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Influence of extender, freezing rate, and thawing rate on post-thaw motility, viability and morphology of coyote (*Canis latrans*) spermatozoa

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Abstract

The objective of this study was to examine the post-thaw effects of three cryoprotective extenders (Tris–fructose–citric acid extender, Tris–glucose–citric acid extender, and lactose extender), three linear freezing rates (−1, −6, and −20 °C/min), and three thawing rates (37 °C water bath for 120 s, 60 °C water bath for 30 s, and 70 °C water bath for 8 s) on coyote spermatozoa. After thawing, the findings supported that cryopreservation of coyote (*Canis latrans*) spermatozoa frozen at a moderate freezing rate (−6 °C/min), in either a Tris–fructose or Tris–glucose extender, and thawed at a slow rate (37 °C water bath for 120 s) or moderate rate (60 °C water bath for 30 s), resulted in a more vigorous post-thaw motility (range, 57.5–44.0%) and viability (range, 64–49.6%) with the least amount of morphological and acrosomal abnormalities.

Keywords: Coyote; Semen cryopreservation; Extender; Freezing rates; Thawing rates

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1. Introduction

A great deal of investigation has been conducted to determine optimal conditions for the cryopreservation of canine spermatozoa. The many factors involved with successful cryopreservation have been studied at length and have been critically reviewed [1–3]. Among the factors most often cited as being essential for success are seminal processing and packaging, type and composition of extender, length of equilibrium time, method and rate of freezing, storage, and method and rate of thawing [4–14].

While the basic knowledge stemming from domestic dog research has proven valuable in the development of cryopreservation techniques for canine spermatozoa, there has been relatively little interest shown thus far in using these techniques on wild canids [15,16]. In that regard, most members of the wild canid family breed well in the wild or in captivity, leading those who work for their conservation to show little interest in using cryopreservation [15,16]. However, there are numerous species within the family Canidae, including the red wolf (*C. rufus*), Mexican wolf (*C. lupus baileyi*), Ethiopian wolf (*C. simensis*), maned wolf (*Chrysocyon brachyurus*), African wild dog (*Lycaon pictus*), San Joaquin kit fox (*Vulpes macrotis*), and Northern swift fox (*V. velox hebes*), which are imperiled and could benefit from this type of basic research [17]. Increasing the knowledge regarding basic wild canid reproductive biology, more specifically surrounding the development and implementation of sperm cryopreservation protocols, could prove an invaluable resource for future preservation efforts of these species [18].

In the present study, the coyote (*Canis latrans)* was utilized to determine the effectiveness of various cryopreservation techniques. This information increases the knowledge of cryopreservation of coyote spermatozoa and could be used to improve the application of assisted reproductive technologies on endangered canines. The aim of this study was to compare the effects of three types of cryoprotective media and nine freeze–thaw protocols on post-thaw progressive motility, survival, and changes in coyote sperm morphology.

2. Materials and methods

2.1. Animals

Semen was collected from 10 sexually mature male coyotes (2–6-year-old), between 10 and 14 kg body weight, from the captive breeding colony at the United States Department of Agriculture, National Wildlife Research Center, Predation Ecology and Behavior Field Station in Millville, UT, USA. The NWRC, Institutional Animal Care and Use Committee approved all procedure in this study as protocol QA-862. These animals were housed in individual kennels (4.3 m^2) and identified by ear tags and subcutaneous microchips. The coyotes were fed a daily ration of meat slurry, and water was provided ad libitum. They were born in captivity and hand-reared by staff to reduce stress from routine handling. Nine semen collections were obtained from each coyote during the 2002 breeding season, specifically, between 24 January 2002 and 24 March 2002.

2.2. Anesthesia and electroejaculation procedure

Coyotes were fasted the day of semen collection and were transported to an indoor collection site. They were then anesthetized with 100 mg ketamine (Ketaved, Vedco Inc., St. Joseph, MD, USA) and 30 mg xylazine (Tranquived, Vedco Inc.) administered i.m. by hand syringe. The penis was cleaned and catheterized with a 5 FR 16-cm long polyproylene catheter (Kendall, Mansfied, MA, USA), and the bladder was drained of urine and flushed with 50 mL of 0.9% saline solution to remove any remaining urine.

Semen collection was conducted in a dedicated surgical suite under sterile conditions. A P.T. Electronic Model ejaculator and a No. 4 (1.6 cm diameter, 25.4 cm length) rectal probe (P.T. Electronics, Boring, OR, USA) were used in conjunction with standard electroejaculation procedures to collect semen [19]. Electroejaculation consisted of five sets of stimulations, with each set consisting of multiple on–off stimuli (∼30–40), and a 5-min rest between sets. The voltage for each stimulus ranged from 2 to 5 V, with the voltage required for ejaculation varying among individual coyotes. The samples were collected in a warm sterile glass tube.

2.3. Semen evaluation

Immediately after collection, the ejaculate was placed in a 37 °C waterbath and volume and pH were recorded. The percentage of progressively motile spermatozoa was estimated by microscopic examination at 400× magnification on a prewarmed slide (37 °C), and a subjective assessment of the progressive status was recorded (0 = no motility, 1 = slight side-to-side movement with no forward progression, 2 = moderate side-to-side movement with occasional slow forward progression, 3 = side-to-side movement with slow, steady forward progression, 4 = steady and moderately rapid forward progression, 5 = steady rapid forward progression) [20]. Sperm concentration was measured using standard hemocytometer methods (Hausser Scientific, Horsham, PA, USA). The percentage of viable spermatozoa was estimated by viewing 200 spermatozoa under 1000× magnification using an eosin–nigrosin stain. To evaluate morphologic and acrosomal abnormalities, a drop of each ejaculate was stained with Spermac® (Stain Enterprises, Wellington, South Africa) and 200 cells were visually examined at 1000× magnification [21]. Morphological abnormalities were visually classified as head, midpiece, and principle piece defects. Morphological characteristics were noted and the percentage of normal spermatozoa and of each abnormality was calculated. The Spermac® stain permitted differentiation of the acrosome (green) and the post-acorosome (pink), allowing for ready microscopic identification of acrosome damage and partial or total acrosome removal.

2.4. Selection of semen for freezing

All ejaculates included in this study contained $\geq 70 \times 10^6$ spermatozoa/mL, exhibited ≥60% motility with an average progressive status ≥4.0 and contained ≥75% morphologically normal spermatozoa.

2.5. Semen processing and freezing

After collection and evaluation, semen samples were diluted 1/4 (v/v) in one of three extenders: extender 1 (Tris–fructose–citric acid extender: 6.056 g of tris hydoxymethyl aminomethane, 3.400 g of sodium citrate monohydrate, 2.500 g of fructose, 16 mL of glycerol, 40 mL of egg yolk, 200,000 IU of penicillin, 0.2000 g of dihydrostreptomycin, and 184 mL of distilled water, pH 6.82, 1708 mOsm) [22]; extender 2 (Tris–glucose–citric acid extender: 6.056 g of tris hydoxymethyl aminomethane, 3.400 g of sodium citrate monohydrate, 2.500 g of glucose, 16 mL of glycerol, 40 mL of egg yolk, 200,000 IU of penicillin, 0.2000 g of dihydrostreptomycin, and 184 mL of distilled water, pH 6.85, 1657 mOsm) (modified [22]) and extender 3 (lactose extender: 28.75 g of lactose, 12.5 mL of glycerol, 40 mL of egg yolk, 125,000 IU of penicillin, 0.1250 g of dihydrostreptomycin, and 180 mL of distilled water, pH 6.34, 1252 mOsm) (modified [23]) (all chemicals were obtained from Sigma–Aldrich, St. Louis MO, USA). Each extender was prepared prior to cryopreservation and frozen in small aliquots that could be thawed and warmed to 37 °C before the dilution of the semen. Immediately before use, the extender was centrifuged and the supernatant was clarified by filtration through a 0.8 μm filter.

Once extended, 0.5 mL of the semen was packaged in 0.5 mL French Straws (IMV Technologies, L'Aigle, France) and allowed to cool to 5 °C over a 2 h period. After this equilibrium period, straws from each extender group were randomly assigned to one of the three linear freezing rates: −1 °C, −6 and −20 °C/min. The straws were cooled by forced LN_2 vapor to −80 °C in a R204 Series II programmable freezer (Planer Products Ltd., Sunbury-on-Thames, UK) and then immersed and stored in liquid nitrogen.

2.6. Thawing

Samples from each of the extender-freezing rate combinations were randomly assigned to one of the three thawing rates: (1) 37 °C water bath for 120 s, (2) 60 °C water bath for 30 s, or (3) 70 °C water bath for 8 s. Ten straws were obtained for each of the 27 treatments, one from each of the 10 male coyotes (total of 270 samples). A post-thaw evaluation, including motility, morphology, viability, progressive status, and acrosomal damage, was conducted on the semen samples immediately after thawing.

2.7. Statistical analysis

Data were analyzed using SAS (Version 8.2, SAS Institute Inc., Cary, NC, USA). A mixed model ANOVA was used to evaluate the effects of the three factors (cryoprotective extender, freezing rate, and thawing rate) and their interaction on sperm motility, survival, and morphology. Significant main effects and interaction means were compared using a Tukey Kramer multiple comparison. Standard errors were estimated by the analysis of variance model under the assumption of homogeneity of variance and equal sample size; thus, standard errors for all interaction treatment groups were equal. Overall differences in sperm cell characteristics (sperm motility, survival, and morphology) between fresh and frozen–thawed semen were assessed using a paired Student's t test. Values were presented as mean ± S.D., and were considered significant at $P \leq 0.05$.

3. Results

The mean electroejaculate volume was 1.6 ± 0.53 mL (range, 0.5–3.0 mL), with a mean concentration of $397 \times 10^6 \pm 207.2$ spermatozoa/mL (range, 94×10^6 to 1143×10^6). The mean percentages of motile and viable spermatozoa, immediately after collection, were 83.5 ± 4.9 and $89.3 \pm 4.4\%$, respectively. Mean percentage of morphologically normal spermatozoa was $86.1 \pm 5.5\%$, with $96.6 \pm 1.8\%$ of spermatozoa containing intact acrosomes immediately after collection. After freezing and thawing, there was an overall decline in percent motility ($18.9 \pm 23.1\%$, $P \leq 0.001$), viability ($23.8 \pm 24.6\%$, $P \leq 0.001$), normal spermatozoa ($77.1 \pm 4.8\%$, $P \leq 0.001$), and acrosomal integrity ($65.6 \pm 4.5\%$, $P \leq 0.001$), regardless of extender, freeze rate, or thaw rate (Fig. 1). The mean percentages of pre-freeze and post-thaw progressive motility and viability of spermatozoa for each individual coyote, averaged across extender, freeze rate and thaw rate, are given in Table 1.

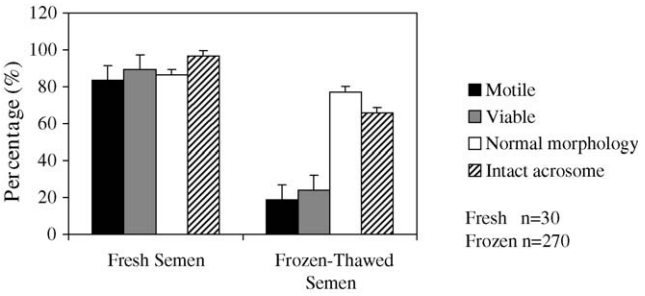

Fig. 1. Overall mean (±S.E.M., %) motility, viability, normal morphology, and intact acrosomes for fresh and frozen–thawed coyote semen. All means were different ($P \leq 0.05$) from fresh and frozen–thawed semen.

Table 1
Mean (±S.E.M.) percentage of pre-freeze and post-thaw progressive motility and viability of spermatozoa for each individual coyote, averaged across extender, freeze rate and thaw rate

Coyote	Motility		Viability	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
1	80.3 ± 3.4^{b}	24.4 ± 25.8^{a}	88.6 ± 2.1^{a}	30.9 ± 26.3^{a}
2	84.3 ± 4.7^{a}	21.9 ± 24.8^{ab}	90.0 ± 5.8^{a}	27.2 ± 26.4^{a}
3	83.4 ± 9.1^{ab}	14.9 ± 14.6^{c}	87.6 ± 6.4^{a}	20.9 ± 17.2^{b}
4	86.3 ± 2.5^{a}	23.9 ± 25.8^{a}	91.6 ± 2.4^{a}	28.9 ± 26.9^{a}
5	85.3 ± 1.7^{a}	19.0 ± 26.7^{b}	89.3 ± 0.4^{a}	22.2 ± 29.0^{b}
6	82.6 ± 3.4^{b}	10.6 ± 16.4^{d}	90.0 ± 4.1^{a}	13.2 ± 18.5^{c}
7	80.3 ± 1.7^{b}	9.2 ± 13.4^{d}	88.6 ± 4.5^{a}	14.6 ± 16.7^{c}
8	86.0 ± 4.1^{a}	19.4 ± 29.8^{b}	91.6 ± 2.4^{a}	23.2 ± 31.3^{b}
9	79.1 ± 2.4^{b}	21.7 ± 23.8^{ab}	85.6 ± 3.3^{a}	29.0 ± 24.5^{a}
10	87.6 ± 2.5^{a}	23.6 ± 19.8^{a}	89.7 ± 5.6^{a}	28.6 ± 21.5^{a}
Mean ± S.E.M.	83.5 ± 4.9	18.9 ± 23.1	89.3 ± 4.4	23.8 ± 24.6

Within columns, values with different superscripts (a–d) are different ($P = 0.05$).

Table 2
Mean (±S.E.M.) percentage of post thaw progressive motility of coyote spermatozoa frozen using different extenders, freezing rates, and thawing rates

Thaw rate (°C)	Freeze rate								
	Lactose[a]			Tris–glucose[a]			Tris–fructose[a]		
	−1 °C	−6 °C	−20 °C	−1 °C	−6 °C	−20 °C	−1 °C	−6 °C	−20 °C
37	1.5 ± 2.9	19.3 ± 16.6	2.5 ± 3.1	8.4 ± 6.5	45.6 ± 29.9	16.0 ± 13.3	10.4 ± 8.1	57.5 ± 15.8	14.2 ± 13.1
60	1.9 ± 4.0	17.6 ± 20.8	8.2 ± 6.8	14.0 ± 1.5	44.0 ± 31.7	33.6 ± 20.4	14.9 ± 8.2	55.6 ± 34.6	27.8 ± 19.2
70	0.1 ± 0.3	10.8 ± 14.9	2.1 ± 2.9	13.7 ± 9.4	29.6 ± 32.0	9.3 ± 9.1	14.1 ± 8.9	25.7 ± 33.5	11.8 ± 9.6

Each treatment group contains 10 straws, 1 from each coyote, resulting in 270 total samples.
[a] Extender.

Table 3
The combined effects of extender and freeze rate across thaw rates on mean (±S.E.M.) post-thaw progressive motility of coyote spermatozoa

Freeze rate (°C)	Extender		
	Lactose	Tris–glucose	Tris–fructose
−1	1.6 ± 3.4^{a}	12.0 ± 3.4^{a}	13.1 ± 3.4^{a}
−6	15.9 ± 3.4^{b}	39.8 ± 3.4^{b}	46.3 ± 3.4^{b}
−20	4.3 ± 3.4^{ab}	19.6 ± 3.4^{a}	17.9 ± 3.4^{a}

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).
Each treatment group contains 30 straws; 3 from each coyote, 1 from each thaw group.

The method of cryopreservation affected the post-thaw quality of coyote semen. The effects of extender, freeze rate and thaw rate all independently affected sperm motility and survival ($P \leq 0.001$), while the only interactions exerting a significant influence on motility and survival were extender × freeze rate ($P \leq 0.05$) and freeze rate × thaw rate ($P \leq 0.05$). The effects of extender, freeze rate and thaw rate on mean post-thaw motility, estimated using the raw data, are shown in Table 2. When averaged across thaw rates (Table 3), to show the extender × freeze rate interaction, motility was higher ($P \leq 0.05$) with a freeze rate of −6 °C/min (15.9% for lactose, 39.8% for Tris–glucose and 46.3% for Tris–fructose) compared with that of −1 °C/min (1.6% for lactose, 12.0% for Tris–glucose and 13.1% for Tris–fructose; $P \leq 0.05$) or −20 °C/min (4.3% for lactose, 19.6% for Tris–glucose and 17.9% for Tris–fructose) ($P \leq 0.05$). Post-thaw motility was also greater ($P \leq 0.001$) in both the Tris–glucose and Tris–fructose extender when compared to the lactose extender.

Post-thaw motility was averaged across extenders to show the interaction between freeze rates and thaw rates (Table 4). Again, motility was higher ($P \leq 0.05$) with a freeze rate of −6 °C/min. Rapid thawing (70 °C water bath for 8 s) was inferior (22.2% for −6 °C/min and 7.7% for −20 °C/min) to both the slow thawing ($P \leq 0.01$, 40.8% for −6 °C/min and $P \geq 0.05$, 10.9% for −20 °C/min) and moderate thawing ($P \leq 0.01$, 39.1% for −6 °C/min and $P \leq 0.01$, 23.2% for −20 °C/min) under the −6 and −20 °C/min freeze rate. However, there was no effect ($P \geq 0.05$) of thawing rate on post-thaw motility at the −1 °C/min freeze rate.

The effects of extender, freeze rate, and thaw rate on mean post-thaw viability, estimated using the raw data, are shown in Table 5. When averaged across thaw rates (Table 6), the interaction between extender × freeze rate showed that viability was greater ($P \leq 0.05$)

Table 4
The combined effect of freeze rate and thaw rate across extenders on mean (±S.E.M.) post-thaw progressive motility of coyote spermatozoa

Thaw rate (°C)	Freeze rate		
	−1 °C	−6 °C	−20 °C
37	6.8 ± 3.4^{a}	40.8 ± 3.4^{a}	10.9 ± 3.4^{ab}
60	10.2 ± 3.4^{a}	39.1 ± 3.4^{a}	23.2 ± 3.4^{b}
70	9.3 ± 3.4^{a}	22.1 ± 3.4^{b}	7.7 ± 3.4^{a}

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).
Each treatment group contains 30 straws; 3 from each coyote, 1 from each extender group.

Table 5
Mean (±S.E.M.) percentage of post-thaw viability of coyote spermatozoa frozen using different extenders, freezing rates, and thawing rates

Thaw rate (°C)	Freeze rate								
	Lactose[a]			Tris–glucose[a]			Tris–fructose[a]		
	−1 °C	−6 °C	−20 °C	−1 °C	−6 °C	−20 °C	−1 °C	−6 °C	−20 °C
37	3.6 ± 5.8	25.1 ± 20.6	5.9 ± 5.0	16.0 ± 9.5	51.2 ± 30.8	21.1 ± 15.1	15.4 ± 9.9	64.0 ± 15.3	20.4 ± 14.9
60	6.5 ± 8.2	22.9 ± 24.2	12.8 ± 9.6	19.9 ± 12.7	49.6 ± 31.7	41.9 ± 24.6	22.1 ± 7.9	61.1 ± 36.0	33.1 ± 20.4
70	0.5 ± 1.3	14.4 ± 16.7	4.2 ± 5.9	19.6 ± 10.6	33.6 ± 34.1	13.2 ± 11.2	20.1 ± 9.5	29.5 ± 35.8	17.1 ± 12.2

Each treatment group contains 10 straws, 1 from each coyote, resulting in 270 total samples.
[a] Extender.

Table 6
The combined effects of extender and freeze rate across thaw rates on mean (±S.E.M.) post-thaw viability of coyote spermatozoa

Freeze rate (°C)	Extender		
	Lactose	Tris–glucose	Tris–fructose
−1	3.5 ± 3.7^{a}	18.5 ± 3.7^{a}	19.2 ± 3.7^{a}
−6	20.8 ± 3.7^{b}	44.8 ± 3.7^{b}	51.5 ± 3.7^{b}
−20	7.6 ± 3.7^{ab}	25.4 ± 3.7^{a}	23.5 ± 3.7^{a}

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).
Each treatment group contains 30 straws; 3 from each coyote, 1 from each thaw group.

with a freeze rate of −6 °C/min (20.8% for lactose, 44.8% for Tris–glucose, and 51.5% for Tris–fructose) compared with that of −1 °C/min (3.5% for lactose, 18.5% for Tris–glucose, and 19.2% for Tris–fructose; $P \leq 0.05$) or −20 °C/min (7.6% for lactose, 25.4% for Tris–glucose, and 23.5% for Tris–fructose; $P \leq 0.05$). Post-thaw viability was also higher ($P \leq 0.001$) in both the Tris–glucose and Tris–fructose extender when compared to the lactose extender.

Post-thaw viability was averaged across extenders to show the interaction between freeze rates and thaw rates (Table 7). Post-thaw viability was superior ($P \leq 0.05$) with a freeze rate of −6 °C/min. Rapid thawing (70 °C water bath for 8 s) was inferior (25.8% for −6 °C/min and 11.5% for −20 °C/min) to both the slow thawing ($P \leq 0.01$, 46.8% for −6 °C/min and $P \geq 0.05$, 15.8% for −20 °C/min) and moderate thawing ($P \leq 0.01$, 44.5% for −6 °C/min and $P \leq 0.01$, 29.3% for −20 °C/min) under the −6 °C/min and −20 °C/min freeze rate. However, there was no effect ($P \geq 0.05$) of thawing rate on post-thaw viability under the −1 °C/min freeze rate.

Sperm morphology and acrosomal integrity after freezing and thawing did not differ between any of the combinations of extender, freeze rate and thaw rate. Freeze rate alone had the only significant effect on morphology and acrosome integrity, with the fastest freezing rate causing the greatest disruption in acrosomal integrity and changes in morphology ($P \leq 0.05$). Table 8 shows the mean percentage of sperm cell abnormalities from pre-freeze and post-thaw coyote semen averaged across extender, freeze rate and thaw rate, while the mean percentage of morphologically normal sperm cells and intact acrosomes pre-freeze and post-thaw for each individual coyote averaged across extender, freeze rate and thaw rate, is given in Table 9.

Table 7
The combined effect of freeze rate and thaw rate across extenders on the mean (±S.E.M.) post-thaw viability of coyote spermatozoa

Thaw rate (°C)	Freeze rate		
	−1 °C	−6 °C	−20 °C
37	11.7 ± 3.7^{a}	46.8 ± 3.7^{a}	15.8 ± 3.7^{ab}
60	16.2 ± 3.7^{a}	44.5 ± 3.7^{a}	29.3 ± 3.7^{b}
70	13.4 ± 3.7^{a}	25.8 ± 3.7^{b}	11.5 ± 3.7^{a}

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).
Each treatment group contains 30 straws; 3 from each coyote, 1 from each extender group.

Table 8
Mean (±S.E.M.) percentage of sperm cell abnormalities from pre-freeze and post-thaw coyote semen, averaged across extender, freeze rate and thaw rate

Characteristic	Pre-freeze	Post-thaw
Morphological abnormalities		
Detached head	3.0 ± 2.4^{a}	5.3 ± 3.7^{b}
Macrocephalic head	0.03 ± 0.1^{a}	0.0 ± 0.0^{a}
Microcephalic head	1.1 ± 0.9^{a}	2.5 ± 0.9^{a}
Bicephalic	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Bent midpiece with cytoplasmic droplet	0.1 ± 0.1^{a}	0.4 ± 0.3^{a}
Bent midpiece without cytoplasmic droplet	0.4 ± 0.3^{a}	1.1 ± 0.4^{b}
Coiled flagellum	1.7 ± 1.4^{a}	3.9 ± 0.9^{b}
Bent flagellum	1.6 ± 1.1^{a}	4.2 ± 1.0^{b}
Biflagellate	0.0 ± 0.0^{a}	0.02 ± 0.0^{a}
Proximal cytoplasmic droplet	1.4 ± 0.9^{a}	2.8 ± 0.6^{a}
Distal cytoplasmic droplet	1.0 ± 1.1^{a}	2.5 ± 0.7^{a}
Acrosomal abnormalities		
Damaged acrosome	1.4 ± 1.2^{a}	12.4 ± 2.4^{b}
Partial acrosome removal	1.2 ± 0.7^{a}	10.0 ± 2.4^{b}
Total acrosome removal	0.8 ± 0.9^{a}	11.9 ± 2.5^{b}

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).

Table 9
Mean (±S.E.M.) percentage of morphologically normal sperm cells and intact acrosomes pre-freeze and post-thaw for each individual coyote, averaged across extender, freeze rate and thaw rate

Coyote	Normal morphology		Intact acrosomes	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
1	88.0 ± 6.4^{a}	78.1 ± 3.3^{a}	96.3 ± 2.1^{a}	67.8 ± 3.6^{a}
2	87.3 ± 3.8^{a}	78.1 ± 2.3^{a}	97.3 ± 2.0^{a}	64.6 ± 4.6^{a}
3	82.6 ± 7.3^{b}	74.5 ± 3.9^{b}	95.1 ± 3.1^{a}	65.5 ± 4.0^{a}
4	89.6 ± 4.2^{a}	78.0 ± 2.5^{a}	97.5 ± 1.1^{a}	65.7 ± 5.2^{a}
5	84.2 ± 4.9^{b}	73.7 ± 11.1^{b}	96.6 ± 0.8^{a}	64.9 ± 4.4^{a}
6	80.2 ± 4.1^{b}	75.2 ± 3.8^{ab}	96.5 ± 1.2^{a}	63.8 ± 4.5^{a}
7	87.5 ± 3.7^{a}	76.1 ± 2.8^{a}	97.3 ± 1.3^{a}	66.0 ± 5.1^{a}
8	86.7 ± 4.8^{a}	78.1 ± 2.2^{a}	96.3 ± 2.5^{a}	64.6 ± 4.7^{a}
9	87.0 ± 3.6^{a}	80.2 ± 2.2^{a}	96.0 ± 0.7^{a}	65.5 ± 3.5^{a}
10	88.2 ± 3.3^{a}	79.1 ± 2.3^{a}	96.7 ± 0.6^{a}	67.4 ± 3.3^{a}
Mean ± S.E.M.	86.1 ± 5.5	77.1 ± 4.8	96.6 ± 1.8	65.6 ± 4.5

Within columns, values with different superscripts (a and b) are different ($P = 0.05$).

4. Discussion

Motility is an important parameter to monitor for evaluating the potential fertility of post-thaw semen. Taken alone, however, this can lead to an imprecise approximation of fertility. Pursel et al. [24] verified that post-thaw spermatozoa could be motile but incapable of fertilization due to acrosomal damage. In this study, sperm motility and viability were coupled with morphology and acrosomal integrity to assess the effects of extender, freeze rate and thaw rate on cryopreservation of coyote semen.

Various extenders have been tested on canine semen by a number of authors using many different compositions. The most commonly used laboratory prepared extenders contain main components consisting of lactose [6,8,13,25], Tris–fructose–citric acid [4,6,12,13,22,26], or Tris–glucose–citric acid [6,8,13,27]. In this study, using electroejaculated semen, the lactose-based extender provided the lowest post-thaw motility and post-thaw viability results, regardless of freeze rate or thaw rate, when compared with both the Tris–fructose and Tris–glucose extenders. This has several possible explanations. Post-thaw motility and viability may have been affected by the ability of spermatozoa to glycolyse glucose and fructose, while lactose, with its higher molecular weight and lower permeability, is limited as an energy source because of its inability to cross plasma membranes readily [1]. The lack of an additional buffering agent in the lactose-based extender might have also affected the post-thaw motility and viability of coyote spermatozoa. If the pH of the lactose extender was lowered due to the metabolic activity of the spermatozoa, motility and viability could also have been reduced [1,28]. Olar et al. [8] obtained similar results comparing a Tris–glucose based extender to a lactose based extender, while Ivanova-Kicheva et al. [6] reported a higher post-thaw motility using a lactose-based extender than both a Tris–fructose based and Tris–glucose based extender. The discrepancy between the two different outcomes might be a consequence of using different packaging systems in conjunction with different freezing methods.

Our results also indicated a slight difference between the effects of the Tris–fructose and Tris–glucose based extenders on the post-thaw motility and viability of coyote semen. While the difference was not significant, it was noted that semen samples frozen using the Tris–fructose based extender regularly resulted in a higher percentage of post-thaw motility and viability when compared with the Tris–glucose based extender. This is in agreement with Yildiz et al. [13] who reported that post-thaw motility and viability was greater in a fructose based Tris–citric acid extender when compared to a glucose-based Tris–citric acid extender. Ponglowhapan et al. [29] also noted, in chilled canine semen, that a Tris–fructose extender maintained higher sperm motility than extenders prepared with glucose or a mixture of both sugars. However, this increased beneficial effect of fructose over glucose was not noted in fresh semen where Rigau et al. [30] reported that there were no significant differences in extenders that were prepared with fructose or glucose on the percentage of motile spermatozoa. However, their findings indicated that the use of fructose in semen extenders resulted in a more linear motility pattern while motility patterns for glucose-based extenders were more oscillatory. These differences might be explained by the sugar metabolism of fresh spermatozoa when compared to that of chilled and frozen spermatozoa.

The largest challenge to spermatozoa during cryopreservation is surviving the intermediate temperature zone (-10 to -30 °C) through which the cell must pass during the cooling and thawing stages [31]. During freezing, the spermatozoa must avoid the formation of intracellular ice crystals [32]. It must be cooled slowly enough to prevent growth of intracellular ice crystals but quickly enough to prevent the accumulation of high concentrations of solutes that can weaken the cell membrane and cause subsequent pH disturbances [31,33,34]. While a cooling rate that is too fast or too slow can cause cell death, the underlying mechanism that causes cell damage varies.

Several studies have attempted to determine the optimal freezing rate for domestic dog semen [1,2,8,14,27]. In our study, the highest post-thaw motility was obtained when spermatozoa were frozen at a moderate rate (−6 °C/min) across the critical temperature range (−10 to −30 °C) regardless of the extender or thaw rate. These findings are similar to those found in domestic dog epididymal spermatozoa by Yu et al. [14]. They reported that the optimum linear cooling rate ranged from −3 to −11 °C/min and was superior to both fast rates (−58 and −209 °C/min) and slow rates (−0.5 °C/min) [14]. These findings, along with those reported by this study, conflict with Hay et al. [27] who found no difference in post-thaw motility using linear cooling rates of −12 and −28 °C/min on naturally ejaculated semen. However, they did find that both a slow freeze rate (−0.5 °C/min) and rapid freeze rates (−99 and −214 °C/min) had detrimental effects upon post-thaw motility and acrosomal integrity [27]. Some investigators [8,10,12,26,35,36] have used a variable freeze rate rather than a linear freeze rate in their studies, making comparison between our results and theirs somewhat difficult. They noted that cooling at a slow to moderate rate (−2 to −13 °C/min) from 5 °C to −10 °C, or −15 °C, followed by a rapid freezing rate (−20 to −50 °C/min) over the critical temperature zone (−10 °C to −30 °C) yielded a higher post-thaw motility [8,10,12,26,35,36].

The thawing phase is as important to post-thaw motility and survival as is the freezing phase [31]. An excessively slow thawing rate can result in recrystallization of intracellular ice crystals, resulting in a reduction in cell survival. Conversely, a rapid thaw does not allow the penetrating cryoprotectant to leave quickly enough, thus disrupting the osmotic equilibrium and causing the spermatozoa to swell from the influx of water [37].

Several researchers looking at thaw rates in domestic dog semen obtained similar results to this study, using thaw rates of a 37 °C water bath for 15 s [12,25] and 30 s [13,14], which were comparable to those utilized in this study. Other researchers, however, have obtained higher post-thaw motility results through a faster thawing (70 and 75 °C water bath for 6 s [27], 6.5 s [26], 8 s [9], and 12 s [8]. In this study, we also noted that thawing in a 60 °C water bath for 30 s seemed to maintain motility and viability better than the 37 °C water bath for 120 s for both the slowest (−1 °C/min) and fastest (−20 °C/min) freeze rate, but vice versa for the medium freeze rate (−6 °C/min) which maintained a higher post-thaw motility and viability in the 37 °C water bath for 120 s.

The differences observed in our study and those indicated by other investigators might be explained by the pairing of the freezing rate and thawing rate. It is recognized that to optimize cell survival, the rate at which the cell was frozen must be matched with a corresponding thaw rate, to reverse the osmotic balance and rehydrate the cell, while preventing intracellular ice formation [31]. It should be noted that to maximize the post-thaw results for coyote semen, the optimal combination of freezing and thawing rates may lie somewhere between those utilized in this study.

A degree of post-thaw morphological and acrosomal damage to coyote spermatozoa was also observed across all protocols in this study, with freeze rate exerting the only significant effect. Post-thaw morphological damage, though not drastic, was comprised of a large proportion of secondary defects such as bent or coiled flagellum. This secondary damage was potentially due to cold or osmotic shock, similar to what Watson [38] and England and Plummer [39] observed in ram, bull, and canine semen. Utilizing the same technique for evaluating acrosomal integrity (Spermac®), a higher percentage of intact acromsomes (65%)

were observed in the coyote when compared to previously reported values (>20%) [40] and (24%) [27] in the domestic dog. To determine whether a pronounced decrease in acrosomal integrity will affect fertilizing capability of coyote spermatozoa, it will be necessary to first confirm the outcome of the Spermac® staining using fluorescent staining in conjunction with flow cytometry [41], and address the ability of spermatozoa to interact with and bind to an oocyte, either through artificial insemination trials or in vitro approaches [27,42–44].

There might be substantial differences between the results of other investigators using the domestic dog and our findings in the coyote. Similar studies using wild canids have reported differences in the ability of domestic dog, blue fox (*A. lagopus*), silver fox (*V. vulpes*), and red wolf (*C. rufus*) semen to tolerate the cooling phase of cryopreservation [2,15,45,46]. Another potential source of variation between our results and those of other investigators may have stemmed from our use of electroejaculation for collection of semen. While most investigators have used digital manipulation to obtain ejaculates, there were obvious safety reasons to indicate use of electroejaculation for coyotes. It has been shown in the beagle that collecting semen samples by electroejaculation, when compared to digital manipulation, resulted in lower total sperm count and concentration while still yielding similar sperm motility, viability, and morphology [47,48]. These differences in semen characteristics must be taken into account when comparing post-thaw results from electroejaculated semen and semen samples obtained by digital manipulation. Along with species and collection differences, we noted in our study that there were slight male-to-male differences in the response of spermatozoa to freezing and thawing. These types of effects have been noted in the domestic dog [14,37,41] and have lead investigators to hypothesize possible causes for these individual differences, including age differences between the animals collected and possible genetically inherited differences in the animals [14,49]. Identifying these types of differences will play a critical role in understanding and improving the cryopreservation of spermatozoa.

In conclusion, our findings established that cryopreservation of coyote spermatozoa, frozen at a moderate freezing rate (−6 °C/min) in either a Tris–fructose or Tris–glucose extender, and thawed at slow rate (37 °C water bath for 120 s) or moderate rate (60 °C water bath for 30 s), resulted in more vigorous post-thaw motility, a higher level of viability, and a larger percentage of morphologically normal cells with intact acrosomes. There was, however, a marked decrease in motility, viability, appearance of normal morphology, and percentage of intact acrosomes after freezing and thawing. Therefore, a more in-depth study addressing each stage of cryopreservation is needed to gain further understanding of where and when damage occurs in coyote spermatozoa before these techniques can be effectively utilized.

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