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A two-step dilution tris-egg yolk extender containing Equex STM significantly improves sperm cryopreservation in the African wild dog (*Lycaon pictus*)

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ABSTRACT

Conservation management of endangered African wild dogs (AWD; *Lycaon pictus*) can benefit greatly from development of sperm freezing and artificial insemination. Previous freezing attempts yielded nearly 0% motile sperm within 2 h of thawing. In this study, two canine freezing protocols were tested: Protocol 1: a one-step dilution in TRIS-20% egg yolk containing 8% glycerol; and Protocol 2: a two-step dilution in TRIS-20% egg yolk containing a final extender concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution. Semen was collected by electroejaculation from n = 24 AWDs, of which eight ejaculates of sufficient quality (four good quality with initial sperm motility of 75.0 ± 4.4% and four poor quality; showing rapid decrease in sperm motility to 3.3 ± 3.3% prior to freezing) were frozen. For good quality samples, motility and sperm motility index persisted for up to 8 h for Protocol 2, and was higher between 2 and 6 h after thawing with a decrease from 4 h of incubation. Motility dropped to nearly 0% after 2 h incubation for Protocol 1. Viability was higher for Protocol 2 throughout the 8 h of incubation, with a decrease after 6 h, compared to 4 h for Protocol 1. Acrosome integrity was higher for Protocol 2 throughout post-thaw incubation, with a decrease after 2 h for both protocols. Protocols did not differ in normal sperm morphology or DNA integrity. Poor quality samples yielded similar results, except for acrosome integrity, which declined for Protocol 2. In conclusion, a two-step dilution in TRIS-egg yolk-glycerol extender containing Equex STM yields significantly improved post-thaw quality and longevity of AWD spermatozoa, making it suitable for sperm banking and artificial insemination initiatives.

1. Introduction

Over the last four decades, there has been a drastic 58% decrease in vertebrate wildlife numbers around the globe [69]. The primary cause for this decline is human interference, particularly habitat loss and destruction [8,23,27]. For many species, captive breeding has become extremely important to ensure their survival and to maintain their genetic diversity. However, the success of captive breeding programs is often limited by husbandry related issues such as species-specific enclosure requirements, nutritional deficiencies, and abnormal group structures that can impede natural breeding and/or reproductive health [19,38]. Assisted reproductive techniques are an important aid in overcoming some of the limitations inherent to the captive breeding of non-domestic animals [19,38]. Artificial insemination (AI) and sperm freezing are the most accessible and commonly used techniques, and are already well-established tools for the breeding management of domestic animals [6,14,26]. Progressively, these techniques have been incorporated into the captive breeding programs of a wide range of wildlife species [38]. The major advantages of sperm freezing include: (i) securing genetic diversity; (ii) extending the fertility of a genetically

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Abbreviations

AWD	African wild dog
AI	artificial insemination
EY	egg yolk
SDS	sodium dodecyl sulphate
PSA	Pisum Sativum Agglutinin
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
PVP	polyvinylpyrrolidone

important animal beyond its lifespan; and (iii) allow easy transport of semen across the globe [48,62].

The African wild dog (AWD, *Lycaon pictus*), once roaming most of sub-Saharan Africa, has disappeared from most of its original range with the current population now estimated at a mere 6600 mature animals [67]. Sperm freezing, and development of AI techniques, can aid species management and conservation of the AWD [62]. Sperm freezing in this species has been attempted previously by either diluting semen 1:3 in TEST (TES and Tris) buffer containing 7.5% glycerol and 15% egg yolk (EY) [11]; or by two-step dilution with the first dilution in Tris-citrate buffer containing 20% EY, and the second dilution after cooling to 5 °C in the same extender but containing 8% glycerol [16]. Initially, both protocols gave good immediate post-thaw sperm motility of up to 40% and 31.8 ± 5.8% respectively. However, within 2 h of thawing, sperm motility declined rapidly to nearly 0%. AI using spermatozoa with such a short motile lifespan is unlikely to lead to successful fertilization in the ampullae of the oviducts.

In domestic dogs, sperm motility and normal morphology are parameters typically used to determine the quality of frozen-thawed samples used for AI [25,30,58]. However, other sperm quality parameters such as viability, acrosome integrity and DNA integrity should be evaluated with equal importance. The sperm acrosome is crucial for zona pellucida penetration and fertilization [1], however, it is often damaged during freezing but such damage is not detected by a corresponding decline in motility [32]. Moreover, spermatozoa with damaged DNA do not necessarily show a decline in other sperm quality parameters, but such damage can significantly impair embryo development and cause pregnancy loss [21,39,45,53].

There are species-, breed- and even individual differences in the resistance of the sperm cell to cold shock and the freeze-thaw process [13,64]. However, when developing sperm freezing techniques for a new species, it is advisable to start with testing semen extenders and freezing protocols that give good results in closely related species [4]. The AWD belongs to the Canidae, but is taxonomically quite distinct (the only species in the genus *Lycaon*) and relatively distantly related to the other canids [66]. However, the domestic dog can be used as an important model for the development of sperm freezing protocols. In

Table 1
Animal details and semen quality of African wild dogs (*Lycaon pictus*) included in this study.

Pack	ID - Name	Date of birth	Date of collection	Body weight (kg)	Semen volume (ml)	Total spermatozoa ejaculated (x 10^6)	Sperm motility (%)	Frozen	Comments/reason for not freezing
ABQ	2393 - Mooseface	8 Nov 2006	8 Aug 2014	31.8	5.43[a]	58.3	83	Yes[d]	–
	2394 - Digger	8 Nov 2006	7 Aug 2014	32.0	0.00	–	–	No	No ejaculate
	2395 - Growlly	8 Nov 2006	7 Aug 2014	32.4	4.25[a]	142.4	74	Yes[d]	–
BRK	2494 - Nar	25 Nov 2010	21 Aug 2014	31.2	2.85 (urine)	3.8	1	No	Urine contamination, low motility
	2499 - Jack	25 Nov 2010	21 Aug 2014	34.2	4.40 (urine)	107.6	12	No	Urine contamination, low motility
BIN	2428 - Blacktail	24 Oct 2007	17 Sep 2014	31.6	1.70[a]	27.3	74	No[d]	Motility lost after centrifugation
	2383 - Victor	16 Oct 2006	18 Sep 2014	33.2	1.80[a]	71.9	80	Yes	–
	2427 - Verizon	24 Oct 2007	17 Sep 2014	34.3	0.43	10.8	36	No	Low motility
OCZ	T1 - Dojo	23 Nov 2011	30 Sep 2014	29.9	0.01	0.03	0	No	Low volume, no motility
	T3 - Chipata	23 Nov 2011	30 Sep 2014	31.3	0.08	0.8	55	No	Low volume, low motility
	T2 - Juma	23 Nov 2011	30 Sep 2014	27.7	0.02	0	–	No	No spermatozoa
BRU	M1 - Brutus	unknown	17 Nov 2015	35.3	0.41	1.3	50	No	Low motility
PLA	M1 - Zevon	unknown	14 Jan 2016	24.5	0.02	0.2	43	No	Low volume, low motility
	M6 - Styx	unknown	16 Jan 2016	27.7	1.58[a]	83.7	(75[b]) 31 (0[c])	Yes[e]	Rapid decrease in motility
	M8 - Harrison	unknown	16 Jan 2016	22.7	0.00	–	–	No	No ejaculate
	M10 - Garfunkel	unknown	15 Jan 2016	25.5	0.15	3.0	(80[b]) 59	Yes[e]	Rapid decrease in motility
	M11 - Lennon	unknown	15 Jan 2016	21.8	0.65	8.5	(90[b]) 79 (0[c])	Yes[e]	Rapid decrease in motility
	M2 - Marley	unknown	14 Jan 2016	24.0	4.36[a]	49.2	(90[b]) 76 (10[c])	No[e,f]	Rapid decrease in motility
	M3 - Zeppelin	unknown	14 Jan 2016	21.0	0.29	1.6	66	No[f]	–
	M4 - Dylan	unknown	14 Jan 2016	25.0	15.00 (urine)	14.6	0	No	Urine contamination, no motility
	M5 - Ozzy	unknown	15 Jan 2016	25.0	0.74	2.0	0	No	No motility
	M13 - Wilson	unknown	16 Jan 2016	24.0	2.55[a]	44.4	(90[b]) 66	Yes[e]	Rapid decrease in motility
SAN	M1	2011	20 Mar 2016	31.0	0.10	0	–	No	No spermatozoa
	M2	2011	19 Mar 2016	26.5	2.68[a]	13.0	63	Yes	–

[a] Samples with large prostate fluid contribution.
[b] Estimated motility immediately after collection.
[c] Estimated motility immediately prior to extender dilution and refrigeration; motility values without superscript indicate values obtained from detailed sperm analysis.
[d] Samples centrifuged after sperm analysis.
[e] Samples had rapid decrease in motility immediately prior to extender dilution.
[f] Samples used to optimise freezing technique.

the dog, the most commonly used laboratory-prepared extenders are still TRIS-citrate-fructose or -glucose based [40,58,63]. EY is added to the semen extenders of most species, usually at a concentration of 20% for domestic dogs [24,51,54]. EY is a complex biological substance containing proteins, phospholipids, vitamins, glucose, antibacterial compounds and antioxidants [7]; presumingly protecting the sperm plasma membrane against cold shock by interacting with the phospholipid bilayer to prevent or restore the loss of phospholipids [7,65]. The type of cryoprotectant added to the semen extender may vary for different species but for most, glycerol yields the best freezing outcomes; although toxic in high concentrations [5,12]. Glycerol replaces intracellular water during dehydration [12], reduces the extracellular freezing point by decreasing the electrolyte concentration, and integrates into the sperm plasma membrane changing its stability and permeability [10,12,20]. In domestic dogs, optimal post-thaw semen quality has been achieved using glycerol concentrations between 2 and 8% [34,42,50].

The addition of sodium dodecyl sulphate (SDS), an anionic detergent, to EY-based extenders was beneficial to post-thaw sperm motility, viability, acrosome integrity and longevity in a wide range of species, including domestic dogs [31,40,43,51,60]. The exact mechanism underlying this beneficial effect is not known, but SDS is believed to interact with molecules present in EY, stabilising the sperm membrane and increasing its permeability, and thereby avoiding osmotic shock [2,51]. SDS has been added to extenders in its pure form [43], or as a component of Equex STM [51], Equex paste [44], or Orvus ES paste [31,60]. However, the beneficial effect of SDS appears to be influenced by additional factors in these additives. When using Equex STM in the domestic dog, an increased sperm longevity and viability was observed during post-thaw incubation, however these effects were absent when using Equex paste at the same dosage [44]. Furthermore, Equex paste did not show any beneficial effects in generic grey and Mexican grey wolves (*Canis lupus* and *C. lupus baileyi)* [71]. By contrast, post-thaw motility, plasma membrane integrity and acrosome integrity of spermatozoa were greatest when Equex STM was added at the end of the equilibration period [40], making a two-step dilution important when using this additive. In addition, this two-step procedure has also been proposed to enable cooling and equilibration of spermatozoa in lower initial glycerol concentrations, thereby minimizing its toxic effect [40].

The aim of this present study was to improve the longevity of AWD spermatozoa after thawing by testing two sperm freezing protocols which routinely yield high quality frozen-thawed spermatozoa in domestic dogs [40,58]: Protocol 1 - a one-step dilution in TRIS - 20% v/v EY extender containing 8% v/v glycerol, without subsequent thawing solution (one-step - thawing solution); and Protocol 2 - a two-step dilution in TRIS - 20% v/v EY extender to a final extender concentration of 5% v/v glycerol and 0.5% v/v Equex STM, coupled with a TRIS-citrate-fructose thawing solution (two-step + thawing solution).

2. Materials and methods

2.1. Animals

Semen was collected by electroejaculation from a total of n = 24 adult AWDs (≥2.8 years old; Table 1) from 4 US zoos: Albuquerque BioPark (ABQ pack; Albuquerque, NM), Brookfield Zoo (BRK pack; Chicago, IL), Binder Park Zoo (BIN pack; Battle Creek, MI), and Oklahoma City Zoo (OCZ pack; Oklahoma City, OK) during the 2014 breeding season (August–September); and from Namibia: Harnas Wildlife Foundation (BRU, PLA and SAN packs, Gobabis) during the 2015–2016 breeding season (November, January and March respectively). AWDs in the US were held in packs containing three males except for OCZ, which also contained a female. Animals were held in outside enclosures during the day with access to inside holding areas overnight, except at BIN in which animals were locked inside their holding area overnight. Animals in Namibia were held in large natural enclosures in mixed-sex packs. All animals had access to water *ad libitum* and in the US were individually-fed with ground horsemeat (Central Nebraska Packing Inc., NE, USA), occasionally replaced by bones, whole pig or goat carcass. In Namibia, animals were group-fed with donkey and horsemeat on the bone or intestines (local slaughter material), occasionally replaced by dog pellets (Hill's Pet Nutrition, Kansas, United States), or goat, sheep or wild game meat.

2.2. Anaesthesia

In the US, AWDs were immobilised with 1.2–4.0 mg/kg tiletamine/zolazepam hydrochloride (40–120 mg IM; Telazol®, Zoetis Inc., MI, USA) with or without 0–6.3 μg/kg medetomidine (0–0.4 mg IM; Medetomidine HCl®, ZooPharm, WY, USA), and maintained with Isoflurane (0.5–5% in 1 l/min O_2; IsoFlo®, Zoetis Inc., MI, USA; Isothesia®, Henry Schein Animal Health, OH, USA; or Isoflurane®, MWI Animal Health, ID, USA). In cases where medetomidine was administered, AWDs were reversed with atipamezole (0.01–0.1 mg/kg, IM; Antisedan®, Zoetis Inc., MI, USA) after intubation and/or at the end of anaesthesia. In Namibia, all animals were sedated with an initial dose of 3.9–7.5 mg/kg tiletamine/zolazepam hydrochloride (100–180 mg IM; Zoletil® 100; Virbac, Carros, France) with, where needed, the addition of another 20–40 mg IM to maintain the level of anaesthesia. In these animals, no medetomidine was administered.

2.3. Semen collection

After positioning the AWD in dorsal or lateral recumbency, the penis was manually extruded from the prepuce and rinsed with sterile saline. The bladder was emptied using a 6 fr or 8 fr canine urinary catheter (Jørgen Kruuse A/S, Langeskov, Denmark) gently passed down the urethra, and flushed several times with sterile saline to avoid urine contamination. Semen was collected using a 20 or 25 mm diameter rectal probe (Beltron Instruments™, Bryan, TX, USA) with three raised longitudinally oriented 30–40 mm strip electrodes connected to a 20 Hz sine wave electroejaculator (CGS Products™ Pty. Ltd., Trafalgar, Victoria, Australia) [37]. The probe was lubricated with KY Jelly™ (Johnson & Johnson, New Brunswick, NJ, USA) and inserted into the rectum with the electrodes oriented ventrally at the level of the prostate as estimated using ultrasound (6.5–17.5 cm deep, depending on animal). The electroejaculation protocol consisted of three series separated by rest periods of approximately 5 min. Each series included 30 stimulations of 4 s, increasing the voltage after every 10 stimuli. During each 4-sec stimulation, the probe was gradually retracted towards the anus. Series 1 was conducted at 2, 3 and 4 V, series 2 at 3, 4 and 5 V, and series 3 at 3, 4 and 5 V or 4, 5 and 6 V depending on the reaction of the animal to stimulation. During stimulation, the penis was placed in a 50 ml or 15 ml pre-warmed plastic tube, which was replaced after each series to avoid urine contamination. Each tube was kept at 37 °C until analysis, which began within 5 min after completion of the last series.

2.4. Semen evaluation

Each fraction was evaluated for volume, colour, presence of motile spermatozoa, and pH (if semen volume was sufficient), then all fractions were combined. To evaluate motility, 10 μl of sample was placed on a pre-warmed glass slide with cover-slip on a slide microscope fitted with a 37 °C warm stage (LEC Instruments Pty. Ltd., Scoresby, Victoria, Australia). Where sperm concentration was high, a 10 μl aliquot was first diluted 1:1 v/v in phosphate buffered saline (PBS) at 37 °C. Motility rating (1–4 scale) and sperm motility index (0–400 scale) were calculated by classifying at least 100 spermatozoa at 400× magnification as grade 0 (non-motile sperm), 1 (motile non-forward progression), 2 (poor forward progression), 3 (moderate straight-line forward progression) or 4 (fast straight-line forward progression) [37]. The

percentage of viable and morphologically normal versus abnormal spermatozoa were evaluated by eosin-nigrosine smear (≥100 cells, 1000× magnification) [15]. Abnormal spermatozoa were grouped by primary defects (abnormalities of the sperm head and intermediary piece) and secondary defects (tail abnormalities and cytoplasmic droplets). Sperm concentration and total number of spermatozoa ejaculated were calculated using a haemocytometer (BLAUBRAND® Neubauer improved bright-line, Brand GMBH, Wertheim, Germany) [68].

Acrosome integrity was evaluated using Pisum Sativum Agglutinin (PSA) conjugated with FITC (Sigma-Aldrich, St-Louis, MO, USA) [49]. Between 20 and 50 µl of semen was washed in PBS and centrifuged (720×*g*, 5 min). The pellet was resuspended in 50 µl 95% ethyl alcohol (Sigma-Aldrich, St-Louis, MO, USA) and refrigerated at 4 °C for at least 30 min. Approximately 20 µl semen was then smeared on a glass slide and air-dried. Glass slides were stored at 4 °C for up to three days then stained in the dark with 20 µl 0.1% w/v PSA-FITC in PBS layered on top of the cells and incubated at 4 °C for 15 min, then were rinsed in distilled water and air-dried. The acrosomes of 200 spermatozoa per male were evaluated at 1000× magnification by fluorescence microscopy. Acrosome-intact spermatozoa displayed an intense green fluorescence over the entire acrosomal region, whereas acrosome-damaged spermatozoa had incomplete or minor fluorescence at the equatorial region (Fig. 1a).

Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) was performed using the *In-Situ* Cell Death Detection Kit, Fluorescein™ (Roche Diagnostics, Basel, Switzerland). This test evaluates DNA integrity by fluorescently labelling free 3′OH termini associated with single and double stranded DNA breaks. Between 20 and 50 µl of semen was washed twice in 0.1% w/v polyvinylpyrrolidone (PVP; Sigma-Aldrich, St-Louis, MO, USA) in PBS by centrifugation (720×*g*, 5 min), and the sperm pellet resuspended in PVP solution to a final concentration of 1–2 × 10^6 spermatozoa/ml. An aliquot of 10 µl was smeared onto a poly-L-lysine-coated micro slide (Sigma-Aldrich, St-Louis, MO, USA), air-dried then fixed with 20 µl 4% w/v paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature and rinsed. Slides were then stored at 4 °C, and subsequently permeabilised and stained within three days of collection. Spermatozoa were permeabilised with 50 µl 0.5% Triton X-100 in 0.1% sodium citrate in PBS (Sigma-Aldrich, St-Louis, MO, USA) for 30 min at room temperature, rinsed with 500 µl of PBS, then incubated with 50 µl TUNEL reaction mixture (Labelling solution and Reaction enzyme solution combined) for 1 h at 37 °C in the dark. After rinsing with another 500 µl of PBS, slides were incubated with 25 µl 1 µg/ml Hoechst 33342 in PBS (Sigma-Aldrich, St-Louis, MO, USA) for 5 min at room temperature to counterstain sperm DNA. Each TUNEL assay included a negative (incubated as above with 50 µl Labelling solution only) and positive control slide. The positive control slide consisted of spermatozoa incubated for 1 h at 37 °C with 50 µl 1000 U/ml DNase I (recombinant; Sigma-Aldrich, St-Louis, MO, USA) in incubation buffer (10 mM Tris-HCl, pH 7.4 containing 10 mM NaCl, 5 mM $MnCl_2$, 0.1 mM $CaCl_2$ and 25 mM KCl) to induce double-stranded DNA breaks. A minimum of 100 cells were evaluated per sample at 1000× magnification by fluorescence microscopy. DNA fragmented spermatozoa showed an intense green fluorescence at the sperm head while DNA-intact sperm cells stained blue (Fig. 1b).

Ejaculates with ≥60% initial sperm motility were selected for freezing. Three US samples were centrifuged (720 *g*, 5 min) to increase sperm concentration prior to freezing, but this caused a decline in sperm motility in one sample, making it unsuitable for freezing (Table 1).

2.5. Sperm freezing

Each semen sample selected for freezing was split into two aliquots in 15 ml Falcon tubes and each aliquot was frozen using a different freezing protocol. For Protocol 1, semen was diluted 1:1 with a Tris-EY extender (0.25 M Tris-HCl; 0.08 M sodium citrate; 0.07 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% EY; and 8% glycerol), immersed in a 37°C water container and cooled over 2.5 h to 4°C. The sample was then loaded into 0.25 ml straws, suspended 4 cm over liquid nitrogen vapour for 10 min, then plunged into the liquid nitrogen. For each AWD, two straws were thawed in a 37°C water bath for 30 s. Protocol 2 consisted of an initial 2:1 v/v dilution (2 volumes of semen diluted in 1 volume of extender 1) with a Tris-EY extender containing a lower glycerol concentration (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% v/v EY; and 3% v/v glycerol), which was cooled over 2.5 h to 4°C similar to Protocol 1. The same volume of a 4°C pre-cooled second extender was then added (3:1 dilution; 1 volume of extender 2 is added to the initial mixture), containing a higher glycerol concentration and Equex STM (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% v/v EY; 7% v/v glycerol; and 1% v/v Equex STM), resulting in a final extender concentration (2 extenders combined) of 5% glycerol and 0.5% Equex STM, and so a concentration of 2.5% glycerol and 0.25% Equex STM after final semen dilution. Thawing of two straws per AWD was also performed in a 37°C water bath for 30 s after which they were emptied 1:1 in a thawing solution (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate). Sperm motility was evaluated at 5 min and every 2 h for 8 h after thawing. Viability, morphology and acrosome integrity was evaluated every 2 h for 6 h, and DNA integrity was evaluated immediately post-thaw. The four poor quality samples frozen from the PLA pack (Table 1) were only evaluated for motility, viability, morphology and acrosome integrity 5 min after thawing.

2.6. Statistical analysis

Percentage data were ARCSIN transformed, and sperm motility index data were $\log_{10}$ transformed. Paired sample t-tests or Sign test (motility rating) were used to evaluate differences between fresh and immediate post-thaw sperm quality for each freezing protocol, and to compare differences between protocols at each time point. Within each freezing protocol, changes in sperm quality over time during post-thaw incubation were evaluated using repeated measures ANOVA and post-hoc Least Significant Difference (LSD). $P \leq 0.05$ was considered significantly different. All data are presented as mean values ± SEM.

3. Results

Among the n = 24 AWDs in which electroejaculation was attempted during the breeding season, n = 17 produced urine-free semen of sufficient quantity to permit sperm analysis. Of these, the mean total spermatozoa ejaculated was 30.5 ± 9.7 × 10^6, motility was 55.0 ± 6.3%, viability was 76.6 ± 3.4%, acrosome integrity was 87.1 ± 2.8% (n = 16), DNA integrity was 99.7 ± 0.1% (n = 13), and normal sperm morphology was 53.8 ± 5.7%; with 23.4 ± 4.6%

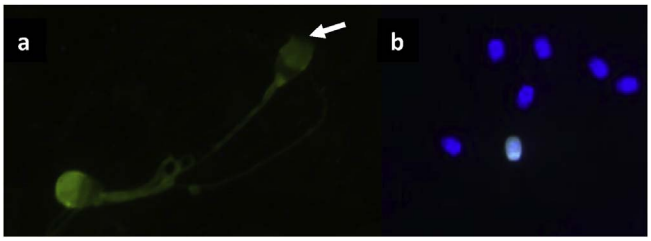

Fig. 1. (a) Acrosome intact (left) and incomplete (right) AWD sperm showing evidence of damaged membranes (arrow) stained with PSA-FITC; (b) DNA fragmented (green - FITC) and intact (blue - Hoechst 33342) AWD sperm evaluated by TUNEL assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

primary defects and 22.9 ± 3.3% secondary defects.

However, based on our selection criteria, initial sperm quality was sufficient for freezing in only 11 samples. One of these samples was excluded due to absent sperm motility after centrifugation; while two other samples were used in pilot trials to optimise the sperm freezing workflow in the field in Namibia (Table 1). Of the remaining eight samples frozen, four exhibited a rapid decline in sperm motility immediately prior to extender dilution and refrigeration (PLA pack: M6, M10, M11 and M13; Table 1). As such, semen was divided into four good quality and four poor quality samples. Among the n = 13 AWDs excluded from freezing, insufficient sperm quality was due to a variety of reasons including: low semen volume and/or low sperm motility (n = 6), urine contamination of the sample (n = 3), azoospermia (n = 2), or aspermia (n = 2; Table 1).

Total number of spermatozoa ejaculated was 71.4 ± 26.8 × 10^6 for good quality (n = 4) and 34.9 ± 16.2 × 10^6 for poor quality samples (n = 4) selected for freezing. Sperm motility, motility index and rating, viability, acrosome integrity, morphology and DNA integrity of fresh and frozen-thawed samples are presented in Table 2. Sperm motility was significantly lower for both freezing protocols immediately after thawing for good quality but not for poor quality samples due to their low pre-freeze value (Table 2). Sperm motility index and rating were only evaluated in good quality samples. No significant decrease in motility rating was found, while motility index decreased significantly for Protocol 2 immediate post-thaw. Viability declined significantly for both protocols after thawing in good and poor quality samples. However, post-thaw viability remained nearly 2-fold higher (nearly 60%) for Protocol 2 than Protocol 1 in both good (significant) and bad quality samples (Table 2). In good quality samples, acrosome integrity did not decline after freeze-thawing using Protocol 2; which was 3-fold significantly higher than Protocol 1 (Table 2). For poor quality samples, however, a significant decline in acrosome integrity was only seen for Protocol 2, although this was most likely due to the highly variable results observed for Protocol 1 (n = 3; Table 2). The percentage of sperm with normal morphology, primary or secondary defects did not change after thawing using either protocol for good or poor quality samples (Table 2). DNA integrity was only measured in good quality samples but did not decline immediately after freeze-thawing using either protocol (Table 2).

Within 2 h of post-thaw incubation, sperm motility dropped to less than 1% for Protocol 1 (Fig. 2a). However, a significant progressive decrease in sperm motility was only seen after 4 h of incubation for Protocol 2, with motility still present for up to 8 h (Fig. 2a). Moreover, motility for Protocol 2 remained significantly higher than Protocol 1 between 2 and 6 h after thawing. Similar results were observed for post-thaw sperm motility index (Fig. 2b). Sperm viability for Protocol 2 remained significantly higher (consistently 2–3.5-fold) than Protocol 1 throughout the post-thaw evaluation period (Fig. 2c). In addition, viability only declined significantly from immediate post-thaw levels after 6 h for Protocol 2, compared to 4 h for Protocol 1 (Fig. 2c). The integrity of the sperm acrosome for Protocol 2 essentially remained significantly higher (≥3-fold) than Protocol 1 throughout the post-thaw evaluation period (Fig. 2d). A small but significant decrease was seen after 2 h of incubation for both protocols (Fig. 2d). The proportion of spermatozoa with normal morphology or primary and secondary defects did not differ between freezing protocols throughout the post-thaw evaluation period (Fig. 2e). However, a small but significant decrease in normal morphology was observed after 2 h of incubation for Protocol 2; with a corresponding increase in secondary defects (Fig. 2e).

4. Discussion

This study demonstrates that a sperm freezing protocol consisting of a two-step dilution in TRIS - 20% EY extender and final extender concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution, yields significantly improved post-thaw quality and longevity of AWD spermatozoa compared to previous studies [11,16]. Importantly, sperm motility persists for up to 8 h and viability remains stable for more than 4 h after thawing. Moreover, DNA integrity and morphology of spermatozoa do not appear to be damaged by the freeze-thaw process. This protocol provides post-thaw spermatozoa of sufficient quality for use in AI, making it suitable for worldwide frozen transport and long-term gene banking for the first time in the AWD.

Protocol 1, using a one-step dilution with a TRIS - 20% EY extender containing 8% glycerol, yielded poor results similar to previous AWD sperm freezing studies. Sperm motility of 40% and 32%, respectively, was reported immediately post-thaw, but plummeted to nearly 0% within 2 h [11,16]. By contrast for our Protocol 2, sperm motility was still > 30% after 2 h and only started to decline significantly from 4 h, with motility present for up to 8 h; which represents a significant improvement. Although spermatozoa can be found at the top of the uterine horn within 1 min after natural mating in the domestic dog [59], it is unlikely that these spermatozoa are involved in fertilization. Sperm transport to the site of fertilization is known to occur in two phases in mammals [35,36], with spermatozoa responsible for fertilization reaching the oviduct during the sustained transport phase, several hours after insemination [36]. In addition, the timing when mature oocytes are ready for fertilization in the domestic dog is variable (60–108 h post ovulation) [47]. Fresh spermatozoa can survive in uterine crypts and the distal part of the uterotubal junction of the female reproductive tract for several days after natural mating or AI, enabling fertilization once the oocytes complete post-ovulatory maturation [47]. Thus, the chance of pregnancy and larger litter sizes increases when using spermatozoa that can maintain motility for long periods.

In domestic dogs, most AI studies using frozen-thawed spermatozoa only evaluate sperm quality immediately post-thaw. A relatively large number of functional spermatozoa are required for successful AI in the domestic dog, necessitating insemination using fresh sperm of good quality. Generally, fresh spermatozoa with motility above 70–75% are selected for freezing, and spermatozoa with a post-thaw motility of at least 40% result in whelping rates of 70% or higher [22,58]. Immediate

Table 2
Sperm quality (mean ± SEM) in fresh and thawed African wild dog (*Lycaon pictus*) semen frozen using Protocol 1 and 2 for good quality samples and poor quality samples (characterized by a rapid decrease in motility just prior to freezing).

	Fresh	Post-thaw Protocol 1	Post-thaw Protocol 2
Good quality samples (n = 4)			
Motility (%)	75.0 ± 4.4^a	18.3 ± 7.1^b	33.3 ± 4.5^b
Motility rating (0–4)	3.3 ± 0.8	1.8 ± 0.8	1.8 ± 0.5
Sperm motility index (0–400)	194.0 ± 28.0^a	31.5 ± 10.9^a	59.4 ± 9.6^b
Viability (%)	90.8 ± 1.3^a	28.3 ± 9.6^c	58.0 ± 10.1^b
Acrosome integrity (%)	93.5 ± 2.0^a	20.6 ± 6.2^b	60.5 ± 10.8^a
Normal morphology (%)	71.0 ± 6.8	42.8 ± 10.3	45.8 ± 10.8
Primary defects (%)	8.0 ± 2.9	11.0 ± 1.8	12.1 ± 1.7
Secondary defects (%)	19.5 ± 4.1	45.3 ± 9.3	41.5 ± 10.1
DNA integrity (%)	99.7 ± 0.3	99.5 ± 0.2	99.4 ± 0.2
Poor quality samples (n = 4)			
Motility (%) (n = 3)	3.3 ± 3.3	0.3 ± 0.3	1.7 ± 1.7
Viability (%)	89.5 ± 1.9^a	29.0 ± 11.0^b	55.8 ± 5.7^b
Acrosome integrity (%) (n = 3)	96.2 ± 1.6^a	42.7 ± 22.7^a	67.3 ± 5.7^b
Normal morphology (%)	66.3 ± 6.2	67.5 ± 7.0	63.1 ± 6.0
Primary defects (%)	20.2 ± 3.5	24.0 ± 6.5	28.0 ± 5.2
Secondary defects (%)	13.6 ± 3.6	8.5 ± 3.7	9.0 ± 1.6

Different superscript (a-c) within a given row indicate significant difference between treatments.

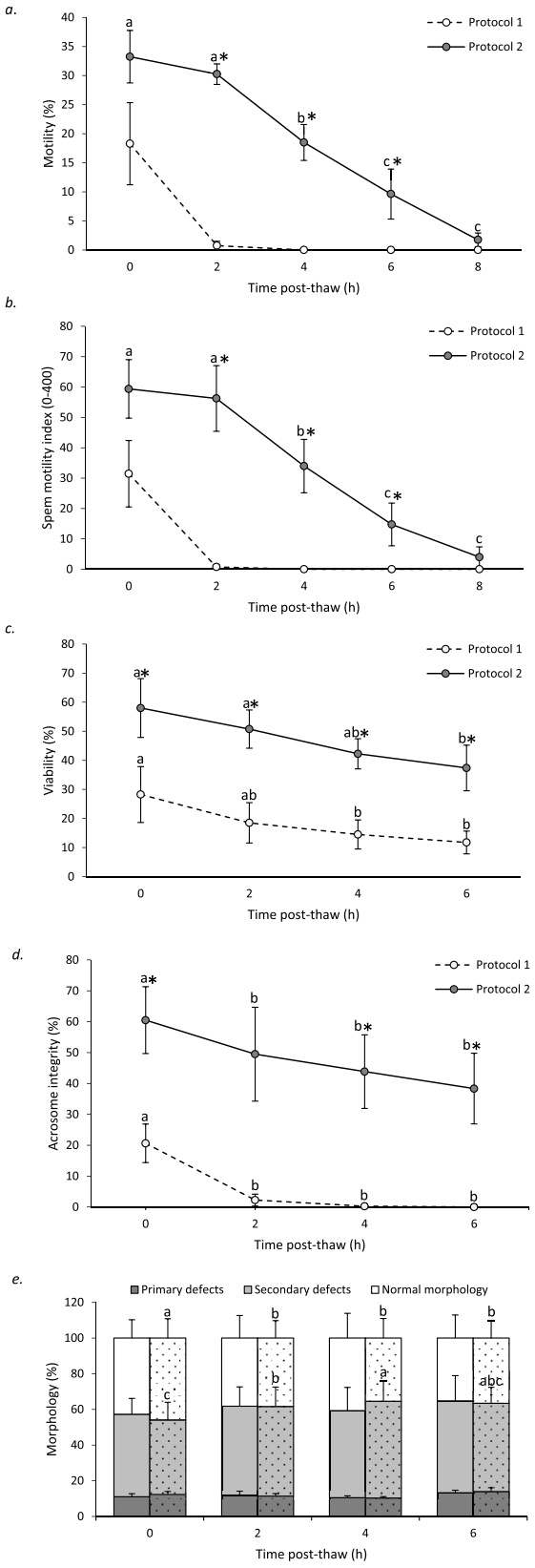

Fig. 2. Post-thaw (a) motility, (b) sperm motility index, (c) viability, (d) acrosome integrity and (e) morphology of African wild dog spermatozoa over a period of 6–8 h after cryopreservation using Protocols 1 and 2.* indicates a significant difference between the 2 protocols at a specific time point. Different letters within each protocol indicate a significant difference between time points. In figure e, plain bars represent Protocol 1 results and dotted bars represent Protocol 2 results.

post-thaw motility of sperm in our study was just below 40%, however the maintenance of motility during post-thaw incubation was either similar or much better than that seen in domestic dog studies [40,43,55].

Using a fluorescent staining technique, Johnston et al. [16] observed 33% viable spermatozoa immediately after thawing, which decreased to 8% after 2 h of incubation. In our study, sperm viability was nearly 2-fold higher (58%) and only decreased 6 h after thawing for Protocol 2. In addition, post-thaw viability seen with Protocol 2 is similar to or better than results typically obtained in domestic dog sperm freezing studies [40,49,51].

In our study, the integrity of the acrosome in good quality spermatozoa did not significantly degrade in response to the freeze-thawing process using Protocol 2, but was highly variable among samples over time. Bad quality samples gave a more variable result using Protocol 1. Protocol 2 yielded acrosome integrity 3-fold higher than Protocol 1 (60% vs. 20%), however it was still much lower than fresh and frozen-thawed AWD spermatozoa reported previously (98.5% and 95.6% respectively) [16]; although the technique used was not described. We used a sensitive fluorescent staining technique to evaluate acrosomes, that may partly explain the difference in the level of acrosome defects detected between the two studies. In domestic dogs, post-thaw acrosome integrity ranges between 45% and 68% [2,43,70]. The level of acrosome integrity observed using Protocol 2 falls within the higher end of this range; ensuring large numbers of fertilization-competent sperm are available post-thaw for AI.

To our knowledge, this is the first time DNA integrity has been evaluated in AWD spermatozoa. We found that DNA fragmentation is typically low, and that freeze-thawing using either protocol, does not appear to induce further damage. In domestic dog spermatozoa, DNA fragmentation increases slightly immediately post-thaw [17], however not in all studies [2,18,61,63]. In all these domestic dog studies, post-thaw sperm DNA fragmentation index was relatively low (< 5%), but different assay techniques were used (acridine orange, sperm chromatin dispersion test, and sperm chromatin structure assay), making it difficult to directly compare with our results. However, given that AWD spermatozoa have less than 1% DNA fragmentation as detected by TUNEL, it appears insensitive to the external stressors intrinsic to the freezing and thawing process. This provides some confidence that long term cryo-storage of spermatozoa from this species will not induce crucial DNA damage, that could otherwise lead to arrested early embryo development due to failed transcription of developmentally important but disrupted genes [21,39,45,53].

Normal morphology, as well as primary and secondary sperm defects did not appear to change immediately after freeze-thawing using either protocol in our study. However, normal sperm morphology decreased slightly with a corresponding increase in secondary sperm defects (tail and cytoplasmic droplet) 2 h after thawing for Protocol 2. Hermes et al. [11] reported an increase in tail defects from 20, 9 and 8% in the fresh sperm fractions to 59, 70 and 25% respectively after thawing. In other wild canids, an increase in tail abnormalities post-thaw was observed in the red wolf (*Canis rufus*) [9] and coyote (*Canis latrans*) [28], but not in generic and Mexican grey wolves [71], or arctic foxes (*Vulpes lagopus*) [56]. Tail defects caused during cooling, freezing and thawing could be due to cold shock or osmotic shock [9], which means that a high increase in morphologically abnormal spermatozoa could indicate that the freezing extender and/or protocol is not optimal. Spermatozoa with an aberrant morphology can reduce pregnancy success, as luminal fluids and epithelial surfaces prevent their migration to higher parts of the female reproductive tract [52].

As we used the same cooling, freezing and thawing rates for both our freezing protocols, the beneficial effect on sperm quality observed using Protocol 2 lies either in the different composition of this extender, or in the two-step dilution and/or thawing solution. The composition of each extender differed in our study with a lower Tris-HCl (0.20 M *vs.* 0.25 M), sodium citrate (0.07 M *vs.* 0.08 M), fructose (0.04 M *vs.*

0.07 M), and final extender glycerol concentration (5% *vs.* 8%), as well as the addition of Equex STM in Protocol 2 compared to Protocol 1. The function of the Tris-HCl-citrate is to buffer the semen extender to an optimal pH. Fructose is generally added as an energy source to semen extenders, and can directly enhance sperm motility [46]. However, the fructose concentration used in our study was lower for the extender showing best post-thaw motility. Moreover, fructose concentrations as low as 0.01 M can maintain sperm motility above 70% for 4 days during refrigerated storage of canine spermatozoa [46].

In the domestic dog, a two-step dilution has been proposed to enable cooling and equilibration with lower glycerol concentrations, minimizing its toxic effect [40]. However, several reports show no beneficial effects of adding glycerol later, indicating it is not toxic at the concentrations used for dog semen freezing [40,42,55]. It is possible however that AWD spermatozoa are more sensitive to the toxic effects of glycerol. Therefore, the lower concentration used in Protocol 2 could have caused part of the improvement observed in our study. A two-step dilution is also known to improve results when Equex STM is added in the second extender [40]. We hypothesize that the addition of Equex STM may largely explain the beneficial effect observed in Protocol 2. The combination of Equex STM with EY and glycerol appears to stabilize the sperm plasma membrane and optimise sperm cell dehydration and rehydration during freezing and thawing [7]. In addition, the higher relative sperm viability, acrosome integrity and longevity we see with Protocol 2 after thawing is similar to the beneficial effects seen in domestic dogs when using Equex STM [40]. However, such a freezing protocol necessitates the use of a thawing solution [2,40,51], which in this case was identical to the Protocol 2 extender minus glycerol, EY and Equex STM. The thawing solution allows dilution of potentially toxic glycerol and SDS [5,12], and could thereby increase sperm longevity. In domestic dogs, a 1:1 dilution improves post-thaw sperm motility, viability and acrosome integrity, however only after 5, 12 and 18 h of incubation respectively [41]. Collectively, this suggests that Equex STM is the main contributor to the beneficial effect observed in Protocol 2.

One important limitation to the successful cryopreservation of AWD spermatozoa encountered in this study was the failure to consistently collect good quality samples; an issue previously reported by other researchers [16]. Such inefficiencies must be considered when planning semen collections for gene banking in this species. Urine contamination, absence of an ejaculate, absence of spermatozoa, low semen volume, and low sperm motility, all contributed to the reduced number of samples available for cryopreservation. Avoiding the using of alpha-2 adrenergic receptor agonists for immobilization, coupled with draining and flushing the bladder prior to electroejaculation, may go some way to avoid urine contamination of samples [16,57]. Moreover, compared to domestic dogs, the total number of spermatozoa ejaculated and sperm quality seen in AWDs in this study are very low, and similar to those reported during the breeding season by some researchers [16], but not others [29]. Although sperm number is known to be lower after electroejaculation compared to manual collection in domestic dogs [33], other sperm quality parameters do not differ [3,33]. Thus, with the exception of sperm number, other sperm quality parameters observed in this study should reflect those inherent during natural mating and ejaculation in AWDs.

In conclusion, a two-step TRIS - EY freezing extender containing Equex STM and glycerol, coupled with a TRIS-citrate-fructose thawing solution, yields significantly improved post-thaw quality and longevity of AWD spermatozoa; greatly improving the chance of successful AI with frozen-thawed spermatozoa. With further refinement, this protocol should be suitable for (i) long-term sperm banking to maintain genetic diversity, (ii) transport of frozen gametes worldwide, and (iii) downstream use in AI for the management and conservation of this highly endangered species. However, AI trials are needed before this sperm freezing protocol can be considered successful for the management and conservation of the endangered AWD.

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