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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.