*) compared to domestic cat (*Felis catus*) ejaculate, Biol Reprod 85 (2011) 473–481.

[26] E.A. McLaughlin, W.C.L. Ford, M.G.R. Hull, The contribution of the toxicity of a glycerol egg-yolk citrate cryopreservative to the decline in human sperm motility during cryopreservation, J Reprod Fertil 95 (1992) 749–754.

[27] J.G. Howard, A.M. Donoghue, L.A. Johnston, D.E. Wildt, Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats, Biol Reprod 49 (1993) 131–139.

[28] J.G. Howard, J.L. Brown, M. Bush, D.E. Wildt, Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones and improvement of spermatozoal motility and morphology after swim-up processing, J Androl 11 (1990) 204–215.

[29] A. Crosier, J. Henghali, J.G. Howard, B.S. Pukazhenthi, K.A. Terrell, L.L. Marker, D.E. Wildt, Improved quality of cryopreserved cheetah (*Acinonyx jubatus*) spermatozoa after centrifugation through Accudenz, J Androl 30 (2009) 298–308.

[30] A. Stendardi, R. Focarelli, P. Piomboni, D. Palumberi, F. Serafini, A. Ferramosca, V. Zara, Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa, Int J Androl 34 (2011) 247–255.

[31] R. Bohnensack, W. Halangk, Control of respiration and of motility in ejaculated bull spermatozoa, Biochim Biophys Acta 850 (1986) 72–79.

[32] A. Minelli, M. Moroni, C. Castellini, P. Lattaioli, I. Mezzasoma, G. Ronquist, Rabbit spermatozoa: A model system for studying ATP homeostasis and motility, J Androl 20 (1999) 259–266.

[33] G. Kamp, G. Busselmann, J. Lauterwein, Spermatozoa: Models for studying regulatory aspects of energy metabolism, Experientia 52 (1996) 487–494.

[34] B.S. Pukazhenthi, K. Neubauer, K. Jewgenow, J.G. Howard, D.E. Wildt, The impact and potential etiology of teratospermia in the domestic cat and its wild relatives, Theriogenology 66 (2006) 112–121.

[35] J.A. Long, D.E. Wildt, B.A. Wolfe, J.K. Crister, R.V. DeRossi, J.G. Howard, Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats, Biol Reprod 54 (1996) 638–646.

[36] J.G. Howard, Semen collection and analysis in carnivores, in: M.E. Fowler (Ed.), Zoo and Wild Animal Medicine: Current Therapy III, WB Saunders Co., Philadelphia, 1993, pp. 390–399.

[37] D.K. Gardner, H.J. Leese, Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro, J Reprod Fertil 88 (1990) 361–368.

[38] B.D. Bavister, Substitution of a synthetic polymer for protein in a mammalian gamete culture system, J Exp Zool 217 (1981) 45–51.

[39] D. Shah, M. Naciri, P. Clee, M. Al-Rubeai, NucleoCounter: An efficient technique for the determination of cell number and viability in animal cell culture processes, Cytotechnology 51 (2006) 39–44.

[40] J.L. Larson, D.J. Miller, Simple histochemical stain for acrosomes on sperm from several species, Mol Reprod Dev 52 (1999) 445–449.

[41] J.V. Passonneau, O.H. Lowry, Enzymatic Analysis: A Practical Guide, Humana Press, Totowa, NJ, 1993.

[42] D.J. McLean, A.J. Feltmann, D.P. Froman, Transfer of sperm into a chemically defined environment by centrifugation through 12% (wt/vol) Accudenz, Poult Sci 77 (1998) 163–168.

[43] J.A. Long, D.C. Bongalhardo, J. Pelaez, S. Saxena, P. Settar, N.P. O'Sullivan, J.E. Fulton, Rooster semen cryopreservation: Effect of pedigree line and male age on postthaw sperm function, Poult Sci 89 (2010) 966–973.

[44] SASSAS/STAT User's Guide: Statistics. Version 9.1, Statistical Analysis System Institute, Inc., Cary, NC, 2002.

[45] D.E. Wildt, M. Bush, J.G. Howard, S.J. O'Brien, D. Meltzer, A. Van Dyk, H. Ebedes, D.J. Brand, Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat, Biol Reprod 29 (1983) 1019–1025.

[46] S.J. O'Brien, M.E. Roelke, L. Marker, A. Newman, C.A. Winkler, D. Meltzer, L. Colly, J.F. Evermann, M. Bush, D.E. Wildt, Genetic basis for species vulnerability in the cheetah, Science 227 (1985) 1428–1434.

[47] A.E. Crosier, L. Marker, J.G. Howard, B.S. Pukazhenthi, J.N. Henghali, D.E. Wildt, Ejaculate traits in the Namibian cheetah (*Acinonyx jubatus*): influence of age, season, and captivity, Reprod Fertil Dev 19 (2007) 370–382.

[48] R. Harrison, I. White, White, Glycolytic enzymes in the spermatozoa and cytoplasmic droplets of bull, boar, and ram, and their leakage after shock, J Reprod Fertil 30 (1972) 105–115.

[49] J.M. Sztein, K. Noble, J.S. Farley, L.E. Mobraaten, Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation, Cryobiology 42 (2001) 28–39.

[50] F. Nawroth, V. Isachenko, S. Dessole, G. Rahimi, M. Farina, N. Vargiu, P. Mallmann, M. Dattena, G. Capobianco, D. Peters, I. Orth, E. Isachenko, Vitrification of human spermatozoa without cryoprotectants, Cryoletters 23 (2002) 93–102.

[51] L.M. Penfold, L. Jost, D.P. Evenson, D.E. Wildt, Normospermic versus teratospermic domestic cat sperm chromatin integrity evaluated by flow cytometry and intracytoplasmic injection, Biol Reprod 69 (2003) 1730–1735.

[52] B.S. Pukazhenthi, D.E. Wildt, M.A. Ottinger, J.G. Howard, Compromised sperm protein phosphorylation after capacitation, swim-up, and zona pellucida exposure in teratospermic domestic cats, J Androl 17 (1996) 409–419.

[53] D.E. Wildt, S.J. O'Brien, J.G. Howard, T.M. Caro, M.E. Roelke, J.L. Brown, M. Bush, Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies, Biol Reprod 36 (1987) 351–360.

[54] D.E. Wildt, J.L. Brown, M. Bush, M.A. Barone, K.A. Cooper, J. Grisham, J.G. Howard, Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: the benefits of physiological surveys for strategic planning, Zoo Biol 12 (1993) 45–80.