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Sperm functional and morphometric differences between Iberian and European ecotypes of capercaillie (*Tetrao urogallus* L.)

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ABSTRACT

The capercaillie (*Tetrao urogallus* L.) is a threatened forest bird with distinct Iberian and European ecotypes. This study examines the functional and morphometric differences between the sperm of these ecotypes. In a first experiment, semen volume, sperm concentration, motility, viability, and DNA integrity were assessed in 'Iberian' and 'European' capercaillies, both maintained at breeding centres in Spain. In a second experiment, morphometric traits were measured in Iberian capercaillies maintained at a breeding centre in Spain, and in European capercaillies also maintained at breeding centres in Spain (EmS birds) and in Poland (EmP birds). In the first experiment, the European males produced larger ejaculate volumes (39.80 ± 3.56 μL) than the Iberian males (29.68 ± 4.64 μL). However, the Iberian males returned significantly higher sperm concentrations ($501.99 \pm 83.90 \times 10^6$ spz/mL vs. $77.66 \pm 26.09 \times 10^6$ spz/mL). In the second experiment, the origin of the birds also affected ($P < 0.001$) sperm head dimensions. These were smaller in the EmP birds compared to the EmS birds ($P < 0.01$), and compared to Iberian males (always maintained in Spain) ($P < 0.001$). Within each of these groups, three sperm subpopulations were identified according to head dimensions, with differences ($P < 0.001$) between these groups in terms of the proportion of each subpopulation. This is the first comprehensive study of sperm morphometric characteristics in these capercaillie ecotypes. These results may provide critical insights into the reproductive and evolutionary strategies of capercaillies and contribute to improving the success of reproductive technologies across different ecotypes and populations.

1. Introduction

The capercaillie (*Tetrao urogallus* L.), the largest grouse species in Europe, is a ground-dwelling, sexually dimorphic bird that

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inhabits the boreal and montane forests of the Palaearctic region (Escoda et al., 2024). Though the species is not considered globally endangered and is currently classified as of Least Concern by the IUCN (IUCN, 2025), some of its localized populations, particularly in Central and Southwestern Europe, have experienced alarming declines in recent decades due to habitat fragmentation, climate change, and anthropogenic pressures. The Iberian populations, historically found in the Cantabrian Mountains and Pyrenees, are now critically endangered. The Cantabrian capercaillie population declined by over 80 % between 1970 and 2019 (Jiménez et al., 2022), while the Pyrenean population saw a 58 % decline between 2000 and 2017 (Gil et al., 2020). These population reductions have been linked to habitat loss, increased predation in fragmented forests (Quevedo et al., 2006), and misaligned breeding timing caused by climate change (Coppes et al., 2021), among other factors. In Poland, too, the capercaillie is one of the most seriously endangered grouse species; over the past half century, the population has become markedly reduced; currently there are just 480–650 individuals left (Łukaszewicz et al., 2011a).

Conservation efforts have traditionally focused on habitat restoration and protection. However, the combination of a small and declining population size, low reproductive success, and genetic bottlenecks has necessitated more direct intervention through *ex situ* strategies, such as captive breeding and assisted reproduction technologies (ARTs) (Kowalczyk et al., 2012; Suárez-Álvarez, 2016). In this context, semen collection, artificial insemination and cryopreservation have emerged as promising tools to help safeguard genetic diversity and increase reproductive success in captivity. These approaches have been successfully implemented in capercaillie populations in Poland (Łukaszewicz et al., 2011b; Kowalczyk and Łukaszewicz, 2015), using techniques originally developed for domestic birds but appropriately adapted.

Despite the increasing use of ARTs in conservation programs, much of the reproductive biology of *T. urogallus* remains poorly understood. Most existing ART protocols were developed for domestic bird species, particularly chickens (Blesbois et al., 2007) but also turkeys (Long et al., 2014) and ganders (Lukaszewicz, 2001), the reproductive physiology and semen characteristics of which differ significantly from those of wild birds. In wild species like capercaillie, semen collection is challenging (Łukaszewicz et al., 2011a) and can induce strong stress responses, with capture myopathy even leading to death. Moreover, the marked seasonality of *T. urogallus* breeding restricts semen availability to a short 7–8 weeks window each year, and semen volumes are often very limited (Ciereszko et al., 2011; Łukaszewicz et al., 2011a), reducing further the feasibility of undertaking extensive functional assessments. Sperm characterization serves multiple purposes in conservation biology. It provides insights into male reproductive fitness, informs the development of cryopreservation and artificial insemination protocols (Boes et al., 2023), and may serve as a biomarker of environmental and physiological stress (Santiago-Moreno et al., 2016).

All European populations of capercaillie belong to the same species, although genetic variation exists among them (Escoda et al., 2024). Sperm cell morphometrics, particularly those of the sperm head - which are likely driven by genetic and other components - certainly seem to influence the resistance to cryodamage that can be suffered during freezing and thawing (Esteso et al., 2006). Smaller head sizes are generally associated with greater cryoresistance due to a favourable membrane surface-to-volume ratio (Esteso et al., 2006), although findings across species are inconsistent (O'Brien et al., 2019). However, it should be emphasized that a larger head size can be disadvantageous in some contexts while conferring advantages in others. Indeed, larger head size seems associated with faster swimming of sperm and a higher capacity to reach and remain the sperm storage tubules in the female tract (Santiago-Moreno et al., 2014, 2015). Therefore, differences in the head size among capercaillie ecotypes might explain the differences noted in the effectiveness of ARTs, such as artificial insemination (Kowalczyk and Łukaszewicz, 2015; O'Brien et al., 2024), and the variable response seen to cryoprotectants (O'Brien et al., 2022), as well as highlight underlying environmental influences.

Genomic analyses have suggested the existence of two significant evolutionarily units among capercaillies, one formed by the two populations of the Iberian Peninsula and the other by the populations across the rest of Europe (Escoda et al., 2024). To date, no comprehensive comparison of sperm traits between these capercaillie ecotypes has been conducted. This study aims to fill this knowledge gap by providing a detailed morphometric and functional characterization of their spermatozoa, contributing to a more solid understanding of this species' reproductive physiology. It is hypothesized that genetic and/or environmental variation among capercaillie populations underlies differences in sperm quality (volume, concentration, motility, viability, DNA integrity) and sperm head size, which, in turn, influence the outcomes of assisted reproductive technologies (ARTs). Such knowledge could help improve reproductive management practices, assist in designing tailored ART protocols, and aid in assessing sperm performance in the face of environmental stressors.

2. Materials and methods

2.1. Animals

The birds examined in the Experiment 1 were 27 capercaillie males. These included 1) a 'European' group of 14 birds aged 1–2 years from the Valsemana Capercaillie Breeding Center (Centro de Cría del Urogallo de Valsemana [CCUV], León, Spain) (the parents of these animals belonged to different private breeders from France, Austria, The Netherlands and Belgium); and 2) an 'Iberian' group comprised of 13 males, of which seven were 3–9 years of age (all from the Centro de Cría de Sobrescobio [CCUS], Asturias, Spain) and six aged 1–2 years from the above CCUV. All birds were kept under the natural photoperiod in elevated, slatted enclosures to prevent ground contamination, with adjacent compartments allowing selective access to females. Handling was performed according to CSIC Ethics Committee regulations (reference Regional Government PROEX 044.4/22) and 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.

The Experiment 2 also involved 13 males (aged 2–6 years), all from Poland and all maintained at the Capercaillie Breeding Centre of the Forestry Wisła District (FWD), which is run in cooperation with the Wroclaw University of Environmental and Life Sciences. These males were housed with or without females, in separate, roofed spaces of 4.0 m x 7.0 m, under natural light and environmental conditions. The FWD has permission (DOPOZGIZ.6401.03.171.2011. km; issued by the General Director of Environmental Protection) for keeping, reproducing and collecting biological materials for experimental purposes at the Capercaillie Breeding Centre. The described experiment was approved by the II Local Ethics Commission for Experiments Carried out on Animals (Permit: 13/2017)

2.2. Experimental design

Experiment 1: Sperm quality differences between Iberian and European capercaillies

Quantitative (volume, concentration, total sperm count) and qualitative (motility, viability, DNA integrity) variables were assessed in the two stated groups of capercaillies, i.e., the European birds (n = 14 individuals all maintained at the CCUV [in Spain]), and Iberian birds (n = 13, all from the CCUV and CCUS [also in Spain]). At least one semen sample was recovered from each bird during the breeding season (April-May).

Experiment 2: Sperm head morphometric differences between Iberian and European capercaillies

In this experiment, three capercaillie groups were studied: 1) Iberian birds (n = 11 from the CCUS in Spain), 2) European birds (n = 11 from the CCUV in Spain [EmS birds]), and 3) European birds (n = 13) from the FWD and maintained in Poland (EmP birds). At least one semen sample was recovered from each bird during the breeding season (April-May). Sperm head morphometry was assessed for a total of 75 smears (one smear per ejaculate, with 1–3 ejaculates analyzed per animal). Samples collected in Poland were stained with the same protocol than in samples collected in Spain. Stained slides were sent to the INIA-CSIC laboratory in Spain, where all samples from this experiment were evaluated for sperm head morphometry using Motic Image Advanced v.3.0 software (see details below).

2.3. Semen collection and sperm analysis

Semen was collected during the natural breeding season using the dorsoventral massage technique described by Burrows and Quinn (1937), as adapted for capercaillies (Łukaszewicz et al., 2011b); the latter involves abdominal massage and cloacal pressure to stimulate ejaculation. Birds were placed in a padded cradle to facilitate handling. Ejaculates were collected using hematocrit capillary tubes (Brand® GMBH + Co KG, Wertheim, Germany), and the contents transferred into Eppendorf tubes pre-filled with 30 μL of LR84 medium (Lake and Ravie, 1984) composed of sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate 4H2O (0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (Mr 10 000; 0.3 g) and 100 mL H2O (final pH, 7.08; final osmolality, 343 mOsm/kg. All these chemicals were research grade chemicals purchased from Panreac Quimica SLU (Barcelona, Spain). Samples were immediately stored at 5°C in darkness until analysis.

Obtaining biological samples from wild species is extremely challenging, which underscores the need to maximize data acquisition and ensure that every collected specimen is utilized efficiently. For this reason, we decided to retain all evaluated parameters, even though in many cases it was not possible to analyse all sperm variables due to the limited amount of sample collected.

Ejaculate volume was estimated indirectly by measuring the semen column length within the hematocrit tube, using a plastic ruler (accuracy ± 1 mm), and referencing a calibration table to convert length to microlitres. Sperm concentration was determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) under phase-contrast microscopy (Nikon Eclipse 50i, Japan). Data were expressed in millions of spermatozoa per mL (spz/mL).

To assess total motility and motility quality, semen samples, slides, and coverslips were all equilibrated at 37°C. A drop of diluted semen was placed on a slide, covered with a coverslip, and examined using phase-contrast microscopy (Nikon Eclipse 50i, objective 40 ×) on a heated stage as described by O'Brien et al. (2022), with observations made in four distinct microscopic fields. Total sperm motility refers to the movement of the tail, which can occur with varying degrees of forward progression. The quality of movement in fresh samples was subjectively scored on a scale of 0–5: 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. All evaluations were performed by the same highly experienced technician. Sperm viability was assessed using eosin-nigrosin (E/N) staining (Vallverdú-Coll et al., 2016). Three microlitres of semen were mixed with 6 μL E/N on a 37°C slide, incubated for 30 s, and smeared. Slides were air-dried and examined under optical microscopy (Motic BA210, Motic Spain, S.L.U. Barcelona, Spain) at 40 × or 100 ×). Viable sperm excluded the stain, while non-viable sperm stained violet. Proportion of viable spermatozoa was recorded by counting 200 spermatozoa per slide.

Sperm DNA fragmentation was evaluated using the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. For this, the kit In Situ Cell Death Detection (Roche, Basel, Switzerland) was used following manufacturer's instructions, with minor changes (Santiago-Moreno et al., 2019). Briefly, each sperm sample was diluted to 12×10^6 spermatozoa/mL in 4 % formaldehyde. Subsequently, 10 μl of this dilution was placed on a glass slide and left to dry. The spermatozoa were permeabilised with 0.1 % of Triton X-100 in PBS. After a wash in PBS, fragmented DNA was nick end-labelled with tetramethylrhodamine-conjugated dUTP by adding 10 μl of the working solution provided by the kit, containing the substrates and the enzyme terminal transferase. The reaction was conducted incubating the slides in a humid box for 1 h at 37°C. After a wash with PBS, the nuclei were counterstained with Hoechst 33342 (excitation 350 nm, emission 460–490 nm) at 0.1 mg/mL in PBS for 5 min in the dark. After an additional wash with PBS, the slides were mounted using Fluoromount-G® (Sigma-Aldrich, MO, USA) and were observed by epifluorescence microscopy (Eclipse E200; Nikon, Japan, 400 ×). Percentages of positive TUNEL spermatozoa (excitation 520–560 nm, emission 570–620 nm) were recorded by counting a minimum of 200 spermatozoa per microscopy. As positive control for TUNEL (TUNEL +), to verify assay specificity and sensitivity, sperm were treated with DNase I, 1 mg/mL, for 60 min at room temperature, fixed on the slides and then treated with TUNEL reaction mixture.

Sperm head morphometry was assessed according to the technique described by Villaverde-Morcillo et al. (2015). Briefly, 5 µL of diluted semen were smeared, air-dried, and stained for 2 min with Hemacolor® (Merck KgaA, Darmstadt, Germany). Slides were mounted using Eukitt (Panreac Química SLU, Barcelona, Spain). Images of 25 sperm heads per slide were captured using a Motic BA210 microscope (Motic Spain, S.L.U. Barcelona, Spain) at 100 × (oil immersion) connected to a Moticam 1SP camera (1.3 MP, Motic Spain S.L, Barcelona, Spain). Measurements were performed using Motic Image Advanced v.3.0 software. The system detected the boundary of sperm heads, and their outlines were displayed as green overlays superimposed on the video image (Fig. 1). Head boundary detections were traced manually by the operator using an editing tool provided by the system. The following variables were recorded: length, width, area and perimeter.

2.4. Statistical analysis

Sperm variables were analyzed using a General Linear Mixed Model (GLMM), including the ecotype as categorical predictor, the age as continuous predictor, and the male (individual) as a random effect, following the statistical model: $yij = \beta 0 + \beta 1Age\ ij + \beta 2\ Ecotype\ ijk + bi + \varepsilon ij$, where yij is the response variable for observation j on male i, β0 is the overall intercept, β1 and β2k are fixed-effect coefficients for age and ecotype, respectively, bi is the random effect of male, and εij is the residual error. Volume data from azoospermic samples were excluded from the statistical analysis. The prevalence of azoospermia in capercaillies from the Iberian and European groups was analysed using a GLMM with a binomial distribution and a logit link function. Sperm head area values were subjected to k-means cluster analysis to identify sperm subpopulations. STATISTICA software uses Lloyd's method to implement the k-Means algorithm. The right number of clusters was determined by a v-fold cross-validation algorithm included in the STATISTICA package. The morphometric descriptors (head sperm area) for the subpopulations (Sp) were identified, and the proportion of sperm within each Sp compared between groups (Iberian, EmS, and EmP birds) using the Chi-squared test. All statistical procedures were conducted using TIBCO STATISTICA™ software (Tibco® Inc., Tulsa, OK, USA).

3. Results

3.1. Experiment 1: sperm quality variables

A total of 94 ejaculates were collected and analyzed: 40 from Iberian and 54 from European birds. Due to limited semen volumes, not all ejaculates permitted assessment of every variable (the number of valid measurements per variable is indicated in Table 1). Detailed information of the samples included are listed in Supplementary Table S.1.

Significant differences were detected between these two ecotypes with respect to ejaculate volume, sperm concentration, and total sperm count ($p < 0.05$). The European males produced larger ejaculate volumes ($p < 0.05$), but the Iberian males returned significantly higher sperm concentrations, and consequently greater total sperm counts per ejaculate ($16.46 \pm 4.18 \times 10^6$ vs. $1.77 \pm 0.69 \times 10^6$; $p < 0.01$). Thus, despite producing lower volumes, Iberian males generated significantly more spermatozoa per unit volume of semen.

No significant differences were observed between the ecotypes in sperm motility variables or sperm viability (Table 1). GLMM results likewise showed no significant differences in sperm DNA integrity between ecotypes.

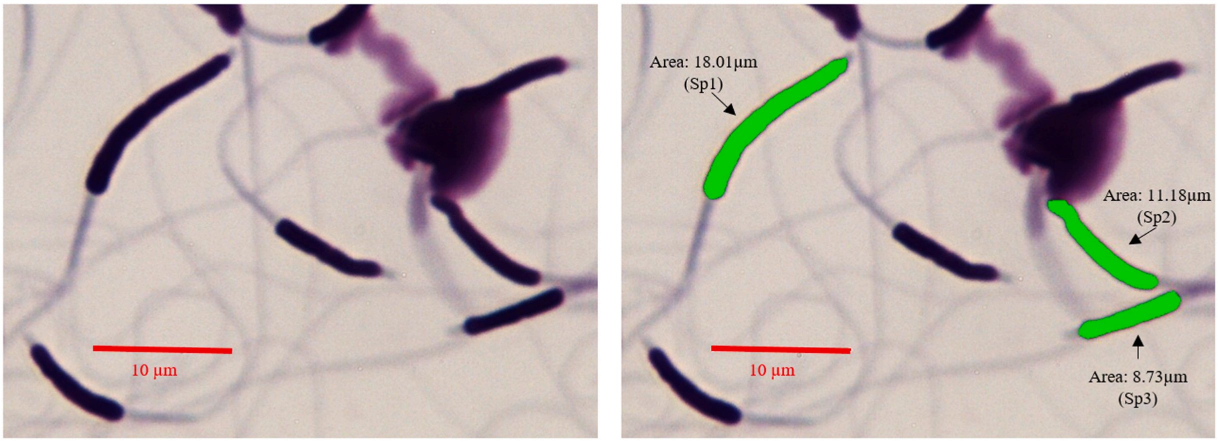

Fig. 1. Image of capercaillie sperm stained with Hemacolor® and measured using computerised morphometric analysis: the outlines and area of the sperm heads were displayed as green overlays superimposed on the video image (x1000). Capercaillie spermatozoa were classified into three distinct subpopulations based on head area: Sp1 (large), Sp2 (medium), and Sp3 (small). (x1000).

Table 1
Comparison of sperm variables between Iberian and European capercaillies. Effect sizes are shown with their 95 % confidence intervals (CI).

Sperm quality	Iberian ($\bar{x} \pm$ SEM)	European ($\bar{x} \pm$ SEM)	95 % CI
Concentration (10^6 spz/mL)	501.99 ± 83.90^a (n = 36)	77.66 ± 26.09^b (n = 26)	0.03 – 0.65
Volume (µl)	29.68 ± 4.64^b (n = 41)	39.80 ± 3.56^a (n = 60)	(-0.48) – (-0.04)
Viability (%)	84.47 ± 2.82 (n = 40)	86.42 ± 2.94 (n = 33)	(-038) – 0.16
Motility (%)	52.57 ± 6.26 (n = 35)	52.95 ± 4.42 (n = 53)	(-0.04) – 0.42
Score (1−5)	2.65 ± 0.23 (n = 33)	2.78 ± 0.13 (n = 53)	(-0.12) – 0.33
DNA integrity (%)	91.65 ± 1.98 (n = 35)	79.00 ± 4.83 (n = 11)	(-0.17) – 0.87

Different superscripts (a,b) indicate significant differences ($p < 0.05$).

Azoospermia (Table 2) was greater in European ejaculates (37.7 %) than in Iberian ejaculates (24.1 %).

3.2. Experiment 2: sperm head morphometrics

The origin of the birds (Iberian ecotype, EmS birds, and EmP birds) had a significant influence ($p < 0.001$) on sperm head length, perimeter and area (Table 3). The length of the sperm heads in the EmP birds was smaller ($p < 0.001$) than that of the EmS birds. No differences in sperm head width was seen among these three groups. The perimeter was smaller in the EmP birds compared to the EmS birds ($p < 0.001$), and compared to the Iberian ecotype ($p < 0.001$). The perimeter was smaller in the Iberian capercaillies ($p < 0.05$) than in the EmS birds. The area was smaller in the EmP birds compared to the EmS birds ($p < 0.01$), and compared to the Iberian ecotype ($p < 0.001$).

Three subpopulations (Sp) according to sperm head area (Fig. 1) were identified in ejaculates from each capercaillie group: Sp1, large heads; Sp2, medium heads; Sp3, small heads. The smallest ($p < 0.001$) Sp1 subpopulation was observed in the EmP birds, and the largest in the EmS birds. The largest ($p < 0.001$) Sp2 was observed in the EmP birds, and the smallest ($p < 0.001$) in the EmS birds. No differences were found among groups in terms of the proportion of Sp3 sperms (Table 4).

4. Discussion

This study is the first comprehensive comparative analysis of sperm morphometric and functional traits in the two capercaillie evolutionary ecotypes (Iberian and European). Differences in sperm characteristics were seen between them, but also among groups of the same ecotype (European) maintained in different areas (Spain and Poland). This suggests that, along with the influence of the ecotype itself, other environmental factors may exert an influence on sperm head dimensions and subpopulations. These ecotypes may be the result of evolutionary influences at work over long periods (Escoda et al., 2024).

In Experiment 1, the European capercaillies in Spain showed significantly larger ejaculate volumes, while the Iberian birds showed markedly higher sperm concentrations and total sperm counts. These differences are likely attributable to the age structure of the study populations. The European males were all between 1 and 2 years old, whereas the Iberian group included older individuals (up to 9 years of age), likely with more fully developed reproductive systems. Previous studies on capercaillies and other avian species support the idea that sperm production improves with age and sexual maturity. For instance, Łukaszewicz et al. (2011a) reported lower sperm concentrations in younger European males compared to older individuals, while in red jungle fowl (*Gallus gallus murghi*), peak sperm output was achieved at around four years of age (Rakha et al., 2017). Therefore, the reduced sperm production observed in European males may reflect the physiological limitations of reproductive immaturity.

Unlike functional aspects of sperm quality (e.g., volume, concentration), morphometric traits appeared unaffected by age. Indeed, no differences were found in sperm head area between adult birds from the Iberian group (3–9 years of age) and the European group maintained in Spain, which also included some 1-year-old individuals. Although a study in another species (e.g., pig) reported that sperm head area was larger in older than in younger boars (Kondracki et al., 2005), the wide age range observed between Iberian and European capercaillies kept in Spain was not associated with differences in sperm head size. Thus, the variations observed in our study are likely attributable to other factors. Certainly, morphometric sperm characteristics may be regulated genetically (Maroto-Morales et al., 2012). For instance, the influence of a genetic component has been described in red legged partridges (*Alectoris rufa*), in which pure animals have sperm head dimensions smaller than those of individuals with genetic introgression from the chuckar partridge

Table 2
Analysis of the prevalence of azoospermia in capercaillies from the Iberian and European groups.

	Odds Ratio	95 % CI	*p*
Ecotype (Iberian: European)	0.18	0.07–0.47	0.0004

Table 3
Mean length, width, area and perimeter of sperm heads in semen samples from birds of the Iberian ecotype, of the European ecotype maintained in Spain (EmS), and of the European ecotype maintained in Poland (EmP).

Ecotype	Length (μm)	Width (μm)	Perimeter (μm)	Area (μm^2)
Iberian	$10.62 \pm 0.22_{AB}$	1.06 ± 0.05	$21.97 \pm 0.36_{ABb}$	$11.67 \pm 0.46_{A}$
European maintained in Spain (EmS)	$11.17 \pm 0.23_{A}$	0.95 ± 0.03	$23.48 \pm 0.46_{ABa}$	$11.37 \pm 0.28_{x}$
European maintained in Poland (EmP)	$10.19 \pm 0.10_{B}$	0.99 ± 0.01	$20.05 \pm 0.25_{C}$	$9.84 \pm 0.13_{By}$

Different superscripts indicate significant differences (a,b: $p < 0.05$, x,y: $p < 0.01$; A,B,C: $p < 0.001$).

Table 4
Sperm subpopulations in capercaillie ejaculates according to sperm head area (μ^2, mean ± SD) in semen samples from the Iberian ecotype, the European ecotype maintained in Spain (EmS), and the European ecotype maintained in Poland (EmP). Proportions of sperm head subpopulations (Sp1: large heads; Sp2: medium heads; Sp3: small heads) are shown in parentheses.

Ecotype	Sp1	Sp2	Sp3
Iberian	14.98 ± 1.65 μ^2 (27.10 %b)	11.71 ± 0.85 μ^2 (34.49 %b)	9.00 ± 0.73 μ^2 (38.39 %a)
European maintained in Spain (EmS)	13.74 ± 0.56 μ^2 (42.66 %a)	11.40 ± 1.24 μ^2 (21.16 %c)	9.75 ± 0.49 μ^2 (36.17 %a)
European maintained in Poland (EmP)	17.44 ± 4.33 μ^2 (0.77 %c)	10.32 ± 0.58 μ^2 (59.57 %a)	8.99 ± 0.50 μ^2 (39.64 %a)

Different letters indicate differences ($p < 0.001$) with respect to sperm head subpopulation proportions.

(*Alectoris rufa* x *Alectoris chukar*) (Santiago-Moreno et al., 2015). Inbreeding among the present European capercaillies should not be ruled out as influencing sperm head dimensions since the parents of these birds are held by private breeders from different areas, in which the selection of individuals that best adapt to captivity conditions may have occurred. Interestingly, the Iberian ecotype showed a great proportion of sperm with DNA integrity (about 92 %), despite the inbreeding levels in some isolated population in the Cantabrian mountains of Spain (Escoda et al., 2024). From a conservation standpoint, these findings have several important implications. First, the high sperm output and high DNA integrity of the Iberian males highlight their potential reproductive value for both *in situ* and *ex situ* breeding programs. The low proportion of DNA damage in these birds supports the assumption that some Iberian subpopulations may maintain adequate levels of heterozygosis (Escoda et al., 2024), reflected as adequate semen quality.

This study examined the same European ecotype under two contrasting environments (Spain and Poland). The differences in sperm head dimensions seen within the same ecotype suggest that other environmental factors might influence spermatogenesis, leading to changes in the proportion of sperm with different head dimensions. Many pesticides can disturb different stages of spermatogenesis and, thus, the final size of avian sperm cells. High concentrations of oestrogenic chemical residues in water, sediment and tissues impair spermatogenesis in humans and certain wild animals (Riana Bornman, Bouwman, 2012), and some pesticides may exert harmful effects on sperm chromatin condensation (Sánchez-Peña et al., 2004) and, in turn, on sperm head size. Even residues of organochlorines (e.g., DDT) can still be detected in capercaillies (including chicks) (Santiago-Moreno et al., 2025) (although they may theoretically have no reproductive effects). Factors with influence on spermatogenesis may also influence sperm head dimension, for instance on the formation of diploid or polyploid spermatids (Carothers and Beatfy, 1975; Eastmond et al., 1997). Finally, climatic conditions (including global warming) may impair spermatogenic activity (Santiago-Moreno et al., 2016; Narayan, 2015; Kanter et al., 2013), suggesting that the results seen for the EmS birds may be the outcome of them not being accustomed to warmer, more Mediterranean conditions.

In comparison with other species, the capercaillie has a medium sperm head size (about 11 μm^2). This is larger than that recorded for the European peregrine falcon (*Falco peregrinus peregrinus,* 8.5 μm^2) but smaller than that reported for chickens (13.9 μm^2) and red legged partridges (14.7 μm^2) (Santiago-Moreno et al., 2015; Villaverde-Morcillo et al., 2015; Villaverde-Morcillo et al., 2017). Sexual strategies such as polygyny and monogamy also influence the morphological and functional characteristics of sperm. In this regard, sperm size is usually related to sperm competence (Santiago-Moreno et al., 2014, 2016). The capercaillie and other polyandrous bird species usually exhibit sperm head dimensions larger that those recorded for the spermatozoa of monogamous species (e.g., falcons, eagles) (Santiago-Moreno et al., 2016).

Morphometric traits influence the success of ARTs. The ability of sperm to withstand the freeze/thaw cycle may, in part, be related to sperm head dimensions. Sperm head size influences water flow across the plasma membrane during the cryopreservation process, and in turn affects cell survival rates. Thus, sperms with smaller heads should better survive freezing (Gravance et al., 1998; Esteso et al., 2006). Ejaculates with a large subpopulation of sperms with large heads (e.g., the Iberian or EmS birds) might show poorer cryosurvival than those with a smaller of sperm with large heads (e.g., the EmP birds). Differences in sperm characteristics after freeze-thawing seem to support this idea (Kowalczyk and Łukaszewicz, 2015; O'Brien et al., 2022). For instance, using a similar EK extender, sperm viability (61 % vs 28 %) and motility (46 % vs 5 %) was greater after freezing-thawing in European capercaillies from Poland compared to these from Spain.

In summary, the sperm characteristics of the capercaillie reflect both ecotype and age, although potential environmental influences cannot be excluded. Three sperm subpopulations according to sperm head dimensions were identified, with variable proportions of them. Sperm morphometric and functional characteristics may provide key information for improving our understanding of reproductive and evolutionary strategies and reproductive technologies in this species. These findings provide may help achieve greater success with ARTs in different capercaillie ecotypes and populations.

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CRediT authorship contribution statement

J. Gómez-Delgado: Writing – original draft, Methodology, Formal analysis. **B. Martínez-Madrid:** Methodology, Investigation. **A. Toledano-Díaz:** Writing – review & editing, Methodology, Investigation. **C. Castaño:** Methodology. **A. Gómez-Crespo:** Methodology. **G. de Pedro Aguilar:** Resources, Methodology. **D. Cubero:** Visualization, Resources. **A. Kowalczyk:** Methodology, Investigation. **E. Łukaszewicz:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **J. Santiago-Moreno:** Writing – review & editing, Validation, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing interest

None of the authors have any conflict of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.108071.

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