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Use of native chicken breeds (*Gallus gallus domesticus*) for the development of suitable methods of Cantabrian capercaillie (*Tetrao urogallus cantabricus*) semen cryopreservation

Emma O'Brien[1] | **Cristina Castaño**[1] | **Adolfo Toledano-Díaz**[1] | **José Néstor Caamaño**[2] | **Carlos Hidalgo**[2] | **Luis Eusebio Fidalgo**[3] | **Ana María López-Beceiro**[3] | **Milagros Cristina Esteso**[1] | **Ramón Balsera**[4] | **Pedro García-Casado**[5] | **Ewa Łukaszewicz**[6] | **Julián Santiago-Moreno**[1]

[1] Departmento de Reproducción Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA-CSIC), Madrid, Spain

[2] Selección y Reproducción Animal-SERIDA, Principado de Asturias, Gijón, Spain

[3] Department of Anatomy, Animal Production and Veterinary Clinical Sciences, Faculty of Veterinary, Universidad de Santiago de Compostela, Lugo, Spain

[4] Servicio de Vigilancia y Control de la Biodiversidad, Dirección General de Medio Natural, Oviedo, Spain

[5] R&D Department, Zoitechlab (Arquimea Group), Madrid, Spain

[6] Division of Poultry Breeding, Institute of Animal Breeding, Wrocław University of Environmental and Life Sciences, Wrocław, Poland

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Abstract

Background: The Cantabrian capercaillie (*Tetrao urogallus cantabricus*) is critically endangered. This subspecies has the lowest genetic variability and it is in regression. It belongs to *Phasianidae* family; therefore, the domestic chicken (*Gallus gallus domesticus*) could be a good model for developing reproductive technologies for use in capercaillie populations with low availability of animals.

Objectives: In this study, we analyzed the response of capercaillie sperm to the freezing–thawing process for contributing to the development of a semen cryobank of Cantabrian capercaillie.

Methods: We used domestic chicken as the animal model in order to obtain the freezing protocol before applying on capercaillie. In the first experiment, two different extenders (EK and LR84) and different concentrations [4% and 6% dimethyl-acetamide (DMA) v:v] of cryoprotectants were evaluated using in-straw freezing method in domestic chickens. A pilot study in capercaillie males, using the same conditions evaluated in chicken, was performed.

Results: In chicken, we found that the LR84-4% DMA media provided the best results for freezing semen. In capercaillie study, LR84 extender seemed to be the most appropriate diluent and 4% was the better dose of DMA cryoprotectant agent. Further, based on previous studies carried out in rooster samples, we also tested the glycerol (8% v/v) as a cryoprotectant for capercaillie semen cryopreservation.

Conclusions: Our results suggest that sperm from both domestic and wild species had a similar response to freezing–thawing processes. Mediterranean chickens may be used as a suitable model for developing sperm freezing protocols that can be extrapolated to threatened capercaillie populations. In addition, LR84 media with glycerol was the most efficient extender to freeze capercaillie sperm native.

KEYWORDS
capercaillie, cryoprotectant, extender, sperm freezing

1 | INTRODUCTION

The endangered Cantabrian capercaillie (*Tetrao urogallus* subsp. *cantabricus*) inhabits deciduous forests of the Cantabrian Mountains of Spain. This species faces a high risk of extinction in its native habitat, not only because of its peripheral location but also due to its small population size, low genetic diversity and low incoming gene flow (Alda et al., 2013). The primary causes of threat are multiple, such as the management of the habitat that involved increasing the density and transformation of forest edges, increasing predators, climate change and hunting. In 2019, the census carried out by the Ecological Transition Ministry of Spain (2019) reveals that there are only 292 specimens of Cantabrian capercaillie that survive in a 350 km^2 range of the ridge Cantabrian.

Conservation programmes and reproductive technologies can help to protect species from becoming extinct. Semen cryopreservation, coupled with artificial insemination, has become the main tool for threatened species in ex situ assisted reproduction. Also preserving their genetic diversity in genome resource banks is essential if the success of captive breeding programmes is to be guaranteed (Prieto et al., 2014).

Previous studies on the optimization of capercaillie semen cryopreservation protocols have been described in central European capercaillie populations (*T. urogallus*). Kowalczyk and Łukaszewicz (2015) reported two methods of freezing capercaillie semen using EK as extender and the permeant cryoprotectants 6% dimethyl-acetamide (DMA) for freezing in pellets and 6% of dimethyl-formamide (DMF) for freezing in straws. Their results showed that the straw method allows obtaining a higher survival rate and number of motile sperm, compared to pellets. However, there are no previous studies of cryoresistance of Cantabrian capercaillie semen. Reproductive techniques for use with a target species could be developed using phylogenetically related model species. Domestic chicken *(Gallus gallus domesticus)*, a *Phasianidae* species as the capercalillie, might provide a good model for developing new reproductive technologies for use with threatened related wild species as capercaille, in which the number of available individuals is very scarce.

Based on the protocols developed in domestic chicken, we described, for the first time, the effect of different extenders and cryoprotectants in-straw freezing protocol of the Cantabrian capercaillie's semen.

2 | MATERIALS AND METHODS

2.1 | Experimental birds and semen collection

A total of five chicken males (Birchen Leonesa breed) and nine capercaillie males were used (two European and seven Cantabrian capercaillies). Birchen Leonesa roosters were raised as part of a genetic resource conservation programme run by our institution. They were housed under natural photoperiod and temperature conditions. Cantabrian capercaillie (*Tetrao urogallus cantabricus*, see an image in supporting information) and European capercaillie (*Tetrao urogallus* L.) males were maintained under natural photoperiod and temperature conditions in different captive breeding and genetic reserve centres.

All semen collections in both chicken and capercaillie were performed using the dorsoabdominal massage technique (Burrows & Quinn, 1937; Łukaszewicz et al., 2011). In capercaillie males, semen was recovered by capillarity using a microhaematocrit tube (Brand GMBH + Co KG, Wertheim, Germany).

2.2 | Extenders composition

The EK media composed of 0.14 g potassium citrate, 0.21 g sodium dihydrogen phosphate, 0.98 g disodium hydrogen phosphate, 0.7 g glucose, 1.4 g sodium glutamate, 0.2 g D-fructose, 0.7 g inositol, 0.1 g polyvinylpyrrolidone (PVP), 0.02 g protamine sulphate per 100 ml of ultrapure water; pH 7.80 and osmotic pressure 385 mOsmol/kg (Kowalczyk et al., 2012). Lake-Ravie medium (LR84) composed of 1.92 g sodium glutamate, 0.8 g glucose, 0.08 g magnesium acetate $4H_2O$, 0.5 g potassium acetate, 0.3 g of PVP, ultrapure water (100 ml); pH 7.08 and osmotic pressure 343 mOsm/kg (Lake & Ravie 1984).

2.3 | Assessment of sperm variables

The sperm concentration was estimated using a Neubauer chamber (Marienfeld, Lauda- Königshofen, Germany). The quality of movement in fresh samples was subjectively scored on a scale of 0–5 (Santiago-Moreno et al., 2011) using a phase contrast microscope (Zeiss, Germany): 0 = no movement, 1 = tail movements but no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement. The objective sperm motility was analyzed using a computer-aided sperm analyses (CASA) system coupled with a phase contrast microscope (Nikon Eclipse model 50i; Nikon Instruments Europe B.V., Izasa S.A.; negative contrast) and employing a Sperm Class Analyzer (SCA, Barcelona, Spain) v.4.0. software (Microptic S.L., Barcelona, Spain) (Santiago-Moreno et al., 2012). Semen was diluted to a concentration of approximately 40 million sperm/ml and loaded onto a warmed (37°C) 20 μm Leja 8-chamber slides (Leja Products B.V., Nieuw-Vennep, The Netherlands). The percentage of motile sperm (MT) and

the percentage showing progressive motility (MP) were recorded. Sperm movement characteristics: curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s) and amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat-cross frequency (BCF, Hz) were also recorded. A minimum of three fields and 200 sperm tracks were evaluated at a magnification of 100× for each sample (image acquisition rate 25 frames/s). DNA integrity was assessed in fresh sperm and after freezing–thawing by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). For this, the kit "In Situ Cell Death Detection" (Roche, Basel, Switzerland) was used following the manufacturer's instructions with minor changes in order to adapt the technique to the analyses of rooster sperm (Santiago-Moreno et al., 2019). Propidium iodide (PI) and SYBR-14 were used as fluorochromes in the examination of membrane integrity (Challah & Brillard, 1998); 200 cells were examined using the epifluorescence microscope at 400× (wavelength: 450–490 nm). Sperm integrity and morphology were examined in nigrosin-eosin smears (Viability E/N) according to the procedure of Vallverdú-Coll et al. (2016). Sperm were morphologically categorized into four classes: normal and head, midpiece and tail morphoabnormalities. The percentage of sperm with an intact acrosome was determined by examining 200 aniline blue-stained cells by phase-contrast microscopy (magnification 1000×) following the procedure of Santiago-Moreno et al. (2009).

2.4 | Semen freezing and thawing

Freezing was done in two steps: from 5°C to −35°C at 7°C/min, and then from −35°C to −140°C at 60°C/min (Santiago-Moreno et al., 2011) by nitrogen vapor [4 min at 17 cm above the surface of liquid nitrogen (3.4 L volume) and 2 min at 1 cm] in an expanded polystyrene bath (31 cm × 31 cm × 30.3 cm). The frozen straws were then plunged into and maintained in liquid nitrogen (at −196°C) until thawing. The freezing rate was monitored using a Ventrix K/J/T thermometer (Ventrix, China) equipped with a probe resistant to freezing. For thawing, the straws were warmed for 30 s in a water bath at 5°C.

2.5 | Experimental design

This study consisted of one experiment and a pilot study involving the cryopreservation of semen from native chicken breed and capercaillie, respectively. Experiment 1 involved the use of two different diluents with two different DMA concentrations in a Birchen Leonesa rooster. The pilot study consisted of the use of capercaillie males, involving the same extenders with DMA as Experiment 1, and a Lake-Revie extender with glycerol as previously tested in native Mediterranean chicken breeds (Abouelezz et al., 2015).

2.6 | Experiment 1: Cryopreservation of semen of the Birchen Leonesa rooster comparing two extenders and two different concentrations of DMA

This experiment studied the effect of two extenders, LR84 and EK, with two different concentrations of the DMA cryoprotectant (4% and 6%) on frozen-thawed rooster sperm variables.

Ten samples of five roosters (two for each animal) about 2 years old of the Birchen Leonesa were obtained. As the volume obtained from each male was not enough to perform the four treatments, half of the samples ($n = 5$) were immediately diluted 1:1 (v:v) using LR extender, and the other half of samples ($n = 5$) were diluted 1:1 (v:v) using EK extender. Then, each aliquot was refrigerated for 1 h at 5°C. After the equilibrated period with the extender, a third volume of the extender with 12% DMA (final concentration 4% DMA) or 18% DMA (6% DMA final concentration) was added and equilibrated for 10 min at 5°C. After equilibration time, the samples were loaded into 0.25 ml French straws and frozen.

For this experiment, the comparison of each experimental condition was made analyzing the motility variables by CASA system, and the viability by fluorescence (PI/SYBR14) of the fresh and frozen/thawed rooster semen.

2.7 | Pilot study: Cryopreservation of capercaillie semen comparing extenders previously studied in chicken

Under the hypothesis that the best conditions observed in roosters could be the same for capercaillie, we carried out this study using the same extenders and cryoprotectant agents previously evaluated in roosters.

2.8 | Pilot study 2.1: Freezing semen of capercaillie using LR84 and EK extenders with 6% and 4% DMA as a cryoprotectant

Semen samples of three capercaillie males were analyzed: one Cantabrian capercaillie male named URS1, and two European capercaillie males named URA1 and URA2. The samples obtained were divided into two aliquots. Both fractions were brought to a 1:1 (v/v) dilution, but each one with a different extender (EK or LR84), and refrigerated for 1 h at 5°C. Then, DMA was added to leave a final 6% or 4% concentration and equilibrated for 10 min at 5°C before freezing.

For this experiment, due to the scarce volume of the collected sample, the fresh sperm analysis consisted only the subjective motility and volume. For frozen/thawed samples, viability (PI/SYBR14), motility (CASA) and sperm DNA integrity (TUNEL) were analyzed.

TABLE 1 Rooster sperm quality variables (mean ± SE) of fresh and frozen-thawed samples with 4% of dimethyl-acetamide (DMA) or 6% of DMA using LR84 or EK extenders

		CPA concentration			CPA concentration	
		6% DMA			4% DMA	
		Frozen-thawed			Frozen-thawed	
Sperm trait	Fresh	LR84	EK	Fresh	LR84	EK
Concentration (10^6 spz/ml)	896.7 ± 322.1				828.3 ± 323.3	
Subject motility (%)	74.3 ± 5.8 A	5.2 ± 1.8 B	2.3 ± 1.1 B	80 ± 2.5 a	14.5 ± 6.4 b	12 ± 3.6 b
Score	3 ± 0.4	1.7 ± 0.6	1.7 ± 0.8	3.5 ± 0.2 a	1.8 ± 0.3 b	1.9 ± 0.2 b
Viability (%)	60.9 ± 4 A	36.3 ± 7.8 B	27.7 ± 9.6 C	63.1 ± 4.9 a	23 ± 3.9 b	16 ± 1.7 b
MP (%)	27.2 ± 8 A	1.9 ± 0.8 B	0.3 ± 0.2 B	52.4 ± 4.2 a	3.4 ± 1.3 b	2.7 ± 1.1 b
MT (%)	66.3 ± 6.1 A	7.8 ± 1.8 B	5.2 ± 1.2 B	87.7 ± 1.7 a	14 ± 4.8 b	12.2 ± 3 b
VCL (μm/s)	62.5 ± 9.7 A	36.4 ± 7.7 B	20.7 ± 4 C*	102.3 ± 6.8 a	47.31 ± 1.4 b*	41.1 ± 3 b
VSL (μm/s)	42.3 ± 8.9 A	23.5 ± 7.7 A	9.9 ± 2.6 B	69.9 ± 6.1 a	30 ± 3.8 b	25.6 ± 2.8 b
VAP (μm/s)	50.5 ± 9.6 A	27.7 ± 7.8 B	13.3 ± 3.4 C*	85.4 ± 6.2 a	35.7 ± 5.1 b*	30.3 ± 3.1 b
LIN (%)	63.7 ± 4.8	56.7 ± 9.9	42.9 ± 6.2	67.7 ± 2.9	63.1 ± 6.7	61.9 ± 3.7
STR (%)	81.4 ± 2.6	78.7 ± 7	72.8 ± 4.5	81.4 ± 2.5 a	78.7 ± 4.3 b	72.7 ± 1.6 b
WOB (%)	77.5 ± 3.5	69.5 ± 7.5	59.1 ± 6.2	83.3 ± 1.4	75.3 ± 4.7	73.2 ± 5.3
ALH (μm)	2.9 ± 0.2 A	1.3 ± 0.4 B	1.1 ± 0.5 B	3.1 ± 0.2 a	2 ± 0.3 b	1.8 ± 0.2 b
BCF (Hz)	8.8 ± 0.2 A	6.4 ± 2.1 A	4.1 ± 1.7 B	8.9 ± 0.1	8.4 ± 0.9	8 ± 0.6

Note: Different letters within each sperm variable indicate significant differences ($p \leq 0.05$) between fresh and frozen-thawed sperm and between diluents (LR84 vs. EK) for each DMA concentration (upper-case letters for 6% DMA and lower-case letters for 4% DMA). Asterisks indicate significant significances ($p \leq 0.05$) between diluents with different DMA concentrations (EK-6% DMA vs. LR84-4%).

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; CPA, Cryoprotectant; LIN, linearity; MP, progressive motility; MT, total motility; STR, straightness; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; WOB, wobble.

2.9 | Pilot study 2.2: Freezing semen of capercaillie using LR84 extender and glycerol as a cryoprotectant

The glycerol (GLY) as cryoprotectant was previously checked in native Mediterranean chicken samples (Abouelezz et al., 2015), returned higher sperm viability, intact acrosome and motility values. Thus, we tested the freezability of the Cantabrian capercaillie sperm with 8% GLY in the in-straw freezing method.

Samples from four of six Cantabrian capercaillie males (male identification: 31-C, 55-C, 61-C and 70-C) were obtained. Only the semen volume obtained from two of them was enough for freezing (31-C and 55-C). Both samples obtained were brought to a 1:1 (v/v) dilution in LR84 extender for 1 h at 5°C. Then, the glycerol was added directly to diluted sperm samples to reach the final concentration of 8% (v/v).

For this experiment, the fresh sperm analysis consisting of the subjective motility, viability (eosin/nigrosin), sperm morphoabnormalities, acrosome status and DNA integrity were performed. For frozen/thawed samples, viability (PI/SYBR14 and E/N), motility and sperm DNA integrity (TUNEL) were analyzed.

2.10 | Statistical analysis

The influence of the interaction semen media (LR84 and EK) × cryoprotectant concentration (DMA 4% and 6%) on sperm variables was analyzed by two-way analysis of variance (ANOVA), following the statistical model $xijk = m + Ai + Bj + ABij + eijk$, where $xijk$ is the measured sperm variable, m is the overall mean of variable x, Ai is the effect of media ($i = 1–2$), Bj is the effect of DMA concentration ($j = 1–2$); $ABij$ is the interaction between A and B and $eijk$ is the residual ($k = 1–10$). A *post hoc* Newman–Keuls test was performed to compare differences between extenders. Comparisons between fresh and frozen-thawed sperm variables were made using a paired t test. Data were expressed as means ± SE. All statistical calculations were made using Statistica software for Windows v.12 (StatSoft Inc., Tulsa, OK, USA).

3 | RESULTS

In roosters, the values of all frozen/thawed sperm variables, except for motility score, linearity (LIN), straightness (STR) and wobble (WOB) were lower ($p < 0.05$) than those recorded for fresh sperm when the semen was cryopreserved using the 6% DMA (Table 1). In addition, the values of all frozen/thawed sperm variables except for LIN, WOB BCF were lower ($p < 0.05$) than those recorded for fresh sperm when the semen was cryopreserved using the 4% DMA (Table 1). Frozen-thawed sperm viability, curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were greater ($p < 0.05$) using LR84-6%DMA extender than EK-6% DMA extender. There were no

TABLE 2 Capercaillie sperm quality variables of frozen-thawed samples using LR84 or EK extenders with 4% of dimethyl-acetamide (DMA) (Cantabrian capercaillie URS1) and 6% of DMA (European capercaillie URA1 and URA2)

	Frozen-thawed samples					
	URS1		URA2		URA1	
Sperm trait	LR84	EK	LR84	EK	LR84	EK
Subject motility (%)	5	5	2	0	0	0
Score	2	2	1	0	0	0
Viability (%)	7	5	15	19	3	1
TUNEL+ (%)	0	4.5	7.5	–	2.4	–
MP (%)	4.3	1.7	0	0	0	0
MT (%)	18.3	5.1	4.3	0	0	0
VCL (μm/s)	59	84.6	31.1	0	0	0
VSL (μm/s)	31.2	55	20.1	0	0	0
VAP (μm/s)	48.7	72.8	24.1	0	0	0
LIN (%)	55	65	64.6	0	0	0
STR (%)	64.1	75.5	82.3	0	0	0
WOB (%)	82.6	86.1	78.5	0	0	0
ALH (μm)	1.4	2.2	0	0	0	0
BCF (Hz)	4.3	5.2	0	0	0	0

Note: See the sperm traits of the fresh samples in Section 3.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; CPA, Cryoprotectant; LIN, linearity; MP, progressive motility; MT, total motility; STR, straightness; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; WOB, wobble.

differences for any frozen-thawed sperm variables between LR84-4% DMA extender than EK-4% DMA extender (Table 1). Two-way ANOVA revealed a significant interaction ($p < 0.05$) semen media × cryoprotectant concentration on rooster sperm variables. Comparisons between extenders with different DMA concentrations revealed that VCL was greater ($p < 0.05$) in samples frozen with LR84-4% DMA than in samples frozen with EK-6% DMA. VAP was greater ($p < 0.05$) in samples frozen with LR84-4% DMA than in samples frozen with EK-6% DMA (Table 1).

The fresh semen values from the three capercaillie samples using LR84 and EK extenders with 6% and 4% DMA as a cryoprotectant were as follows: Cantabrian male URS1: volume 23.9 μl, concentration 30.8×10^6 sperm/ml, motility 80% and motility score 3; European male URA2: volume 74.8 μl, concentration 33.5×10^6 sperm/ml, motility 75% and motility score 3.5; European male URA1: volume 30.1 μl, concentration $<5.0 \times 10^6$ sperm/ml, motility 5% and motility score 1. In the European capercaillie URA2, there were no differences between LR84-6% DMA and EK-6% DMA diluents for sperm viability, but motility kinetic variables fall to zero values with EK-6% DMA (Table 2, URA2 male). Also as expected, in the oligospermic European male URA1 post-freezing sperm variables had practically zero values (Table 2, URA1 male). On the other hand, in URS1 Cantabrian capercaillie male, the percentage of total motility and progressive motility seems to be higher in samples frozen with LR84-4% DMA. However, the kinetic variables of motility (VCL, VSL, VAP, STR and ALH) were greater than using EK extender. In addition, the percentage of DNA fragmentation observed is higher in EK (4.5%) than LR84 extender (0%) (Table 2, URS1 male).

Regarding the capercaillies semen samples frozen with LR84 extender and glycerol, the fresh samples of Cantabrian 55-C male were taken having volume of 17.7 μl, concentration of 60×10^6 sperm/ml, motility 80%, motility score 3.5, viability (E/N) 80%, intact acrosome 93%, total abnormalities 7% (1% head, 4% midpiece and 2% tail) and DNA fragmentation 28%. The fresh samples of Cantabrian 31-C male were taken having volume of 15.6 μl, concentration of 160×10^6 sperm/ml, motility 75%, motility score 3.5, viability (E/N) 77%, intact acrosome 92%, total abnormalities 29% (4% head, 7% midpiece and 18% tail) and DNA fragmentation 19%. The fresh samples of Cantabrian 70-C male were taken with volume of 8.3 μl, concentration of 90×10^6 sperm/ml, motility 70%, motility score 3, viability (E/N) 84%, intact acrosome 90% and total abnormalities 9% (2% head, 2% midpiece and 5% tail). The fresh samples of Cantabrian 61-C were taken with volume of 4.0 μl, concentration of 28×10^6 sperm/ml, motility 55%, motility score 3.5, viability (E/N) 85%, intact acrosome 97%, total abnormalities 18% (5% midpiece and 13% tail) and DNA fragmentation 22%. Only two samples (55-C and 31-C) were adequate to be frozen, using LR84-8% glycerol as a cryoprotectant. The sperm of 31-C male showed the best response to the freezing–thawing process, maintaining a subjective motility of 20% (17.9% total motility according to the CASA system) and the viability determined by means of vital staining nigrosin-eosin (67%) (Table 3). The percentage of viable sperm after freezing was greater than those observed using DMA as a permeant cryoprotectant.

TABLE 3 Cantabrian capercaillie sperm quality variables of frozen-thawed samples using LR84 extender with 8% of glycerol

	Frozen-thawed samples	
Sperm trait	**55-C**	**31-C**
Subject motility (%)	7	20
Score	1.5	3
Concentration (10^6 spz/ml)	7.2	22.6
Viability E/N (%)	44	67
TUNEL+ (%)	0	7
MP (%)	3.4	2.7
MT (%)	9.6	17.9
VCL (μm/s)	46.1	42.3
VSL (μm/s)	36.4	18.7
VAP (μm/s)	41.4	30.8
LIN (%)	79.1	44.2
STR (%)	88	60.8
WOB (%)	69.9	72.9
ALH (μm)	1.8	2.2
BCF (Hz)	1.6	6.7
Viability (%)	16	24

Note: See the sperm traits of the fresh samples in Section 3.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; CPA, Cryoprotectant; LIN, linearity; MP, progressive motility; MT, total motility; STR, straightness; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; WOB, wobble.

4 | DISCUSSION

In this study, we obtained and described the ejaculate of both Cantabrian and European capercaillie. It should be noted that semen collection in wild species is complicate due to the intractability of most species. In the present study, dorsoabdominal massage, without chemical restraint, followed by semen collection in a microhaematocrit tube was a suitable method without risks to health and welfare of capercaille.

In chicken, the results of EK media improved when DMA concentration decreased from 6% to 4%. Similarly, frozen Cantabrian capercaillie sperm (URS1 of pilot study 2.1) with a lower concentration of DMA (4%) in both EK and LR84 showed better quality after freezing than European capercaillie sperm (URA2 and URA1 of pilot study 2.1) frozen with a higher concentration of DMA (6%). This negative effect of high DMA concentration was also reported in Mediterranean native chicken (Abouelezz et al., 2015), conversely with studies in red jungle fowl (*Gallus gallus murghi*) in which 6% DMA maintained higher post-thaw quality and fertility (Rakha et al., 2017). Hence, it should be noted that the sperm of native Mediterranean breed of chicken are not very tolerant of DMA, and concentrations lower than 6% are recommended. Despite suitable results have been found in semen European capercaillie frozen with 6% DMA (Kowalczyk et al., 2012), our results recommend that the lower DMA concentration should be used in future studies with Cantabrian capercaillie. The method of freezing may also influence the results. The pellet method has been recommended when DMA is used as cryoprotectant (Tselutin et al., 1995). Indeed, first studies in capercaillie with DMA used the semen freezing in pellets (Kowalczyk et al., 2012). However, further studies by Kowalczyk and Łukaszewicz (2015) did not find differences between straw and pellet methods with 6% DMA as the cryoprotectant.

Previous findings (Abouelezz et al., 2015) showed that glycerol was more effective than DMA to cryopreserve sperm from native chicken breeds. The advantages of using glycerol as a cryoprotectant in chicken semen have also been established and recommend in the conservation of rare genetic resources (Thélie et al., 2019). In order to study if similar findings were found in capercaillie, we evaluated the effect of the glycerol (8%) in LR84 media on the freezability of Cantabrian capercaillie sperm (pilot study 2.2). The values of freezing–thawing Cantabrian capercaillie sperm variables using LR84-8 % glycerol were better than the results with DMA in both LR84 and EK media. The viability values of freezing–thawing Cantabrian capercaillie sperm of both 55-C (from 80% to 44%, fresh vs. frozen-thawed) and 31-C (from 77% to 67%, fresh vs. frozen-thawed) samples were similar to those observed in European capercaillie by Kowalczyk et al. (2012) using EK medium and 6% DMA in pellets method. In a subsequent study, using in-straw protocol and EK medium with 6% DMF, the authors reported 61% of total living cells (Kowalczyk & Łukaszewicz, 2015). Our data showed that frozen-thawed sperm quality variables of the 31-C male were better than the 55-C male. In this regard, it is well known that the sperm ability to be cryopreserved can vary between individuals. Moreover, other earlier studies in European capercaillie indicated that the quality of ejaculates depends on individual male properties and their age but not the way of male management (Kowalczyk & Łukaszewicz, 2015).

The initial quality of fresh semen sample plays a relevant role in freezing–thawing suitability. The Cantabrian capercaillie volumes were lower in comparison with previous studies in European capercaillie (Ciereszko et al., 2011; Łukaszewicz et al., 2011). Also, sperm concentrations obtained in Cantabrian capercaillie were lower than those found in European capercaillie by Kowalczyk et al. (2012) and Kowalczyk and Łukaszewicz (2015). Perhaps, the volumes and concentration of the Cantabrian capercaillie ejaculated are naturally less than what is seen in European capercaillie located in central Europe, or maybe the bottleneck of the Cantabrian population determines a strong inbreeding that finally affects the semen quality.

The high DNA damage levels in fresh Cantabrian capercaillie semen samples of the pilot study 2.2 are remarkable (from 19% in 31-C to 28% in 55-C). Regarding this, a link has been reported between sperm DNA damage and inbreeding in ungulates (Ruiz-Lopez et al., 2010). Our data also showed that semen quality from European capercaillie males of the zoological garden also showed low quantitative characteristics, which may reveal inbreeding of this captive population.

Conversely, the quality of the fresh samples for Cantabrian capercaillie (URS1 male of the pilot study 2.1 and all males of the pilot study 2.2) was similar to those observed in European capercaillie in previous studies. While we observed 55%–80% of total motility in fresh semen, Kowalczyk and Łukaszewicz (2015) observed 82% average motility and

Ciereszko et al. (2011) reported 71% motility after 2 h of short-term storage in EK extender. Fresh sperm viability, ranging between 77% and 85% in Cantabrian capercaillie, was similar to European capercaillie semen (Kowalczyk & Łukaszewicz, 2015; Kowalczyk et al., 2012; Łukaszewicz et al., 2011).

5 | CONCLUSION

In conclusion, our results suggest that sperm from both domestic and wild species had a similar response to freezing–thawing processes. Mediterranean chickens may be used as a suitable model for developing sperm freezing protocols that can be extrapolated to threatened capercaillie populations. In addition, LR84 media with glycerol was the most efficient extender to freeze capercaillie sperm native. Although the sample size of this study is small and it is necessary to carry out more studies to obtain consistent and comparable results, it should be noted that this is the first description of semen freezing in Cantabrian capercaillie for the future development of a germplasm bank in this subspecies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page have been adhered to. Animals were handled according to procedures approved by the INIA Ethics Committee (reference number ORCEEA 2016-001) and were used in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 2010/63/UE regarding the protection of animals used in scientific experiments.

AUTHOR CONTRIBUTIONS

Emma Oť Brien: Formal analysis; Writing original draft; Writing review & editing. Cristina Castaño: Methodology; Writing original draft. Adolfo Toledano: Methodology; Writing original draft. José Néstor Caamaño: Methodology. Carlos Hidalgo: Methodology. Luis Fidalgo: Methodology. Ana López-Beceiro: Methodology; Writing original draft. Milagros C Esteso: Investigation; Methodology. Ramón Balsera: Methodology. Pedro García-Casado: Resources; Writing review & editing. Ewa Łukaszewicz: Writing review & editing. J. Santiago-Moreno: Conceptualization; Funding acquisition; Investigation; Project administration; Writing original draft; Writing review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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