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# Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa

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**Abstract**

The improvement of freezing extenders is critical when defining sperm cryopreservation protocols for wild species, in order to create germplasm banks. The aim of this study was to evaluate the effect of additives (Equex Paste and EDTA) supplementation, egg-yolk (10 and 20%) and glycerol (4 and 8%) concentrations and extender osmolality (300 and 320 mOsm/kg) on the post-thawing quality of brown bear semen. Semen was obtained from 20 adult males by electroejaculation, and centrifugated individually (600 × g for 6 min). The pellets were diluted 1:1 in the corresponding extender TTF (TES-Tris-Fructose with the aforementioned variants) and cooled to 5° C. Then, it was diluted down to $100 \times 10^6$ spz/mL, loaded in 0.25 mL straws and frozen at −20° C/min. After thawing (in water at 65° C for 6 s.), the semen samples were assessed for motility (CASA), viability (SYBR-14 with propidium iodide), acrosomal status (PNA-FITC with propidium iodide) and mitochondrial activity (JC-1). Extender supplementation with additives rendered significantly higher results for these sperm parameters. Comparing the two percentages of egg yolk, 20% egg yolk showed the highest motility results, percentages of viable spermatozoa and viable spermatozoa with intact acrosome. No differences were detected among samples frozen using 4 or 8% glycerol. For extender osmolality, 300 mOsm/kg showed higher values of VAP, VCL, VSL, and ALH than 320 mOsm/kg. Based on the best performance of sperm motility, viability and acrosome status, we conclude that the most suitable extender to cryopreserve brown bear spermatozoa was TTF adjusted to 300 mOsm/kg, supplemented with 20% egg yolk, 4–8% glycerol, and the additives 1% Equex paste and 2% EDTA.


*Keywords:* Brown bear; Cryopreservation; Spermatozoa; Extender; Germplasm banks

## 1. Introduction

Genetic resource banks (GRBs) are a valuable tool for the conservation of threatened or endangered species and valuable breeds [1,2]. Some populations of brown bear are currently endangered, and are a possible target for germplasm banking in order to aid their protection. This could be the case of grizzly bears (*Ursus arctos horribilis*), considered threatened outside of Alaska by the Threatened Species Conservation Act of the USA and Cantabrian brown bear (*Ursus arctos*), which is considered to be at risk of extinction (Royal Decree 439/1990 of the Spanish law, regulation of the

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National Catalogue of Endangered Species), and only survives in two small isolated populations in the Cantabrian mountains (Northern Spain).

Sperm cryopreservation is usually the first approach in establishing a germplasm bank for the preservation of this species [3]. However, the most important challenge for establishing GRBs is the lack of knowledge on specific sperm traits, which are critical for an adequate protocol design [4].

According to Anel et al. [5], brown bear sperm can be considered that freezes well. The adjustment of a cryopreservation protocol requires the study of several factors affecting the success of the freezing process [6] particularly with regard to the formulation of a specific extender to the characteristics of brown bear ejaculates. There are a few reports on bear semen cryopreservation employing extenders already used in other species, which rendered good results: Tris-buffered-egg-yolk in Hokkaido brown bear sperm [7], Tris-citric acid–glucose-egg yolk in Japanese black bear sperm [8], egg yolk-lactose [9] or TES-Tris-egg yolk [10,11] in giant panda sperm. Nevertheless, due to the great biological value and scarcity of sperm samples obtained from wild, endangered animals, it is necessary to optimize these extenders for the species, in order to improve post-thawing sample quality [5].

Extender composition includes buffer systems, cryoprotectants, sugars, and other additives, which are supposed to protect spermatozoa, providing energy substrates and preventing deleterious effects of changes in pH and osmolality [12,13]. Among the most common components of semen diluents for mammals are egg yolk and glycerol. Egg yolk is beneficial to sperm cryopreservation because it protects against cold shock [12], and has been included routinely in most extenders for frozen semen from domestic animals as well as several wild species at different concentrations (in Cuvier's gazelle semen from 5% to 20% [14]). However, several studies revealed that higher egg yolk concentrations in some species negatively affected preservation of motility and acrosome integrity during cryopreservation [15] or reduce post-thawing viability [16]. In bear sperm, egg yolk has been used at different concentrations: 15% [7] or 20% [17].

Concentrations of glycerol ranging from 2 to 10% have been used to cryopreserve mammalian spermatozoa. Glycerol, despite its value as cryoprotectant, can be toxic to spermatozoa at high concentrations [18]. For bear sperm cryopreservation, glycerol is the main cryoprotectant used in extenders and the cryoprotective effect of concentrations from 4% to 8% has been assayed in different bear species [7,8,9,10,11,19].

To develop an optimal extender, we must also consider the special characteristics of brown bear ejaculates. A high incidence of sperm agglutination was documented by Kojima et al. [20] and Anel et al. [5]. Agglutination makes semen assessment and handling difficult and can influence sperm freezability [5], although it has been reported that it did not interfere with fertility results in boars [21]. Therefore, the extender composition needs to be improved in order to reduce this problem. One of the options explored in the present study was the addition of a surfactant (Equex paste) and a calcium chelator (EDTA) to the freezing diluents. Studies in other species have shown the beneficial effects of these substances on semen: dog [22–25], cat [26], boar [27], stallion [28], bull [29], and deer [30].

Media osmolality is another important factor that can influence the outcome of the cryopreservation process. The adjustment of extender osmolality is of great importance because of its influence on the dehydration, rehydration, and intracellular crystal formation to which spermatozoa are subjected during the cryopreservation process [30].

Anel et al. [32] suggested that an extender used for ram semen freezing (TES-Tris-fructose-10% egg-yolk-4% glycerol) rendered good results for motility and viability of brown bear spermatozoa. In fact, this extender was successfully used for freezing semen from different wild species: somalia wild ass [33], iberian red deer [34], blue wildebeest [35]. In this regard, based on a preliminary study carried out by our group in brown bear ejaculates with the above mentioned extender, we propose to assay four variants of that extender, aiming at adjusting its composition to the characteristics of brown bear ejaculates. In a first experiment we conducted three different assays to evaluate: the use of Equex paste and EDTA, two concentrations of egg-yolk (10 and 20%) and two glycerol concentrations (4 and 8%). In a second experiment we carried out a study of the use of two extenders with different osmolality (300 mOsm/kg and 320 mOsm/kg).

## 2. Material and methods

All the chemicals were at least of reagent grade, and they were obtained from Sigma (Madrid, Spain), unless otherwise stated.

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### 2.1. Animals and sample collection

Animal handling was performed in accordance with Spanish Animal Protection Regulation RD223/1998, which conforms to European Union Regulation 86/609. Sixty semen samples from 20 sexually mature male brown bears were obtained by electroejaculation during the breeding season (end of April to early July). The animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m), and fed on a diet based on chicken meat, bread, and fruits.

The males were immobilized by teleanaesthesia, using zolazepam HCl, tiletamine HCl (Zoletil100®; Virbac, Carros, France) 7 mg/kg, and ketamine (Imalgene 1000®; Rhone-Mérieux, Lyon, France) 2 mg/ kg. After immobilization, the males were weighed and monitored (pulse, oxygen saturation, and respiration). Prior to electroejaculation, the prepucial area was shaved and washed with physiological saline serum, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average). The ejaculates were collected by fractions in graduated glass tubes. The fresh semen samples of brown bear yielded an average number of spermatozoa of 423.0 ± 53.7 × $10^6$ (mean ± SD). All ejaculates used in the present study have been selected to be urine-free, as explained below.

### 2.2. Experimental design

Semen cryopreservation was performed according to Anel et al. [32]: extender TES-Tris-Fructose with egg yolk, glycerol and antibiotics; cooling rate: −0.25° C/min and freezing rate: −20° C/min.

First, three different assays were carried out to test the addition of additives (Equex paste and EDTA), two glycerol concentrations and two egg-yolk concentrations, respectively. The effect of osmolarity was then evaluated in a fourth assay. The scarce number of total spermatozoa obtained by ejaculated, due to the loss of cells during centrifugation and the number of cells necessary for semen evaluation, made it impossible to design a multifactorial experiment to study these four parameters (additives, glycerol, egg-yolk, and osmolality). All semen samples were evaluated and frozen individually.

#### 2.2.1. Experiment 1

This experiment was based on previous data that we obtained for brown bear ejaculates and on bibliographic reports [7,8,10,22], therefore we used as base an extender composed by TES-Tris–Fructose [32]. In each assay, we used this extender analyzing two different combinations of each basic component: assay 1.1: addition or not of additives (1% Equex paste and 2% EDTA); assay 1.2: two percentages of egg-yolk (10-20%) and assay 1.3: two glycerol concentrations (4-8%). Antibiotics (penicillin and streptomycin) were added to each extender formulation. In all the assays, semen was divided in two aliquots to evaluate each combination.

*Assay 1.1: Additive supplementation*

Fifteen ejaculates obtained from eleven different bears were studied to evaluate the benefits of Equex paste (Minitüb, Germany) and EDTA added to the sperm cryopreservation extender. Ejaculates were divided in two aliquots (in all assays) and frozen either in the TTF extender [with 20% egg-yolk and 8% glycerol at 320 mOsm/kg] supplemented with additives or without additives (A and NA, respectively).

*Assay 1.2: percentages of egg-yolk*

To determine the best concentration of egg-yolk in the cryopreservation extender we used fourteen brown bear semen samples from seven males, which were frozen in the TTF extender [with 8% glycerol and 1% Equex paste + 2% EDTA at 320 mOsm/kg] supplemented with 10% egg-yolk (Y10) or with 20% egg-yolk (Y20).

*Assay 1.3: glycerol concentrations*

The effect of two glycerol concentrations on the post-thawing quality of brown bear spermatozoa was evaluated. Eleven ejaculated sperm samples were collected from 10 different bears and cryopreserved with each of two extenders: TTF [with 20% egg-yolk and 1% Equex paste + 2% EDTA at 320 mOsm/kg] supplemented with 4% glycerol (G4) or with 8% glycerol (G8).

#### 2.2.2. Experiment 2

According to our results in Experiment 1, we defined an optimal extender: TTF, 20% egg-yolk, 8% glycerol and 1% Equex paste + 2% EDTA, which was applied in a final assay to test the effect of osmolality.

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*Assay 2.1: Extender osmolality*

Twenty ejaculates from fifteen males were used to test the effect of extender osmolality on brown bear sperm cryopreservation. Samples were processed with TTF above mentioned either adjusted to 300 or to 320 mOsm/kg (E300 and E320, respectively).

### 2.3. Semen cryopreservation

Immediately after collection, the volume of each ejaculate was recorded, osmolality was measured using a cryoscopic osmometer (Osmomat-030, Gonotec ™; Berlin) and the pH value was determined by pH meter (CG 837-Schott; Germany). For each ejaculate, sample motility was assessed with a phase contrast microscope and urospermia was evaluated by means of a rapid urea test (Merckgnost Urea Rapid Screening test, Merck, Barcelona, Spain). Samples of low motility (<50%) or urine contaminated samples (>80 mg urea/dL) were rejected [36]. The selected samples were divided into two aliquots and centrifuged at 600 × g during 6 min. The supernatant was discarded and each pellet was diluted with an equal volume of the corresponding extender, depending on the experiment, at room temperature. Tubes with the diluted semen were put in glasses containing 100 ml of water at room temperature and transferred to refrigerated container to 5° C, so temperature decreased slowly to 5° C (70–80 min). Once at 5° C, the samples were diluted again 1:1 with the same diluents prepared with a higher glycerol concentration (6% for the G4 assay and 12% for the others), in order to achieve a final glycerol concentration for each extender (4% and 8%, respectively). The samples were diluted with the original extender (4% glycerol for the G4 assay and 8% for the others) to yield a final concentration of $100 \times 10^6$ spermatozoa/mL. After equilibration for 1 h at 5° C, the semen was packaged into 0.25 mL plastic straws, and frozen in a programmable biofreezer (Kryo 10-16 II PlanerTM) at −20° C/min down to −100° C, and then transferred to liquid nitrogen containers. The cryopreserved samples were stored in liquid nitrogen for a minimum of one week. Thawing was performed by plunging the straws in water at 65° C for 6 s and the sample are evaluated immediately at room temperature.

### 2.4. Semen evaluation

Before freezing and after thawing, the semen samples were analyzed for sperm agglutination, motility, viability, acrosomal status and mitochondrial activity.

Sperm agglutination was evaluated on a microscope slide by subjective observation under a phase contrast microscope and rated on a scale from 0 to 4: 0-absence of agglutination; 1-low degree of agglutination (<10% agglutinated sperm); 2-intermediate level of agglutination (10–30%); 3-high degree of agglutination (30–50%) and 4-very high degree of agglutination (>50%).

Motility parameters were assessed by means of a computer-assisted sperm analysis system (CASA) (Sperm Class Analyzer; Microptic, Barcelona, Spain). Samples were diluted ($10–20 \times 10^6$ cells/mL) in an 1% buffer (HEPES 20 mmol/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 300mOsm/kg), and warmed on a 37° C plate for 5 min. Then, 5μL sperm sample was placed in a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and analyzed using a negative phase objective (×10) in a contrast microscope with a warmed stage (38° C). The standard parameter settings were as follows: 25 frames/s; 5 to 80 μm [2] for head area; VCL >10 μm/s to classify a spermatozoon as motile. At least fields and 200 spermatozoa were saved and analyzed afterwards. Reported parameters were total motility (TM), progressive motility (PM; VCL>25, STR>80), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) and amplitude of lateral head displacement (ALH, μm). A detailed explanation of these motility parameters has been provided elsewhere [37].

To evaluate sperm viability, the double stain SYBR-14 with propidium iodide (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain) using flow cytometer was applied. Sperm samples were diluted with PBS down to $5 \times 10^6$ spermatozoa/ml, and 300 μL were transferred to a polypropylene tube to which we added 3 μL PI (24 μM) and 1.5 μL SYBR-14 (100 nM). The tubes were kept at 37° C for 20 min in the dark. We detected three populations corresponding to percentage of live spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red). Only live spermatozoa (named VIAB) were recorded.

For acrosomal status, we used the double stain PNA-FITC (PNA-FITC) and IP in a stock solution in PBS at 1 μg/mL and 24 μM, respectively. PNA is a lectin from *Arachis hypogaea* that binds to beta-galactose moieties associated with the outer acrosomal membrane of spermatozoa, indicating acrosome damaged cells. Sperm samples were diluted in PBS ($5 \times 10^6$ spermatozoa/mL), and 300 μL were transferred to a polypropylene tube to which we added PI (24 μM) and PNA-FITC (1 μg/mL). Flow cytometer rendered the percentage of

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viable spermatozoa with intact acrosome (non red and non green fluorescence, ACR).

JC-1 (Invitrogen, Barcelona, Spain) was used to assed mitochondrial status, identifying mitochondria with high mitochondrial potential. Samples were diluted in 300 $\mu$L of PBS ($5 \times 10^6$ sperms/mL), adding JC-1 at 6.8 $\mu$M. After 30 min at 37° C, we obtained by flow cytometer the percentage of orange stained sperm (high membrane mitochondrial) named MIT.

Evaluation of flow cytometer parameters (viability, acrosomal status and mitochondrial status) was carried out using a FACScalibur flow cytometer (Becton Dickinson Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). 10,000 events with a flow rate of 200 cells/s were analyzed per sample.

### 2.5. Statistical analysis

Data were analyzed using the SAS™ V.9.0. package. The parameters of semen quality were the dependent variables and a GLM procedure was used to evaluate the effect of various factors: addition or not of additives, egg-yolk concentration, glycerol concentration and extender osmolality. Least-squares means were computed for each effect listed and P-values for differences of the multiple comparisons were calculated by Tukey's test. Changes on the frequencies of the agglutinated spermatozoa classification depending on additive supplementation were analyzed using a FREQ procedure. Values were considered to be statistically significant at $P < 0.05$.

Table 1
Post-thawing semen quality (mean ± SEM) for supplementation or not with additives (Equex paste® and EDTA); (TTF* with or without additives corresponds to A and NA, respectively).

| Parameters | A | NA | P |
|---|---|---|---|
| TM (%) | 53.9 ± 3.5 | 36.5 ± 3.9 | 0.021 |
| PM (%) | 28.1 ± 3.3 | 17.4 ± 2.9 | 0.018 |
| VAP (μm/s) | 60.0 ± 3.4 | 45.2 ± 3.6 | 0.005 |
| VCL (μm/s) | 107.2 ± 6.3 | 81.2 ± 7.1 | 0.010 |
| VSL (μm/s) | 45.3 ± 3.3 | 33.4 ± 3.0 | 0.011 |
| ALH (μm) | 4.5 ± 0.2 | 3.5 ± 0.3 | 0.011 |
| VIAB (%) | 60.0 ± 1.4 | 44.1 ± 2.4 | <0.001 |
| ACR (%) | 63.5 ± 1.9 | 46.5 ± 2.3 | <0.001 |
| MIT (%) | 62.35 ± 4.6 | 45.6 ± 4.3 | 0.011 |

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
P indicates the difference between columns.
* TTF extender with 20% egg-yolk and 8% glycerol at 320 mOsm/kg.

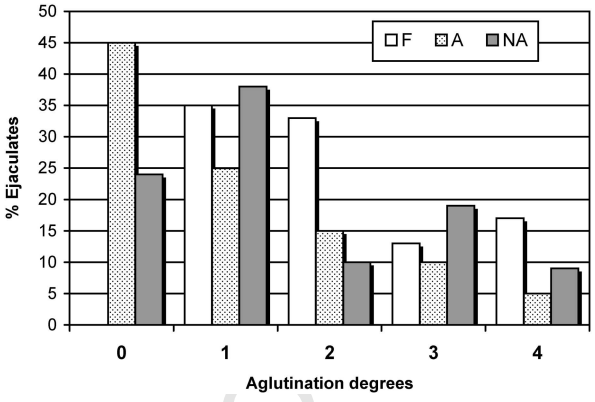


Fig. 1. Distribution (%) of agglutination degrees (0–4) in fresh semen (F) and post-thawed semen in extender with or without additives (A and NA, respectively).

Scale from 0–4: 0-absense of agglutination, 1-low degree of agglutination (<10% agglutinated sperm), 2-intermediate level of agglutination (10–30%), 3-high degree of agglutination (30–50%) and 4-very high degree of agglutination (>50%).

## 3. Results

### 3.1. Experiment 1

The quality of post-thawed sperm was improved by extender supplementation with additives. Table 1 show that TTF with additives (A) rendered significantly higher results for all sperm parameters analyzed. Moreover, when we studied the effect of additives we observed that all of the fresh samples (F) showed agglutination (Fig. 1): most of the samples presented grades 1 or 2 of agglutination (68%) and the rest of the ejaculates showed grades 3 or 4 (32%). A gradual trend to decreasing agglutination levels in post-thawed samples was observed when we used the extender with additives (A), thus 45% of the samples did not present agglutination and 25% manifested grade 1 agglutination (Tables 2 and 3). However, most of post-thawed samples frozen with the extender without additives (NA) showed grade 1 agglutination (38.1%).

As in the case of supplementation with additives, there were significant differences between Y10 and Y20. Samples frozen with Y20 showed better post-thawing mean values for all motility parameters, being significantly higher for all of them except: VCL ($P = 0.057$) and ALH ($P = 0.155$). With regard to fluorescence parameters, Y20 showed significantly higher re-

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Table 2
Post-thawing semen quality (mean ± SEM) for the two egg-yolk concentrations (TTF * with 10% or 20% egg-yolk; Y10 and Y20, respectively).

| Parameters | Y10 | Y20 | P |
|---|---|---|---|
| TM (%) | 22.2 ± 3.5 | 53.6 ± 4.6 | <0.001 |
| PM (%) | 7.4 ± 1.6 | 25.2 ± 3.0 | <0.001 |
| VAP (μm/s) | 42.8 ± 5.3 | 61.2 ± 3.7 | 0.009 |
| VCL (μm/s) | 87.5 ± 10.6 | 112.4 ± 6.7 | 0.057 |
| VSL (μm/s) | 28.6 ± 3.9 | 42.3 ± 3.1 | 0.010 |
| ALH (μm) | 3.8 ± 0.4 | 4.5 ± 0.3 | 0.155 |
| VIAB (%) | 31.1 ± 5.1 | 58.4 ± 3.8 | <0.001 |
| ACR (%) | 33.7 ± 4.5 | 62.5 ± 3.7 | <0.001 |
| MIT (%) | 30.5 ± 8.3 | 55.8 ± 9.3 | 0.053 |

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
P indicates the difference between columns.
* TTF extender with 8% glycerol and 1% Equex paste + 2% EDTA at 320 mOsm/kg.

sults for VIAB and ACR, whereas significance was not found for MIT (P = 0.053).

### 3.2. Experiment 2

For the extender osmolality experiment, osmolality of electroejaculated samples was 308 ± 38 mOsm/kg (mean ± SD).

The comparison of the effect of the two extenders, E300 and E320, with different osmolalities, on quality parameters of post-thawed brown bear semen is shown in Table 4. We observed that post-thawed semen parameters were significantly lower for E320 than for E300 extender: VAP (P = 0.019), VCL (P = 0.022), VLS (P = 0.021) and ALH (P = 0.032). For the other parameters, the results were similar for both extenders.

Table 3
Post-thawing semen quality (mean ± SEM) for the two glycerol concentrations (TTF* with 4% or 8% glycerol, G4 and G8, respectively).

| Parameters | G4 | G8 | P |
|---|---|---|---|
| TM (%) | 45.8 ± 7.1 | 48.6 ± 5.4 | 0.758 |
| PM (%) | 25.3 ± 4.6 | 25.1 ± 4.6 | 0.976 |
| VAP (μm/s) | 87.5 ± 6.5 | 84.5 ± 8.0 | 0.769 |
| VCL (μm/s) | 158.3 ± 12.3 | 164.7 ± 14.9 | 0.747 |
| VSL (μm/s) | 72.0 ± 6.0 | 67.7 ± 7.4 | 0.661 |
| ALH (μm) | 6.3 ± 0.5 | 7.0 ± 0.6 | 0.412 |
| VIAB (%) | 34.9 ± 3.0 | 43.7 ± 4.1 | 0.097 |
| ACR (%) | 41.2 ± 2.9 | 48.3 ± 3.8 | 0.152 |
| MIT (%) | 43.6 ± 3.0 | 40.9 ± 5.6 | 0.670 |

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
p indicates the difference between columns.
* TTF extender with 20% egg-yolk and 1% Equex paste + 2% EDTA at 320 mOsm/kg.

Table 4
Post-thawing semen quality (mean ± SEM) for the two extender osmolalities (E300 and E320 correspond to TTF* adjusted to 300 or 320 mOsm/kg).

| Parameters | E300 | E320 | P |
|---|---|---|---|
| TM (%) | 55.5 ± 2.9 | 51.5 ± 3.2 | 0.360 |
| PM (%) | 28.8 ± 2.2 | 23.7 ± 2.1 | 0.095 |
| VAP (μm/s) | 73.1 ± 3.7 | 60.7 ± 3.5 | 0.019 |
| VCL (μm/s) | 133.3 ± 7.0 | 111.4 ± 6.2 | 0.022 |
| VSL (μm/s) | 53.5 ± 3.5 | 42.8 ± 2.8 | 0.021 |
| ALH (μm) | 5.4 ± 0.3 | 4.6 ± 0.2 | 0.032 |
| VIAB (%) | 61.6 ± 2.4 | 56.7 ± 3.2 | 0.218 |
| ACR (%) | 63.5 ± 2.3 | 61.1 ± 2.6 | 0.483 |
| MIT (%) | 55.9 ± 5.8 | 56.7 ± 5.9 | 0.923 |

TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: the straight line velocity; ALH: amplitude of lateral head displacement. VIAB: sperm viability; ACR: sperm with no damage acrosome within VIAB; MIT: spermatozoa mitochondrial membrane potential.
p indicates the difference between columns.
* TTF extender with 8% glycerol, 20% egg-yolk and 1% Equex paste + 2% EDTA.

## 4. Discussion

The choice of adequate extenders and freezing protocols is a crucial point for the success of semen cryopreservation in any species. Extender composition includes buffer systems, cryoprotectants, sugars, and other additives, which interact with the cell membrane during the freezing-thawing process in a very specific way. Because of this, several combinations were proposed for the different species.

Different extenders formulated for other species were used for bear sperm cryopreservation [7,8,9, 10,19]. Although the results of sperm quality obtained were satisfactory, it is necessary to adjust the extender composition to bear semen characteristics. In the present work, the use of an extender manufactured in our laboratory with a known composition allowed the levels of its constituents to be easily adjusted [32].

To our knowledge, no studies have been carried out to analyze the effect of additives on bear semen quality during cryopreservation. In order to improve the post-thawing quality of these samples we proposed that the extender be supplemented with additives (1% Equex paste and 2% EDTA) and we observed a significant

improvement in sperm sample quality. Equex paste is a commercially available additive for use with semen extenders. It contains a detergent, sodium dodecyl sulphate (SDS), which might interact with the egg-yolk structure and could increase its protective effect against cold shock and freezing injury [27]. It has been proposed that Equex STM Paste improves post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell membranes, particularly acrosome membranes, and to protect spermatozoa against the toxic effects of glycerol during the freezing-thawing process [38]. This substance, added to diluents at 0.5–1.5% v/v, disperses egg yolk components and allows better interaction with the sperm plasma membrane surface [39]. Addition of EDTA to semen extenders as a chelating agent blocks the action of calcium as a mediator of sperm capacitation and the acrosome reaction [23].

Previous studies have reported that the use of Equex STM Paste on semen freezing extenders benefits post-thaw motility [25], viability [22], and longevity [24] of ejaculated canine spermatozoa. Also, addition of Equex to freezing extenders protects the acrosomes of cat epididymal spermatozoa during the freezing-thawing process. In this regard, Ponglowhapan and Chatdarong [38], reported that supplementation with Equex Paste in the semen extender was effective for cryopreserving canine epididymal spermatozoa because it protected acrosome integrity against damage induced by freezing and prolonged post-thaw sperm motility during *in vitro* incubation at 37° C. Moreover, Aisen et al. [23], reported higher percentages of cells with intact acrosome in the post-thawing evaluation of ram spermatozoa when extenders containing EDTA was used.

We observed that the use of additives could also reduce the agglutination level. In boar, the incidence and degree of agglutination was affected by individual, ejaculatory and seasonal variations. It was also reported that sperm agglutination exerted a negative effect on motility but was largely reduced after dilution in semen extender, although it did not interfere with fertility results [21].

Egg yolk was routinely included in semen crypreservation protocols of domestic as well as wild species and seemed to help spermatozoa in resisting against cold shock [40,41].

Egg yolk concentrations of 15% (Hokkaido brown bear [7]) and 20% (Japanese black bear [42]) have been used for freezing the bears semen. In our study, 20% egg yolk appeared as the most suitable egg yolk concentration for preserving brown bear spermatozoa, since it improves sperm motility, viability and acromosomal status. Although some authors have reported negative effects of high concentrations of egg yolk on the preservation of motility and acrosome integrity (in Mohor gazelle [16]) we noted that it has been suggested that egg yolk's effects differ depending on the composition of the extender buffer [43] .Thus, the higher egg yolk concentration in a raffinose-based diluent resulted in a better preservation of motility and membrane integrity during the cooling of spermatozoa in Cuvier's gazelle [14] or in red deer [44].

Concentrations of glycerol ranging from 2 to 10% have been applied to cryopreserve mammalian spermatozoa [31]. For ungulates, optimal glycerol concentration for sperm cryopreservation usually ranges between 4% and 8% [39]. Glycerol concentrations used in sperm of different bear species vary between 4–8% [7,8,9, 10,11,19]. In this regard, our results coincide with those for the glycerol concentrations used in other ursids. Frozen-thawed sperm motility was influenced by different glycerol concentrations in some species, (stallion [44]; boar [45], ram [46] and Rhesus monkeys [47]. However, in Japanese black bear sperm motility was not affected by different glycerol concentrations (4–12%) but the percentage of viability and intact acrosomes were higher for sperm frozen with 4 and 6% glycerol [17]. In our study, sperm motility, viability, acrosome integrity and mitochondrial activity were not significantly affected by the two glycerol concentrations. This suggested that the adequate glycerol concentration for freezing brown bear sperm could be between 4–8%.

The role of extender osmolality on the quality of frozen-thawed brown bear spermatozoa was analyzed in present study. Once we observed the benefits of using additives and the most suitable glycerol and egg yolk concentrations (additives supplementation, glycerol–8%, egg yolk–20%), we prepared two extenders with this composition adjusted to 300 and 320 mOsm/kg (E300 and E320). For brown bear spermatozoa, extender E300 showed significantly higher results for some kinetic parameters (VAP, VCL, VSL, and ALH).

Environment osmolality is a significant factor when cryopreserving semen. Not only do anisoosmotic solutions induce cellular stress, but they also influence the outcome of the cryopreservation process. However, this influence could be positive since the osmolality of the extender modifies water flux through spermatozoa membrane [48]. In this regard, we must consider the average osmolality of the sperm samples used in this experiment (308 ± 38 mOsm/kg) to interpret our results.

According to this, the use of an E300 extender with an osmolality similar to that expected for the sample might be the best option. So, the hypothesis that a nearly isoosmotic extender could provide the best environment for brown bear spermatozoa was confirmed. This finding did not agree with reports documented in other species (bull [49], ram [23], and deer [34]) which showed that moderately hyperosmotic extenders might protect spermatozoa better than isoosmotic ones. In this context, spermatozoa of each species are affected in a different way by changes in the osmolality of the media, so this is a decisive factor to obtain acceptable cryopreservation results [49]. For instance, whereas osmotic tolerance of ram sperm in hypoosmotic conditions is much lower than bull sperm, it is higher than boar sperm [50]. Several reports have revealed information on the osmotic effects of glycerol addition and removal on post-thawing motility and acrosome integrity of ram spermatozoa [51].

Considering the lack about knowledge of sperm cryobiology in bear, our findings can contribute to developing specific extenders for an efficient cryopreservation of brown bear spermatozoa.

In conclusion, we found that the best extender for cryopreserving brown bear sperm was a TES-Tris-Fructose buffer with an osmolality of 300mOsm/kg, and supplemented 20% egg-yolk, 4–8% glycerol and additives (EDTA and Equex paste). In order to further refine extender composition, new studies must be developed in order to improve cryopreservation results.

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