Semen cryopreservation and radical reduction capacity of seminal fluid in captive African lion (*Panthera leo*)

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Abstract

Optimizing cryopreservation protocols for nondomestic felids contributes to the successful development of assisted reproduction techniques and genetic resource banking. In this study, we describe a simple cryopreservation procedure for African lion (*Panthera leo*) ejaculates, which was tested with different packaging options and different sperm numbers per dose. By applying urethral catheterization and electroejaculation, 17 ejaculates with greater than 20% motile and greater than 5% progressively motile sperm were collected. A lyophilized extender (a modified egg yolk-Tes-Tris-fructose-glycerol medium) was rehydrated and added in one step at ambient temperature (~25 °C) to semen, which was prediluted in cell culture medium M199. After slow cooling of insulated samples to 15 °C in a refrigerator (4 °C), the samples were fast frozen over the surface of liquid nitrogen or in a dry shipper. Aliquots of 300 µL containing 20×10^6 sperm were frozen in cryovials and in 0.5-mL straws. Differences were observed in the total motility after thawing between vial (31.5 ± 14.1%) and straw freezing (20.1 ± 8.6%). However, the subpopulations of vital (22.7 ± 7.8% for vial and 19.8 ± 8.5% for straw) and progressively motile (10.0 ± 7.9% for vial and 10.0 ± 6.4% for straw) sperm after washing and 1 hour incubation at 38 °C were of similar magnitude, velocity, and linearity for both packaging options. After freezing of five ejaculates with 20, 60, and 100×10^6 sperm per dose, best results were achieved at the lowest concentration. In general, post-thaw results were highly variable (2.2% and 56.5% total motility) and not correlated to motility or morphology of the fresh semen. To further characterize semen quality, we assessed the protective potential of seminal fluid against oxidative stress, which might be challenged on freeze thawing. The capacity of seminal fluid to reduce radicals was measured in 10 semen samples by electron spin resonance spectroscopy and a spin-labeled fatty acid as a radical probe. Moreover, we determined the lysophosphatidylcholines (LPC) as potential lipid oxidation products in the sperm and erythrocytes of the males. Individuals with a high radical reduction capacity in the seminal fluid and a low LPC content in their erythrocytes showed a better cryosurvival of sperm. This

is a first indication that seminal fluid may affect the freezing potential of African lion ejaculates.

Keywords: Lion ; Semen collection ; Cryopreservation ; Radical reduction ; Seminal fluid

1. Introduction

Maintaining healthy wildlife populations is only possible when adequate genetic pools are maintained [1]. Small population sizes and habitat fragmentation are threatening the diversity in many species including large cats such as African lions (*Panthera leo*) [2–4]. Lions, similar to most wild felids are listed on the IUCN red list: African lion as vulnerable and Asian lion (*Panthera leo* persica) as endangered. *Ex situ* breeding programs and the implementation of assisted reproduction techniques (ART) potentially contribute to sustain the genetic diversity in endangered species conservation [5,6]. Research into ART in felids has already resulted in offspring through the use of artificial insemination (e.g., [7–10]) or embryo transfer [11]. However, the success of ART is still rare and inconsistent in felids, and it is of utmost importance to develop adequate methods for assisted reproduction in time to have them available should captive breeding become inevitable for the survival of a species. This has been successfully demonstrated by the captive breeding program of the Iberian lynx (*Lynx pardinus*), the most endangered felid worldwide [12].

Sperm collection and cryopreservation are the most widely applied methods of ART used in combination with artificial insemination, *in vitro* fertilization (IVF), or intracytoplasmatic sperm injection into oocytes. Sperm cryopreservation is still challenging in many wildlife species because the knowledge of cryopreservation-relevant sperm properties is limited. Also, the design of field-friendly methods for sperm collection and cryopreservation to be implemented into conservation practice is of great importance. Few reports are available on lion semen cryopreservation. Malo et al. [13] froze epididymal sperm from a pride of lions after castration. Motility dropped from 15% in freshly prepared sperm to 5% after freezing. However, 45% of the thawed cells rejected the dye propidium iodide, a marker for dead cells, and 49% of sperm had still active mitochondria after thawing [13]. Improved post-thaw motility results (20.5 ± 6.3% and 30.4 ± 18.0%) were achieved with electroejaculated semen of higher original motility (85%–95% and >40%) by Stander-Breedt et al. [14] and Patil [15], respectively.

Based on these results, we first aimed to test a field-friendly cryopreservation method for African lion (*Panthera leo*) semen collected by urethral catheterization (UC) or electroejaculation (EE). The method was successfully applied on epididymal sperm of domestic cats [16] and comprised a one-step addition of glycerol together with the freezing extender to sperm at ambient temperature. The diluted semen was also packaged at ambient temperature either in vials or straws to compare both options. Before freezing over liquid nitrogen or in a dry shipper, samples were insulated and slowly cooled to ~15 °C, which was achieved after 40 minutes in a conventional refrigerator at 4 °C. Handling of semen at 4 °C was not required. In regard to artificial insemination with frozen semen, more than 20×10^6 sperm per dose could be beneficial, particularly, if the expected proportion of

vital sperm after thawing is low. Therefore, we tested the extender efficiency for cryopreservation of different sperm numbers.

The optimization of freezing methods depends on species-specific properties; for instance, membrane composition, permeability for cryoprotectants, and cryoprotectant cytotoxicity. However, an intraspecies variability of semen properties and the lack of a selection of male individuals for cryoperformance of their sperm still complicate the process. A potential challenge in the cryopreservation of sperm is the degradation of the sample by oxidative stress. Although the capacity of mitochondria to produce reactive oxygen species (ROS) is rather reduced during freeze-thawing, increased lipid peroxidation was observed and is regarded detrimental for sperm function in domestic species (for review see [17]). The dilution of seminal fluid antioxidants, an osmotically driven loss of sperm's cellular water, and a resultant disturbance of antioxidative enzyme functions probably affect the available antioxidant capacity in semen during cryopreservation [17,18]. Whether the defense system against ROS becomes insufficient under challenging conditions depends on the basic antioxidant equipment, and a variable ability of individual semen samples to cope with ROS could be one reason for the different cryosurvival of sperm. Therefore, we measured the capacity of seminal fluid to reduce free radicals by electron spin resonance (ESR) spectroscopy and correlated this protective competence with cryosurvival of sperm. Deviations from putative (in healthy condition) equilibrium between ROS production and their detoxification may also occur in case of pathologic metabolic situations. An accumulation of oxidation products in body tissues and cells including erythrocytes or sperm could be the result. An extensive accumulation of lysophospholipids as degradation products of phosphatidylcholines (PC) after lipid oxidation was, for example, recently observed in erythrocytes and fresh semen of severely obese men [19,20]. To assess any handicap in our samples, we looked at the occurrence of lysophosphatidylcholines (LPC) in sperm as well as in erythrocytes of the respective individuals as PCs represent the most abundant cellular membrane phospholipids.

2. Material and methods

2.1. Animals, ejaculates, and experimental design

Captive bred, male African lions kept in two facilities in South Africa were examined in the years 2012 and 2014. The age of the animals ranged between 2.5 and 8.0 years (mean ± SD: 5.0 ± 1.7 years). A total of 27 ejaculates were obtained from 30 semen collection attempts in 29 different lions (one lion produced two ejaculates, one in 2012 and one in 2014). Only ejaculates with greater than 20% motile and greater than 5% progressively motile sperm were cryopreserved which left a total of 17 samples (2012: N = 6, 2014: N = 11) from 16 different males, which were known proven breeders. To compare the packaging methods, all 17 ejaculates were cryopreserved in both, vials and straws. Straws were frozen in a dry shipper (2012) or in a polystyrene box over liquid nitrogen (2014). Vials were always frozen in a polystyrene box over liquid nitrogen. To assess the maximum sperm concentration for cryopreservation, five ejaculates with sufficient sperm numbers were frozen in vials at different sperm concentrations (20, 60, and 100 × 10^6 in 300 µL).

Before cryopreservation, original sperm parameters (volume, concentration, morphology, motility) were characterized and the ejaculates were treated as described below. Ten ejaculates collected in 2014, were additionally analyzed for the radical reduction capacity in seminal fluid and the LPC content in fresh sperm. Of the 10 respective lions, the LPC content was also measured in erythrocytes. After thawing of cryopreserved samples as well as after subsequent centrifugation and 1 hour incubation, sperm motility and vitality were determined.

The experimental protocols were approved by the Research Ethics and Scientific Committee of the National Zoological Gardens of South Africa (NZG P12/17). Sample transfer to Germany was approved by CITES authorities (permits 125614, 138022). Unless otherwise stated, chemicals were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA or Steinheim, Germany).

2.2. Semen and blood sample collection

Semen was collected during general anesthesia by two methods, urethral catheterization (UC) as described previously for lions [21], or electroejaculation (EE). In case no semen was obtained by UC or the volume was too small, EE was applied subsequently. Twelve of the cryopreserved ejaculates were collected by UC, four by EE, and one using both UC and EE.

A portable cattle machine with the probe originally designed for sheep and goat with a diameter of 3.5 cm and three electrodes of 10.0-cm length were used (El Toro 3, Electronic Research Group, Midrand, Johannesburg, South Africa). The probe was placed at the depth of the prostate into the rectum and three to four sets of 10 low stimuli (1 second on–0.5 seconds off) were applied (5–10 V), then the probe was slightly moved caudal and the procedure repeated (2 seconds on–2 seconds off) to allow the ejaculate to be pushed outside by urethral contraction. The UC and/or EE ejaculates were collected into 1.5-mL tubes at African field temperatures (>30 °C) and one or two fractions initially assessed for volume, color, and motility before pooling.

Blood was drawn from the jugular or medial saphenus vein and sampled in EDTA-coated blood vials, carefully tilted from side to side at least 10 times and stored at 4 °C for a maximum of 12 hours until further handling.

2.3. Cryopreservation and thawing

Cryopreservation of semen was performed according to the recently published protocol for domestic cat epididymal sperm [16]. A Tes-Tris-fructose-buffer (Test) was prepared according to Schmehl et al. [22] with addition of 15% (v:v) of the water-soluble fraction of hen's egg yolk (containing low-density lipoproteins) according to Lengwinat and Blottner [23]. To facilitate transportation and enable longer storage of extender at 4 °C, 2-mL aliquots of Test were lyophilized. Resolubilization was performed by adding Aqua bidest. After supplementation with 7% (v:v) glycerol, the complete extender (TestG) was used.

If not stated otherwise, sperm concentration was adjusted to 100×10^6/mL with M199 (Hepes modification, Sigma-Aldrich M7528). One volume of sperm in M199 was slowly

diluted with two volumes of TestG at ambient temperature (~25 °C) and diluted sperm samples (300 µL each) were pipetted into cryovials (2-mL round bottom, Greiner, Germany) or loaded into 500-µL straws (IMV Technology, France). Equilibration was performed in a refrigerator at 4 °C. Vials and straws were thermally insulated in a water bath or polystyrene box, respectively. After 40 minutes, a temperature of ~15 °C was reached and vials were frozen in the vapor of liquid nitrogen in a polystyrene box [16]. Straws were frozen rapidly either by lowering them to the bottom of a container (dry shipper) or in liquid nitrogen vapor. Straw freezing in a dry shipper used a fully charged but empty dry shipper (Voyageur 5, Air Liquide). Straws were placed in the canister (precooled to 4 °C), which was lowered rapidly (within 1–2 seconds) to the bottom of the dry shipper in one step. After 10 minutes in the dry shipper, straws were plunged into liquid nitrogen for storage [24]. For the liquid nitrogen vapor method, straws were placed horizontally on a rack and lowered slowly through liquid nitrogen vapor until floating 4 cm above the surface of the liquid nitrogen. After 10 minutes, straws were plunged into liquid nitrogen. The freezing rate was about −22 K/min [25].

Thawing of samples was performed by slewing a single vial or straw for 90 seconds in a 5-L circulating water bath (LAUDA, Lauda-Königshofen, Germany) at 38 °C. Note that thawing in a water bath of smaller volume reduces the thawing speed. The sperm suspension was transferred into a prewarmed 2-mL tube and slowly diluted with 300-µL prewarmed M199. After 10 minutes incubation at 38 °C, sperm were centrifuged at ×500*g* for 5 minutes, resuspended in 300-µL fresh prewarmed M199, and incubated at 38 °C for 1 hour.

2.4. Evaluation of sperm quality before and after freezing

Semen volume was measured using a pipette. A 5-µL aliquot of semen was fixed with formol (1% in PBS w/o Ca/Mg) and used to determine the sperm concentration in a counting chamber. Sperm morphology of fixed sperm was evaluated in a wet drop mounted on a slide with coverslip under a microscope with phase-contrast optics (× 1000 magnification).

Sperm motility of fresh semen was subjectively estimated by two competent observers under a microscope equipped with a heating stage and phase-contrast optics (× 100 magnification) and the mean for total and progressive motility in a small sample diluted with prewarmed M199 was documented. After thawing, sperm motility was first analyzed after dilution with M199 and 10 minutes incubation at 38 °C. A second analysis was performed with washed samples after further incubation (after centrifugation and 1 hour at 38 °C). A 5-µL aliquot of sperm suspension was loaded into a prewarmed (38 °C) Makler chamber (Sefi-Medical Instruments, Haifa, Israel) and placed under a phase-contrast microscope (× 20 objective). After a first check by eye though the oculars, sperm motility was evaluated with the Computer Assisted Sperm Analysis (CASA) system SpermVision (Minitüb, Germany). Measurement was repeated in a second chamber and a total of ~450 sperm per sample was recorded. The percentages of motile and progressively motile sperm are presented as well as the mean velocity (VCL: velocity curve line, velocity of the sperm head along its actual path) and the mean linearity (VSL/VCL with VSL: velocity along a straight line from its first to its last position) of progressively motile sperm.

Sperm vitality was evaluated in thawed samples after centrifugation using rhodamine 123 (R123, Invitrogen, Molecular Probes, Eugene, Oregon, USA) and propidium iodide (PI, Invitrogen, Molecular probes, Eugene, Oregon, USA). A 250-µL aliquot of thawed and washed sperm (~2.5 × 10^6 sperm) was stained with 1-µL R123 (5 mg/100 mL stock in Aqua bidest.; final concentration 20 ng/10^6 sperm) and 2.5-µL PI (1 mg/mL stock in Aqua bidest; final concentration 1 µg/10^6 sperm). Stained sperm were incubated in the dark for 20 minutes at 38 °C. 10 µL of stained sperm suspension (~0.25 × 10^6 sperm) were diluted in 2-mL PBS (Sigma-Aldrich D8662) at 38 °C for measurement in a flowcytometer (CyFlow space and FlowMax software, Partec, Germany) equipped with a 50-mW solid-state laser (Ex 488 nm), a 515–560 nm band-pass for R123 (green), and a 620-nm long-pass filter for PI (red). The system was triggered on the forward light scatter, and 15,000 cells were characterized per sample for their fluorescence at a flow rate of about 200 cells per second. Sperm with active mitochondria accumulate R123 and show bright green fluorescence, whereas dead sperm allow PI to enter the nucleus and appear red. R123-positive sperm were considered to be vital.

2.5. Analysis of LPC in sperm and erythrocytes

To separate sperm and seminal fluid, aliquots of ejaculates were centrifuged within 30 minutes after collection at ×1000*g* for 5 minutes. Small and highly concentrated samples were diluted as necessary by PBS (Sigma-Aldrich D8662), which was afterward considered by the corresponding dilution factor. To remove residual sperm from seminal fluid, it was centrifuged again at ×12,000*g* for 3 minutes and then frozen at −20 °C. The sperm pellet was carefully resuspended in 1-mL PBS and centrifuged at ×1000*g* for 5 minutes. This washing step was repeated once and afterward the sperm pellet was resuspended in 300-µL PBS and frozen at −20 °C.

To isolate the erythrocytes, EDTA blood was centrifuged at ×1000*g* for 15 minutes and the upper phase and buffy coat were removed. The resulting cell pellet was washed twice in PBS at ×1000*g* for 5 minutes. The cell pellet was resuspended in 1-mL PBS and frozen at −20 °C. A 5-µL aliquot of the cell suspension was fixed with formol (1% in PBS) and used to determine the concentration of erythrocytes and residual leukocytes in a counting chamber. All samples were transported in liquid nitrogen and further stored at −80 °C until processing.

Lipid extraction of sperm and erythrocytes was performed according to the protocol of Bligh and Dyer (1959). The cell suspension (400 µL containing at least 15 × 10^6 cells) was mixed with 800-µL chloroform/methanol (1:1, v:v) and then centrifuged at ×3000*g* for 5 minutes to establish complete phase separation. The lower chloroform phase was removed by a glass pipette and the solvent evaporated at 40 °C under a nitrogen stream. Samples were stored at −20 °C until analysis. The extracts were redissolved in chloroform and subsequently mixed (1:1, v:v) with the matrix solution of 0.5-M 2,5-dihydroxybenzoic acid (Acros Organics, distributed by Fisher Scientific GmbH, Nidderau, Germany) in methanol. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) was performed on a Bruker Autoflex MS device (Bruker Daltonics, Bremen, Germany). Raw data were processed with the software “Flex Analysis” version 2.2 (Bruker Daltonics). The proportion of LPC was calculated from the respective peak intensities for LPC and PCs according to the formula: LPC [%] = 100 × LPC/(PC + LPC).

2.6. Determination of the radical reduction capacity in seminal fluid

For measuring radical reduction capacity in seminal fluid, a spin-labeled fatty acid (SL-FA, 4-doxylpentanoic acid) was used, which was synthesized as described by Fellmann et al. [26]. SL-FA can be measured by ESR spectroscopy, and the potential of seminal fluid to eliminate free radicals due to reducing components results in a decrease of signal intensity. SL-FA dissolved in chloroform/methanol (1:1, v:v) as 1-mM stock solution was dried under a nitrogen flow and solubilized to a final concentration of 100 µM in HBS (HEPES-buffered solution, 150-mM NaCl, 5-mM HEPES, pH 7.4) containing 1% BSA which binds the SL-FA and, therefore, mediates its accessibility in aqueous solution. 25-µL SL-FA solution was mixed with 5-µL HBS and 20-µL seminal fluid at 23 °C and immediately filled into glass capillaries (Blaubrand intraMark Mikropipetten, 50 µL, BRAND GmbH & Co KG, Wertheim, Germany).

ESR spectra were recorded at different times over 30 minutes with the following parameters: modulation amplitude 2.5 G, power 20 mW, scan width 60 G, 1× accumulated. From the spectra, the intensities of the mid-field peak were determined and related to those in the absence of seminal fluid. Kinetics of signal reduction were monoexponentially fitted (SigmaPlot, Version 10.0, Systat Software Inc.) to determine the rate constant of reduction. The latter was multiplied by the amount of applied SL-FA and related to the sperm number in a 20-µL volume of the original semen sample (sperm concentration in the ejaculate) to calculate the radical reduction capacity per sperm cell.

2.7. Statistical analysis

All statistical analyses were performed by PASW Statistics 18 (SPSS Inc.). Nonparametric tests were used because of the low number of cases. To compare freezing in vials versus straws, the nonparametric Wilcoxon's signed-rank test was used for paired data sets (motility parameters and vitality after thawing) determined in 2012 and 2014. For every parameter, the Spearman rank correlation between results achieved in vials and straws was calculated. To compare the outcome of three different sperm concentrations, differences of parameter distributions for motility and vitality after thawing were analyzed by Friedman's univariate ANOVA on ranks of related data followed by respective post-hoc tests. Spearman correlations were also determined between initial sperm properties (motility, morphology, radical reduction capacity in seminal fluid, and LPC in sperm), LPC in erythrocytes of the donor male, and sperm quality after thawing (motility parameters, vitality). The criterion for statistical significance was an error probability of 0.05 (two-sided).

3. Results

3.1. Initial sperm quality

The initial sperm quality of the 17 cryopreserved ejaculates was variable ranging from 20% to 95% motile sperm (median 60%), 5% to 60% progressively motile sperm (median 22%), and a total sperm count of 77 to 3950 × 10^6 (median 968 × 10^6). The proportion of morphologically normal sperm (presence of an acrosome and absence of midpiece or tail deformations) ranged from 11% to 64% (median 44%). Between 5% and 67% of plasma

droplets (median 28%) were observed. The predominant abnormality was a coiled tail at a frequency of 9% to 56% (median 24%).

3.2. Cryopreservation in vials and straws

When comparing sperm quality after thawing of split samples frozen either in cryovials or straws, only few parameters differed significantly (Table 1). Only in 2014, the total motility directly after thawing was significantly higher in vials than straws. It should be noted that the maximum values in the broad range of sperms' total and progressive motility directly after thawing were in both years higher in vials than in straws. After centrifugation and 1 hour incubation, any differences in the proportion of motile and progressively motile sperm disappeared because the motility of sperm frozen in vials decreased to similar values as was found for straws (Table 1). The percentages of sperm with active mitochondria approximate to the total percentages of motile sperm. The velocity of the progressively moving sperm was significantly higher after freezing in straws than in vials (directly after thawing in 2012, after centrifugation and incubation in 2012 and 2014).

Table 1. Motility and vitality data of African lion sperm after cryopreservation.

Parameters	Year	Median (min–max)		Wilcoxon comparison
		Vials	Straws	P
Motility (%) after thawing	2012	31.2 (18.2–51.8)	18.4 (7.4–38.2)	0.080
	2014	28.3 (2.2–56.5)	18.6 (5.5–32.7)	**0.013**
Progressive motility (%) after thawing	2012	14.7 (1.9–32.9)	11.8 (4.4–25.1)	0.345
	2014	8.5 (0.0–36.2)	9.1 (0.6–21.6)	0.182
Progressive velocity VCL (µm/s) after thawing	2012	108.9 (75.6–124.1)	120.5 (105.7–145.1)	**0.043**
	2014	92.6 (19.8–131.2)	112.9 (51.1–140.2)	0.169
Progressive linearity after thawing	2012	0.57 (0.48–0.64)	0.63 (0.55–0.73)	0.225
	2014	0.55 (0.42–0.72)	0.55 (0.47–0.62)	0.645
Motility (%) after centrifugation, 1 h 38 °C	2012	26.2 (12.9–37.2)	16.6 (6.7–35.4)	0.080
	2014	19.6 (5.7–35.5)	14.8 (8.6–27.8)	0.248
Progressive motility (%) after centrifugation, 1 h 38 °C	2012	8.8 (0.5–22.9)	10.4 (4.3–19.7)	0.345
	2014	8.5 (0.6–26.2)	6.4 (2.8–22.0)	0.929
Progressive velocity VCL (µm/s) after centrifugation, 1 h 38 °C	2012	90.3 (49.0–113.0)	107.2 (94.9–119.5)	**0.043**
	2014	90.5 (43.7–135.4)	98.2 (61.3–128.8)	**0.021**
Progressive linearity after centrifugation, 1 h 38 °C	2012	0.60 (0.50–0.80)	0.57 (0.53–0.71)	0.893
	2014	0.60 (0.45–0.70)	0.58 (0.46–0.68)	0.722
Vitality (%) after centrifugation	2012	25.4 (20.6–39.9)	24.5 (7.6–32.6)	0.225
	2014	20.2 (6.0–28.6)	19.8 (8.8–29.1)	0.314

Split sperm samples were frozen either in vials or straws. Straws were frozen in a dry shipper (2012: N = 6) or in a polystyrene box over liquid nitrogen (2014: N = 11).

Median, minimum, and maximum of the respective data are given for vials and straws in the respective year.

Bold P-values (Wilcoxon's signed-rank test) indicate significant differences in sperm parameters after thawing and centrifugation between vials and straws.

Figure 1 illustrates the results for correlations between both packaging methods. For all sperm parameters, a broad spectrum of sperm quality was observed. For the 11 samples of 2014, sperm motility data and the percentage of vital sperm with active mitochondria, but not the linearity of progressive sperm, were significantly correlated between vials and straws (Fig. 1). When all 17 samples were included in the calculation, significant correlations were obtained for all parameters except for vitality (data not shown). A comparison between the samples frozen in straws by different methods (dry shipper in 2012 versus polystyrene box in 2014) revealed only one significant difference (P = 0.039), a higher linearity directly after thawing of progressively moving sperm frozen in a dry shipper.

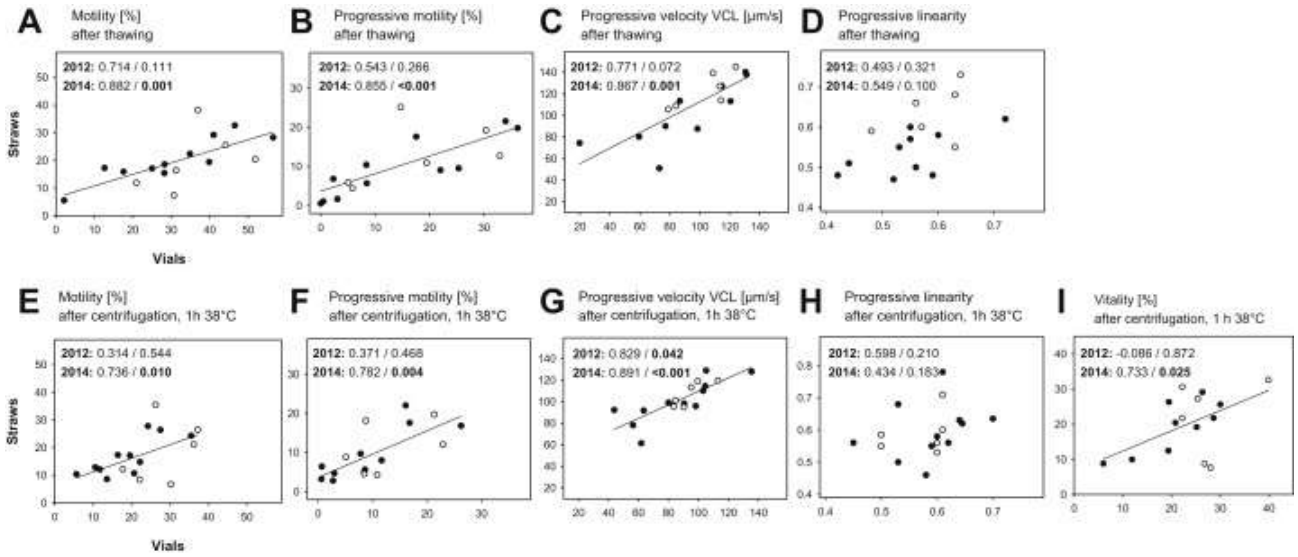

Fig. 1. Motility and vitality data of African lion sperm after cryopreservation. Split sperm samples were frozen either in vials or straws. Corresponding results after thawing and centrifugation are visualized in scatter plots. Spearman correlation coefficients between corresponding data (vials and straws) and significances are given in the upper left corner (r_s/P). Bold P-values indicate significant correlations between vials and straws and linear regression lines were shown in these cases for 2014. Straws were frozen in a dry shipper (2012: N = 6, white circles) or in a polystyrene box over liquid nitrogen (2014: N = 11, black circles, solid lines).

3.3. Cryopreservation of different sperm concentrations

When thawed sperm frozen in vials at three different concentrations (20, 60, and 100 × 10^6 sperm per 300 µL) were compared, except for the linearity of sperm movement, all parameters tended to decline at higher concentrations (Fig. 2). The only significant decline was seen for the proportion of motile sperm directly after thawing when 100 × 10^6 sperm were frozen per dose compared with 20 × 10^6 (P = 0.013, Fig. 2A).

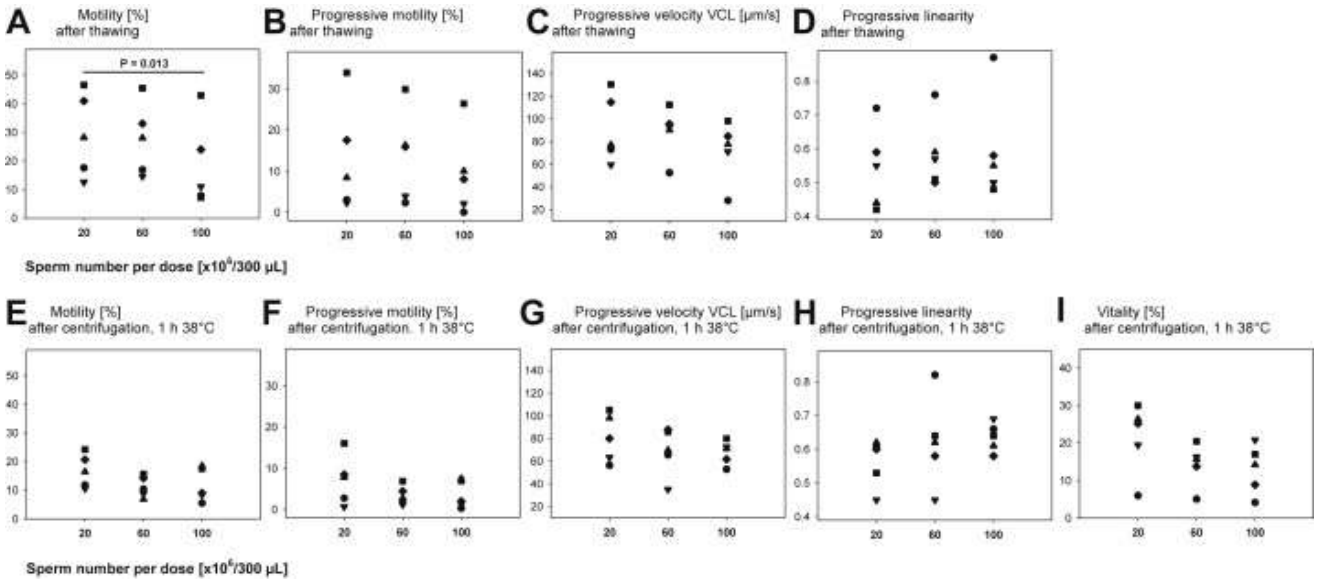

Fig. 2. Motility and vitality data of African lion sperm after cryopreservation. Split sperm samples (N = 5) were frozen in vials at different sperm concentrations (4.7% [v:v] final glycerol concentration). Single data points are displayed per sample. Identical symbols represent the same sample with a total sperm number of 20, 60, or 100 × 10^6 per dose (300 µL). The P-value (Friedman's univariate ANOVA on ranks) of the only significant difference between sperm numbers per vial is given.

3.4. Individual suitability for cryopreservation

The variability of sperm quality after thawing was very large between individual samples (Fig. 1). Since a variable ability of sperm to cope with oxidative stress on freezing might be a potential reason for this variability, the occurrence of degradation products of PC, LPC, was measured in sperm in 10 ejaculates as well as in erythrocytes of the 10 respective lions. The percentage of LPC in sperm ranged from 0% to 14.4% (median 1.5%), and in erythrocytes from 0% to 2.6% (median 0.8%). In addition, the protective capacity of seminal fluid against radical attacks was determined as 0 to 1.2 pmol/(min × 10^6 sperm) (median 0.39). Whereas sperm quality parameters directly after thawing were not correlated to the initial sperm quality (Table 2), three of the parameters (i.e., motility, progressive motility, and vitality) were positively correlated with the capacity of seminal fluid to eliminate radicals (Table 2). This radical reduction capacity in seminal fluid was in turn negatively correlated with the accumulation of LPC in the erythrocytes of males (r_s = −0.824, P = 0.003). Progressive motility and vitality after thawing showed a tendency to correlate negatively with the occurrence of LPC in erythrocytes (Table 2). None of the sperm parameters after thawing was correlated with the percentage of LPC in sperm (Table 2). Only post-thaw data for samples frozen in vials are shown in Table 2; data for straw-frozen aliquots revealed similar results.

Table 2. Correlations of African lion post-thaw sperm quality and fresh sperm properties (motility, morphology, the capacity of seminal fluid to inactivate radical compounds, and proportions of lysophosphatidylcholines (LPC) in sperm and erythrocytes of the males).

Correlation between	Initial motility (%)	Initial progressive (%)	Initial normal sperm (%)	Radical reduction capacity in seminal fluid (pmol/[min × 10^6 sperm])	Lysophosphati-dylcholines (LPC) in fresh sperm (%)	Lysophosphati-dylcholines (LPC) in erythrocytes (%)
Motility after thawing (%)						
r_s	0.311	0.177	0.000	0.659	−0.288	−0.492
P	0.382	0.648	1.000	**0.038**	0.419	0.148
Progressive after thawing (%)						
r_s	0.201	0.068	0.000	0.671	−0.362	−0.602
P	0.577	0.863	1.000	**0.034**	0.304	0.066
Velocity VCL after thawing (µm/s)						
r_s	−0.142	−0.398	−0.142	0.613	−0.153	−0.444
P	0.715	0.329	0.715	0.079	0.695	0.232
Vitality after thawing (%)						
r_s	0.361	−0.109	0.067	0.860	−0.418	−0.619
P	0.339	0.797	0.864	**0.003**	0.262	0.075

Spearman correlation coefficients (r_s) and significances (P) are shown (N = 10).

Bold P-values indicate significant correlations.

4. Discussion

Wild African lion populations have declined as much as 30% over the last 2 decades (www.IUCN.org). Due to progressing spatial isolation of smaller populations and concomitant restriction of male dispersal, genetic variability may continuously decline [2]. This also applies to semi-captive and captive populations in game reserves and zoos [27]. Assisted breeding by artificial insemination with cryopreserved semen could be a realistic means to re-establish genetic heterozygosity, if field-friendly methods are available to provide good-quality semen for preservation in wildlife cryobanks [28].

Field-friendly methods would involve simple procedures such as the addition of cryoprotectant and packaging of diluted semen already at ambient temperature [29]. In previous attempts to freeze lion sperm obtained by EE, semen was either brought to 4 °C before the slow (drop-wise) addition of cryoprotectant to the cooled sperm by Stander-Breedt [14], or the extender was already added in one step after centrifugation of semen and the freezing process was started afterward [15]. The latter protocol has also been successfully applied to epididymal semen of domestic cats [30]. A study from Crosier et al. [31] on cheetah semen revealed that sperm exposure to glycerol for 1 hour at room

temperature (~22 °C) before a cooling phase of 3.5 hours to 5 °C had only a minimal effect on sperm motility after cryopreservation. In the light of this finding, we tested a procedure with a one-step addition of glycerol at ambient temperature, followed by a short equilibration on slow cooling for 40 minutes to only 15 °C before freezing, which had recently been proven to be successful with epididymal semen from the domestic cat [16]. After thawing, we determined 31.5 ± 14.1% (mean ± SD) motile lion sperm (median 31.0, range 2.2%–56.5%) for samples frozen in cryovials. 10 of 17 post-thaw samples had a higher proportion of motile sperm than 30%, and four of them had greater than 40% total motility. Obviously, glycerol exposure (even in one step and at higher temperatures) is well tolerated by lion ejaculates of several individuals. This seems to be also valid in the case of tiger (*Panthera tigris*) and Indian leopard (*Panthera pardus* fusca) semen, which was successfully frozen (24.1 ± 8.4% and 32.1 ± 9.1% post-thaw motility, respectively) when glycerol was added in one step according to the protocol by Patil [15,32].

Directly after thawing, freezing in vials seems to be slightly superior to freezing in 0.5-mL straws when the same sample volume (300 µL) was used. However, due to a more pronounced regression of post-thaw motility in vial-frozen samples after washing and 1 hour incubation, the differences in total and progressive motility disappear between both packaging options. Further, a similar proportion of sperm in both vials and straws had active mitochondria and excluded the dye propidium iodide. This proportion of vital sperm with active mitochondria has turned out to be one valuable predictive parameter for the fertility of liquid-preserved boar semen in a comprehensive field study when measured on the second day of semen storage [33]. Regarding the sperm velocity along the actual path, the straw-frozen progressive sperm swam even somewhat faster and just as linear compared with vial-frozen progressive sperm. Interestingly, sperm velocity and linearity after thawing was in a comparable range with the post-thaw results of Patil [15], who measured moderately lower values in cryopreserved than freshly collected semen. We cannot compare qualitative motion characteristics before and after freezing in our study because initial motility was only subjectively estimated under field conditions. During semen investigation of diverse felid species beyond this study, we revealed that the motion characteristics of the particular subpopulation of progressively motile sperm were similar before and after cryopreservation even if the percentage of progressive sperm dramatically declines (unpublished data).

Cryopreserved sperm can be successfully applied to IVF applications (see [29]). Epididymal lion sperm cryopreserved by our method was recently used to produce lion blastocysts by intracytoplasmatic sperm injection [34]. However, artificial insemination with frozen feline semen is still ineffective and requires high sperm numbers and sperm deposition deep in the uterus or even oviduct [35,36]. Therefore, we assign special importance to the proportion of progressively motile sperm to succeed in passing through the female genital tract particularly during the final steps toward fertilization. This core population of vital and progressively motile sperm which survive cryopreservation seems to be of similar size and quality in both packaging options and would be at disposal for any fertilization attempt.

However, neither the percentage of total nor progressive motile lion sperm after thawing is satisfactory when compared with the outcome of cryopreservation in the domestic cat [16,30] or even more in farm animal species. Swanson [29] proposed a minimum of 10×10^6

motile sperm for one artificial insemination procedure in felids. With only 20×10^6 sperm per dose, a survival rate of 50% would be necessary to meet this requirement. With 60×10^6 sperm per dose, a survival rate of 15% would be sufficient and, finally, more realistic. Therefore, we increased the number of sperm per vial and evaluated their performance after cryopreservation since it is questionable whether the extender ingredients such as glycerol, lipoproteins, lipids, buffer, and fructose are still adequate in the case of a much higher sperm concentration. Only five ejaculates with sufficiently high sperm numbers could be used for this approach. Due to this limited database and not very pronounced differences, the only significant reduction of thawing results could be monitored in the case of 100×10^6 sperm per vial for total post-thaw motility. However, most parameters decreased already at 60×10^6 sperm per vial. Therefore, freezing sperm at the lower concentration and combining sperm pellets after removal of glycerol by centrifugation seems more advisable if artificial insemination is intended.

Within the two captive populations of this study, a large variability of initial sperm quality as well as suitability to cryopreservation was observed between individual samples. We did not use ejaculates with extremely low sperm motility; however, a very good motility was not necessarily indicative for an adequate capability to survive cryopreservation. Since some ejaculates showed quite satisfactory results after thawing, we tried to identify potentially responsible properties which enable a good freeze-thawing success. Besides chemical, osmotic, mechanic, and temperature-related challenges [37], cryopreservation also means oxidative stress to the sperm when the cellular generation of ROS overwhelms antioxidant defense [17,18,38,39]. It is well known that extender supplementation with antioxidative compounds may improve the outcome of cryopreservation in various species including the domestic cat [17,39–41]. Based on the 10 comprehensively analyzed ejaculates, we present first indications that a higher capacity of seminal fluid to remove free radicals might better protect sperm from the attack of ROS during the freezing and thawing process. More motile, progressive, and vital sperm with active mitochondria survive in samples with a high radical reduction capacity per sperm. Interestingly, in individuals with a low radical reduction capacity in seminal fluid (and a poorer cryosurvival of sperm), the accumulation of LPC as lipid degradation product was high in their erythrocytes. An increased amount of LPC may destabilize cellular membranes and is generated when lipid oxidation is accompanied by the loss of the oxidatively modified unsaturated fatty acyl residue [42]. This particularly occurs when antioxidative defense or LPC repair mechanisms are insufficient as for instance under inflammatory conditions [42,43]. A low incidence of LPC in erythrocytes together with an effective radical reduction capacity in a body fluid like semen could reflect a healthy body condition and would, therefore, suggest a role of the global metabolic state of the individual for the cryoperformance of a male's sperm.

In contrast to the findings with erythrocytes, the initial LPC content in fresh sperm was not correlated to the efficiency of radical reduction in the corresponding seminal fluid or to post-thaw sperm survival. Sperm lysolipids are not only produced by the action of ROS [42]. They also play a role in cell signaling after moderate generation by phospholipases as, for instance, in the course of sperm to fertilization [44] and any premature LPC accumulation in sperm should be prevented. However, a partly higher LPC abundance in sperm than erythrocytes indicate insufficient defense and/or repair systems in the epididymis where sperm were stored before collection and contact to the seminal fluid.

4.1. Conclusions

An uncomplicated simple method has been proven suitable for the cryopreservation of African lion sperm. The extender may be shipped as lyophilized powder which is to be reconstituted by distilled water and glycerol. To avoid semen handling at 4 °C, semen is diluted in one step and packaged at ambient temperature (~25 °C). After slow cooling of insulated (water jacket) samples to 15 °C in a refrigerator (4 °C), the samples were frozen over the surface of liquid nitrogen (vials and straws) or in a dry shipper (straws). Vials (easy to fill and label for unskilled persons) and straws revealed similar results. The sperm number should not exceed 60×10^6 sperm per dose (300 µL).

The variability in cryoperformance was high between ejaculates and seems to be at least partly related to the capacity of seminal fluid to reduce radicals and/or ROS. It remains to be tested whether satisfactory thawing results, which were achieved with individual ejaculates, are only accredited to the respective ejaculate, or are a characteristic property of the specific male lion. Causal relationships between antioxidative protection of sperm and the metabolic state of the individual remain to be investigated on the way to identify and support problematical sperm samples to be used for genetic resource banking.

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Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

[1] Lacy RC. Importance of genetic variation to the viability of mammalian populations. J Mammal 1997;78:320–35.

[2] Bjorklund M. The risk of inbreeding due to habitat loss in the lion (*Panthera leo*). Conserv Genet 2003;4:515–23.

[3] Antunes A, Troyer JL, Roelke ME, Pecon-Slattery J, Packer C, Winterbach C, et al. The evolutionary dynamics of the lion *Panthera leo* revealed by host and viral population genomics. PLoS Genet 2008;4:e1000251.

[4] Bauer H, Chapron G, Nowell K, Henschel P, Funston P, Hunter LTB, et al. Lion (*Panthera leo*) populations are declining rapidly across Africa, except in intensively managed areas. Proc Natl Acad Sci U S A 2015;112:14894–9.

[5] Lermen D, Blomeke B, Browne R, Clarke A, Dyce PW, Fixemer T, et al. Cryobanking of viable biomaterials: implementation of new strategies for conservation purposes. Mol Ecol 2009;18:1030–3.

[6] Bruche S, Gusset M, Lippold S, Barnett R, Eulenberger K, Junhold J, et al. A genetically distinct lion (*Panthera leo*) population from Ethiopia. Eur J Wildl Res 2013;59:215–25.

[7] Bowen MJ, Platz Jr CC, Brown CD, Kraemer DC. Successful artificial insemination and embryo collection in the African lion (*Panthera leo*). Congress of American Association of Zoo Veterinarians pro-ceeding, 1982 1982. p. 57–9.

[8] Dresser BL, Kramer L, Reece K, Russell PT. Induction of ovulation and successful artificial insemination in a Persian leopard (*Panthera pardus saxicolor*). Zoo Biol 1982;1:55–7.

[9] Swanson WF. Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids. ILAR J 2003;44:307–16.

[10] Lueders I, Ludwig C, Schroeder M, Mueller K, Zahmel J, Dehnhard M. Successful nonsurgical artificial insemination and hormonal monitoring in an Asiatic Golden cat (*Catopuma temmincki*). J Zoo Wildl Med 2014;45:372–9.

[11] Pope CE, Gomez MC, Dresser BL. In vitro embryo production and embryo transfer in domestic and non-domestic cats. Theriogenology 2006;66:1518–24.

[12] Vargas A, Breitenmoser C, Breitenmoser U. Iberian lynx ex-situ conservation: an interdisciplinary approach. Madrid, Spain: Fundación Biodiversidad; 2009.

[13] Malo AF, Martinez-Pastor F, Olivier F, Spies T, Roldan ERS, Bartels P. Effect of refrigeration and cryopreservation on the quality of lion epididymal spermatozoa. Reprod Fertil Dev 2004;16:223–4.

[14] Stander-Breedt H, Schwalbach LMJ, Greyling JPC, Loskutoff NM. Effect of different cryodiluents and thawing methods on the post-thaw motility of African Lion (*Panthera leo*) spermatozoa. South Afr J Anim Sci 2004;34:74–6.

[15] Patil SB, Jayaprakash D, Shivaji S. Cryopreservation of semen of tigers and lions: computerized analysis of the motility parameters of the spermatozoa. Curr Sci (Bangalore) 1998;75:930–5.

[16] Klaus C, Eder S, Franz C, Muller K. Successful cryopreservation of domestic cat (*Felis catus*) epididymal sperm after slow equilibration to 15 or 10 degrees C. Reprod Domest Anim 2016;51: 195–203.

[17] Yeste M. Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology 2016;85:47–64.

[18] Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2011;2011. http://dx.doi.org/10. 4061/2011/686137.

[19] Pyttel S, Zschornig K, Nimptsch A, Paasch U, Schiller J. Enhanced lysophosphatidyl-choline and sphingomyelin contents are characteristic of spermatozoa from obese men-A MALDI mass spectrometric study. Chem Phys Lipids 2012;165:861–5.

[20] Nimptsch A, Pyttel S, Paasch U, Mohr C, Heinrich JM, Schiller J. A. MALDI MS investigation of the lysophosphatidylcholine/phospha-tidylcholine ratio in human spermatozoa and erythrocytes as a useful fertility marker. Lipids 2014;49:287–93.

[21] Lueders I, Luther I, Scheepers G, van der Horst G. Improved semen collection method for wild felids: Urethral catheterization yields high sperm quality in African lions (*Panthera leo*). Theriogenology 2012;78:696–701.

[22] Schmehl MK, Anderson SP, Vazquez IA, Graham EF. The effect of dialysis of extended ram semen prior to freezing on post-thaw survival and fertility. Cryobiology 1986;23:406–14.

[23] Lengwinat T, Blottner S. In vitro fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. Anim Reprod Sci 1994;35:291–301.

[24] Roth TL, Bush LM, Wildt DE, Weiss RB. Scimitar-horned oryx (*Oryx dammah*) spermatozoa are functionally competent in a heterologous bovine in vitro fertilization system after cryopreservation on dry ice, in a dry shipper, or over liquid nitrogen vapor. Biol Reprod 1999;60:493–8.

[25] Nothling JO, Shuttleworth R. The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. Theriogenology 2005;63:1469–80.

[26] Fellmann P, Zachowski A, Devaux PF. Synthesis and use of spin-labeled lipids for studies of the transmembrane movement of phospholipids. Methods Mol Biol 1994;27:161–75.

[27] Bertola LD, van Hooft WF, Vrieling K, de Weerd DRU, York DS, Bauer H, et al. Genetic diversity, evolutionary history and implications for conservation of the lion (*Panthera leo*) in West and Central Africa. J Biogeogr 2011;38:1356–67.

[28] Pukazhenthi BS, Wildt DE. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev 2004;16:33–46.

[29] Swanson WFS, Magarey GM, Herrick JR. Sperm cryopreservation in endangered felids: developing linkage of in situ – ex situ populations. In: Roldan E, Gomendio M, editors. Spermatology. Not-tingham, UK: Nottingham University Press; 2007. p. 417–32.

[30] Stachecki JJ, Ginsburg KA, Armant DR. Stimulation of cryopreserved epididymal spermatozoa of the domestic cat using the motility stimulants caffeine, pentoxifylline, and 2'-deoxyadenosine. J Androl 1994;15:157–64.

[31] Crosier AE, Pukazhenthi BS, Henghali JN, Howard J, Dickman AJ, Marker L, et al. Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival. Cryobiology 2006;52:169–81.

[32] Jayaprakash D, Patil SB, Kumar MN, Majumdar KC, Shivaji S. Semen characteristics of the captive Indian leopard, *Panthera pardus*. J Androl 2001;22:25–33.

[33] Schulze M, Ruediger K, Mueller K, Jung M, Well C, Reissmann M. Development of an in vitro index to characterize fertilizing capacity of boar ejaculates. Anim Reprod Sci 2013;140:70–6.

[34] Fernandez-Gonzalez L, Hribal R, Stagegaard J, Zahmel J, Jewgenow K. Production of lion (*Panthera leo*) blastocysts after in vitro maturation of oocytes and intracytoplasmic sperm injection. Theriogenology 2015;83:995–9.

[35] Luvoni GC, Kalchschmidt E, Leoni S, Ruggiero C. Conservation of feline semen. Part I: cooling and freezing protocols. J Feline Med Surg 2003;5:203–8.

[36] Swanson WF. Laparoscopic oviductal embryo transfer and artificial insemination in felids–challenges, strategies and successes. Reprod Domest Anim 2012;47:136–40.

[37] Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 1990;11: 73–88.

[38] Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 2009;24:2061–70.

[39] Agarwal A, Durairajanayagam D, du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 2014;12:112.

[40] Luvoni GC, Ruggiero C, Marinoni G, Kalchschmidt E. Effect of taurine-containing diluent for cryopreservation of domestic cat epididymal spermatozoa. Theriogenology 2002;57:466.

[41] Thuwanut P, Chatdarong K, Johannisson A, Bergqvist AS, Soderquist L, Axner E. Cryopreservation of epididymal cat spermatozoa: effects of in vitro antioxidative enzymes supplementation and lipid peroxidation induction. Theriogenology 2010;73: 1076–87.

[42] Fuchs B, Muller K, Paasch U, Schiller J. Lysophospholipids: potential markers of diseases and infertility? Mini Rev Med Chem 2012;12: 74–86.

[43] Aitken RJ, Jones KT, Robertson SA. Reactive oxygen species and

[44] Roldan ER, Shi QX. Sperm phospholipases and acrosomal exocytosis. Front Biosci 2007;12:89–104.