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RESEARCH ARTICLE

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A comparative study on semen quality and cryopreservation ability in Italian native chicken breeds

Michele Di Iorio[a], Stefano Paolo Marelli[b], Emanuele Antenucci[a], Manuela Madeddu[b], Luisa Zaniboni[b], Silvia Belcredito[b], Giusy Rusco[a], Achille Schiavone[c], Dominga Soglia[c], Mauro Penasa[d], Cesare Castellini[e], Arianna Buccioni[f], Margherita Marzoni[g], Lucia Maiuro[a], Nicolaia Iaffaldano[a] and Silvia Cerolini[b]

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ABSTRACT

This study provides a comparative assessment of fresh semen quality and sensitivity to cryopreservation in several Italian chicken breeds. The research involved 145 roosters from 13 breeds. The results showed a wide variability in all the parameters considered among the different breeds, especially in the quantitative variables of fresh semen, such as volume and concentration. For the qualitative characteristics (sperm membrane integrity and motility parameters), the variability across breeds was more pronounced for frozen than fresh semen. Interestingly, apart from total motility in fresh semen, breed had a significant effect on all semen quality parameters in both fresh and thawed ejaculates. Considering the overall qualitative characteristics, the *Robusta maculata*, *Siciliana*, and *Mericanel della Brianza* breeds produced ejaculates with better semen quality compared to other Italian breeds. By evaluating the main parameters of semen quality, our results underline the potential of these traits to influence the reproductive success and genetic conservation. The *Bionda piemontese*, *Bianca di Saluzzo*, *Livorno bianca*, *Pepoi*, and *Siciliana* breeds showed better resilience to cryopreservation, suggesting the need for breed-specific protocols to optimise semen quality after thawing. Importantly, the research highlights the central role of semen quality for both immediate fertilisation success and long-term conservation efforts. Future studies integrating OMICS technologies could elucidate molecular markers influencing breed-specific differences, helping to refine cryopreservation techniques and improve conservation strategies for indigenous Italian chicken breeds. This work contributes valuable insights to global efforts aimed at safeguarding poultry genetic diversity and sustainability.

HIGHLIGHTS

- Significant differences in semen quality were observed among Italian chicken breeds, affecting both fresh and cryopreserved samples.
- Semen quality declined significantly after cryopreservation; however, some breeds demonstrated greater resilience to the freezing and thawing process.
- Knowledge of fresh semen quality helps select males with high fertilising ability, improving reproductive efficiency and genetic diversity.

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Italian chicken breeds; fresh semen quality; cryopreservation sensitivity; breed-specific differences; reproductive success

Introduction

The Italian poultry biodiversity once encompassed a wide array of breeds (90 in total) belonging to different species, spread across various regions of the country, although a significant portion (61%) was recognised extinct in 2001 (Zanon and Sabbioni 2001). Presently, the Italian Herd Book (IHB) lists 22 native chicken breeds still present in rural and fancy farms;

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however, population sizes are limited, and only five breeds are considered not at risk according to a recent survey (Castillo et al. 2021). The decline in poultry biodiversity in Italy is attributed to the rise of intensive farming practices that has been happening since the 1970s and the widespread rearing of highly productive commercial strains (Tallentire et al. 2016; Hartcher and Lum 2020). Consequently, population size of local chicken breeds has significantly decreased, making them vulnerable to inbreeding and reducing genetic diversity within breeds (Castillo et al. 2021; Iaffaldano et al. 2021; Soglia et al. 2021).

In recent years, several projects have begun to promote conservation and valorisation of Italian native poultry breeds, focusing on preserving genetic diversity, cultural significance, and historical value. Moreover, there has been an increasing interest in local breeds and traditional products in many developed countries over the last decade (Franzoni et al. 2021). This trend is driven by the perception that the farming system associated with local breeds, which is often free range with low inputs, is more respectful of animal welfare and environment compared to the intensive farming practices (Soglia et al. 2017, 2020).

The national project 'Conservation of Biodiversity in Italian Poultry Breeds—TuBAvI' (FEASR/MASAF PSRN 2017–2024) is notable among the projects focused on safeguarding poultry breeds. Since 2017, the TuBAvI project has been dedicated to safeguarding, conserving, and valorising the Italian avian genetic heritage by implementing both *in situ* and *ex situ* techniques. One of the major activities of the TuBAvI project is the building up of knowledge on phenotypic traits of existing breeds to address current knowledge gaps and enhance the economic prospects of breed farming. Among a wide range of traits, semen production, and quality have been considered (Pollitaliani n.d.). Assessing semen production and quality in Italian autochthonous breeds is a significant research activity aimed at improving breeder management and reproductive efficiency in conservation plans. Within *in situ* technique such studies are crucial for understanding the fertility and reproductive characteristics of male breeders, as well as identifying any critical issues, thus improving the management of breeders. The *in situ* technique is recognised to be the priority in conservation programs, and the advancement of the complementary *ex situ in vitro* technique is continuously growing. The *ex situ in vitro* technique involves the cryopreservation of genetic material in haploid form (semen and oocytes), diploid (embryos, somatic cells), or DNA sequences (FAO 2012; Mara et al. 2013; Iaffaldano et al. 2021). The establishment of genetic resource cryobanks would serve as a vital link connecting these two techniques, enhancing the effectiveness of conservation programs (Prentice and Anzar 2010; Iaffaldano et al. 2021). In birds, semen cryopreservation is still the most feasible reproductive technology for long term storage of genetic resources (Long 2006; Blesbois 2011; Ehling et al. 2012; Iaffaldano et al. 2021; Sun et al. 2022). Understanding the variability between and within breeds to this biotechnology is crucial for the development of effective conservation and breeding strategies. Accordingly, potential differences among breeds might have implications for the optimising of cryopreservation protocols.

In this context, the present study was carried out with a 2-fold objective: (a) to offer a comprehensive assessment of quantitative and qualitative production of fresh semen in different Italian chicken breeds to provide valuable insights into the inherent reproductive capabilities of male breeders; (b) to assess the sensitivity of semen to the cryopreservation process and evaluate the potential variability among breeds.

Materials and methods

Male breeders' management and semen collection

The study involved 145 roosters belonging to 13 breeds: Ancona (ANC; $n = 7$), Bionda piemontese (BPM; $n = 13$), Bianca di Saluzzo (BSL; $n = 11$), Livorno argento (LVA; $n = 9$), Livorno bianca (LVB; $n = 21$), Livorno nera (LVN; $n = 14$), Livorno collo oro (LVO; $n = 6$), Mericanel della Brianza (MBZ; $n = 8$), Mugellese (MUG; $n = 5$), Pepoi (PEP; $n = 15$), Robusta maculata (RBM; $n = 13$), Siciliana (SIC; $n = 21$), and Valdarnese bianca (VLB; $n = 2$). The birds were raised following standard guidelines for chicken breeders. All roosters were provided unrestricted access to a standard commercial breeder diet (15% CP, 2800 kcal ME/kg) and drinking water. Individual body weight was recorded. The study was conducted during the period March–July 2022. The roosters used in the study were aged between 8 and 11 months.

Semen collection was routinely performed twice weekly using the consolidated technique of the abdominal massage proposed by Burrows and Quinn (1935). Before starting the collection process, donors underwent a training period lasting between 2 and 4 weeks to ensure proficiency in the precise execution of the abdominal massage technique. Before semen collection, birds were fasted to avoid the risk of faeces contamination. Ejaculates were collected into graduated tubes and a preliminary macroscopic

assessment was carried out to estimate semen quality. Ejaculates exhibiting a uniform, homogeneous, white, and opalescent appearance, and displaying high viscosity were selectively retained for subsequent in-depth analyses and further processing *in vitro*. It is worth noting that all bird handling procedures and semen collection methods adhered strictly to the ethical standards outlined in the EU Directive 2010/63/EU, underscoring the utmost consideration given to animal welfare throughout the entire study.

Phenotypic characterisation of fresh semen

The quality of the ejaculates was assessed immediately after collection by measuring several parameters, including volume (VO), concentration (CO), total sperm output (TSO), sperm membrane integrity (SMI), and different motility parameters. The VO (mL) of each ejaculate was carefully measured using a calibrated micropipette, ensuring accurate quantification. Sperm CO ($\times 10^9$/mL) was determined by a photometric approach. After dilution in the ratio of 1:200 with a 0.9% NaCl solution, the diluted sample was subjected to measurement using a calibrated photometer (IMV, L'Aigle, France) set at a specific wavelength of 535 nm (Brillard and McDaniel 1985; Iaffaldano et al. 2021). TSO ($\times 10^9$) was calculated as VO*CO.

The assessment of SMI was carried out using the Muse® Cell Analyser (Luminex Corporation, Austin, TX, USA). The evaluation followed the protocol which was provided by the manufacturer. Semen samples were diluted in phosphate-buffered saline (PBS) to achieve a concentration ranging from 1×10^5 to 1×10^7 spermatozoa/mL. Subsequently, a 20 µL aliquot was mixed with 780 µL of Muse Count & Viability Kit® (dilution factor of 1:40). Following the incubation period of 5 min, the sperm suspension was subjected to flow cytometry analysis. The acquired data were processed and presented using the accompanying software module. Two dot plots were generated:

1. nucleated cells plot: this plot facilitated the identification of cells with a nucleus, distinguishing them from debris and non-nucleated cells;
2. viability plot: a DNA-binding dye was employed to stain cells that had lost their membrane integrity. This dye was able to penetrate the nucleus of dead or dying cells. The viability parameter discriminated between viable cells (which remained unstained) and non-viable cells (including dead or dying cells that exhibited staining).

Sperm motility and motion kinetic parameters were measured with a computer-aided sperm analysis system linked to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyser (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh semen samples were extended with 0.9% NaCl solution to reach a sperm concentration of 50×10^6 sperm/mL and incubated for 20 min at room temperature. Then, 10 µL semen were placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature.

The following motility parameters were assayed: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), and wobble (WOB, %). In each semen sample, a minimum of three microscopic fields and 500 sperm tracks were examined at 100× magnification. The setting used is specifically for chicken semen, provided by the SCA software, and the configurations used were the following: range cell size from 5 to 190 µm^2; frame rate (fps) = 25; motile (VCL) ≥ 13 µm/s; static (VCL) < 13 µm/s; rapid (VCL) > 100 µm/s, progressive (STR) ≥ 70, connectivity (pixels) = 18.

Cryopreservation procedure and post-thaw semen quality

Ejaculates were processed for cryopreservation according to the protocol previously developed (Mosca et al. 2016; Iaffaldano et al. 2021). Briefly, the semen samples were diluted with Modified Pre-Freezing Lake (MFL) diluent to 1.5×10^9 sperm/mL concentration and cooled at 4 °C for 20 min. Then, semen was further diluted to a concentration of 1.0×10^9 sperm/mL using MFL diluent supplemented with N-Methylacetamide (NM) at a final concentration of 2%, cooled at 4 °C for 1 min (equilibration phase), packaged into straws (0.25 mL) and frozen by exposure 3 cm above liquid nitrogen bath for 10 min; finally, straws were plunged into liquid nitrogen (–196 °C) and stored in liquid nitrogen cryotank. The straws were thawed by immersion for 100 s in a thermostatically water bath set to 5 °C. Sperm motility parameters and SMI were assessed after thawing as previously described in fresh semen. At least six straws from three different males were thawed in each breed. The VLB breed was excluded from the post-thawing quality analyses due to an insufficient number of semen doses available for evaluation. The

recovery rates (%) of SMI, TM, and PM after cryopreservation were calculated as [(mean on thawed semen × 100)/mean on fresh semen].

Statistical analysis

Descriptive statistics were calculated for all semen variables assessed in both fresh and frozen/thawed ejaculates. A one-way analysis of variance (ANOVA) was conducted on body weight, sperm variables in fresh and post-thawed semen, and in recovery rates, using the statistical software SPSS (IBM SPSS Statistics 23.0 for Windows, 2020; SPSS, Chicago, IL, USA), considering the breed as source of variation. Duncan's multiple comparison test was employed to compare the least squares means, with significance set at $p < 0.05$. Pearson's correlation coefficients (Pcc) between body weight and different semen variables in fresh and frozen/thawed semen were also assessed. Significance was set at $p < 0.05$.

Two principal component analysis (PCA; variance-covariance matrix; Past 4 software) were carried out including all the analysed quantitative and qualitative parameters as variables in fresh and thawed semen based on breed specific distribution. Two different scatterplots were produced. Screeplot test was used to select principal components to explain the majority of the variation in the datasets.

Results

Body weight

The result of ANOVA showed that the breed affected the body weight of roosters (Table 1; $p < 0.05$). Body weight was not available for VLB and MUG breeds. The mean values are in accordance with the breed standards of the IHB (www.anci-aia.it/il-libro-genealogico-delle-razze-avicole-autoctone/). MBZ is a bantam breed and, as expected, has a body weight, of about 1 kg, which was significantly lower compared to the other breeds. The mean body weight significantly increased to nearly 2 kg in the ANC, LVA, LVB, and PEP breeds. Furthermore, a progressive increase in body weight was observed, with LVN weighing 2.4 kg, LVO 2.5 kg, BSL 3.0 kg, and BPM 3.3 kg. Among all the breeds studied, RBM exhibited the highest significant body weight.

Table 1. Mean body weight ± *SE* (kg) of roosters from Italian breeds used for semen collection.

Breed	*n* of donors	Mean ± *SE*, kg
Ancona	7	2.06 ± 0.07^{e}
Bionda piemontese	13	3.28 ± 0.11^{b}
Bianca di Saluzzo	11	2.96 ± 0.53^{c}
Livorno argento	9	1.93 ± 0.19^{ef}
Livorno bianca	21	1.91 ± 0.64^{ef}
Livorno nera	14	2.40 ± 0.10^{d}
Livorno collo oro	6	2.51 ± 0.09^{d}
Mericanel della Brianza	8	1.05 ± 0.05^{g}
Pepoi	15	1.96 ± 0.05^{ef}
Robusta maculata	13	4.23 ± 0.11^{a}
Siciliana	21	1.74 ± 0.04^{f}

Number of donors is also reported.
[a–g]Different superscript letters indicate significant differences among breeds.

Fresh semen quality: descriptive statistics, analysis of variance, and correlations

Table 2 presents the descriptive statistics for the semen variables recorded in fresh ejaculates. The greatest variability was observed in the quantitative semen parameters, particularly for VO (CV = 61%) and TSO (CV = 92%). In contrast, the lowest variability was noted for SMI (CV = 8%) and the kinetic parameter WOB (CV = 9%). Low variability was also present in TM (CV = 13%) whereas a consistent variability (14–34%) was found in PM and most motion quality parameters.

The results of ANOVA showed that all sperm variables measured in fresh ejaculates, except for TM, were significantly affected by the breed ($p < 0.01$). Mean values of VO, CO, TSO, SMI, TM, and PM per breed are reported in Table 3. The PEP, BPM, and BSL exhibited the highest VO, whereas all other breeds showed significantly lower and similar mean values, ranging from 0.15 mL in the LVB to 0.11 mL in the MBZ breed. Sperm CO was significantly higher in the PEP breed (4.28×10^9/mL) compared to all other breeds. The lowest mean values were

Table 2. Descriptive statistics of semen variables measured in fresh ejaculates of Italian chicken breeds.

Parameters	*n*	Mean	Minimum	Maximum	*SE*	CV, %
VO, mL	144	0.18	0.03	0.61	0.01	60.73
CO, $\times 10^9$/mL	144	2.71	0.42	5.37	0.08	36.47
TSO, $\times 10^9$	143	0.53	0.03	2.95	0.04	92.39
SMI, %	142	89.00	43.50	98.50	0.64	8.55
TM, %	145	83.48	10.10	99.75	0.93	13.40
PM, %	145	26.40	0.50	50.10	0.74	33.97
VCL, µm/s	145	68.38	24.31	128.69	1.42	24.97
VSL, µm/s	145	28.56	7.77	63.11	0.70	29.68
VAP, µm/s	145	42.96	7.62	76.42	1.01	28.18
LIN, %	145	41.80	24.06	70.66	0.64	18.33
STR, %	145	63.58	30.50	93.19	0.72	13.64
WOB, %	145	63.39	49.46	84.03	0.47	8.87
ALH, µm	145	3.33	1.65	5.37	0.05	19.60
BCF, Hz	145	6.60	2.17	9.42	0.09	16.49

VO: volume; CO: concentration; TSO: total sperm output; SMI: sperm membrane integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN (VSL/VCL × 100): linearity; STR (VSL/VAP × 100): straightness; WOB (VAP/VCL × 100): wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

Table 3. Mean ± *SE* and *p*-values of quality parameters measured in fresh ejaculates of different Italian chicken breeds.

	Semen parameters					
Breed	VO, mL	CO, $\times 10^9$/mL	TSO, $\times 10^9$	SMI, %	TM, %	PM, %
ANC	0.13 ± 0.01^{c}	1.80 ± 0.10^{de}	0.23 ± 0.02^{c}	86.98 ± 1.04^{c}	80.93 ± 2.45	$25.10 \pm 3.54^{a–d}$
BPM	0.28 ± 0.05^{a}	2.55 ± 0.30^{bcd}	0.78 ± 0.17^{b}	84.90 ± 2.78^{cd}	83.00 ± 3.12	20.75 ± 1.82^{cd}
BSL	0.25 ± 0.04^{ab}	2.46 ± 0.41^{bcd}	0.76 ± 0.21^{b}	79.25 ± 4.80^{d}	77.48 ± 8.74	17.41 ± 3.13^{d}
LVA	0.15 ± 0.02^{c}	2.49 ± 0.08^{bcd}	0.36 ± 0.05^{bc}	88.83 ± 1.68^{abc}	78.63 ± 1.65	$24.99 \pm 1.40^{a–d}$
LVB	0.15 ± 0.01^{c}	2.68 ± 0.14^{bc}	0.42 ± 0.06^{bc}	87.67 ± 1.12^{bc}	82.25 ± 1.30	30.66 ± 1.57^{ab}
LVN	0.14 ± 0.02^{c}	2.76 ± 0.16^{bc}	0.42 ± 0.06^{bc}	88.01 ± 1.92^{bc}	80.64 ± 2.07	28.47 ± 2.31^{abc}
LVO	0.13 ± 0.02^{c}	2.59 ± 0.17^{bcd}	0.34 ± 0.05^{bc}	86.64 ± 2.91^{c}	84.15 ± 2.43	26.04 ± 3.10^{abc}
MBZ	0.11 ± 0.01^{c}	2.48 ± 0.28^{bcd}	0.27 ± 0.03^{c}	96.28 ± 0.77^{a}	92.98 ± 2.65	30.39 ± 3.32^{ab}
MUG	0.12 ± 0.01^{c}	2.04 ± 0.17^{cde}	0.24 ± 0.01^{c}	86.20 ± 1.83^{cd}	76.48 ± 2.57	21.86 ± 7.06^{bcd}
PEP	0.31 ± 0.03^{a}	4.28 ± 0.20^{a}	1.35 ± 0.18^{a}	92.65 ± 0.81^{abc}	83.75 ± 2.84	21.73 ± 2.18^{bcd}
RBM	0.12 ± 0.02^{c}	1.52 ± 0.17^{e}	0.17 ± 0.03^{c}	95.31 ± 0.71^{ab}	87.55 ± 2.16	32.62 ± 2.05^{a}
SIC	0.15 ± 0.01^{c}	3.28 ± 0.10^{b}	0.50 ± 0.04^{bc}	91.26 ± 0.65^{abc}	88.28 ± 0.88	29.74 ± 1.11^{abc}
VLB	0.16 ± 0.01^{bc}	2.63 ± 0.23^{bcd}	0.43 ± 0.01^{bc}	90.36 ± 3.26^{abc}	81.62 ± 10.67	20.30 ± 7.53^{cd}
p-Value	0.0001	0.0001	0.0001	0.0001	0.1320	0.0001

VO: volume; CO: concentration; TSO: total sperm output; SMI: sperm membrane integrity; TM: total motility; PM: progressive motility; ANC: Ancona; BPM: Bionda piemontese; BSL: Bianca di Saluzzo; LVA: Livorno argento; LVB: Livorno bianca; LVN: Livorno nera; LVO: Livorno collo oro; MBZ: Mericanel della Brianza; MUG: Mugellese; PEP: Pepoi; RBM: Robusta maculata; SIC: Siciliana; VLB: Valdarnese bianca.
[a–e]Different superscripts indicate significant differences among breeds.

Table 4. Mean ± *SE* and *p*-values of sperm kinetic parameters in fresh ejaculates of different Italian chicken breeds.

	Semen parameters							
Breed	VCL, μm/s	VSL, μm/s	VAP, μm/s	LIN, %	STR, %	WOB, %	ALH, μm	BCF, Hz
ANC	$64.14 \pm 6.44^{a–e}$	25.11 ± 2.94^{de}	40.74 ± 3.97^{bc}	38.50 ± 2.16^{cde}	59.30 ± 2.59^{cd}	64.35 ± 1.27^{ab}	3.15 ± 0.31^{bc}	6.22 ± 0.41^{bcd}
BPM	$63.72 \pm 4.85^{a–e}$	25.47 ± 1.68^{de}	39.92 ± 2.87^{bc}	41.03 ± 1.57^{bcd}	$64.54 \pm 1.65^{a–d}$	63.17 ± 0.85^{ab}	3.59 ± 0.16^{abc}	7.19 ± 0.30^{abc}
BSL	$62.90 \pm 6.87^{b–e}$	23.07 ± 2.53^{de}	38.40 ± 4.33^{c}	37.09 ± 1.52^{cde}	$60.71 \pm 1.58^{a–d}$	60.78 ± 1.12^{bcd}	3.54 ± 0.22^{abc}	6.78 ± 0.18^{abc}
LVA	72.05 ± 4.22^{abc}	25.49 ± 2.22^{de}	41.13 ± 2.47^{bc}	35.66 ± 3.16^{de}	$61.30 \pm 3.36^{a–d}$	57.22 ± 1.89^{cd}	3.64 ± 0.14^{ab}	6.13 ± 0.32^{cd}
LVB	72.71 ± 3.58^{abc}	$30.70 \pm 1.75^{a–d}$	46.02 ± 2.53^{abc}	41.38 ± 1.23^{bcd}	66.94 ± 1.59^{abc}	62.67 ± 0.82^{abc}	3.31 ± 0.13^{bc}	6.53 ± 0.21^{bcd}
LVN	$68.98 \pm 1.75^{a–d}$	$30.36 \pm 1.36^{a–d}$	44.68 ± 1.39^{abc}	44.16 ± 2.41^{bc}	$65.60 \pm 2.22^{a–d}$	65.37 ± 2.27^{ab}	3.08 ± 0.14^{bc}	6.72 ± 0.31^{abc}
LVO	73.08 ± 5.03^{abc}	24.54 ± 2.69^{de}	41.78 ± 3.95^{bc}	32.64 ± 1.54^{e}	57.84 ± 1.28^{d}	56.61 ± 1.47^{d}	4.05 ± 0.28^{a}	5.57 ± 0.33^{d}
MBZ	78.20 ± 7.70^{ab}	33.61 ± 3.35^{ab}	52.07 ± 5.03^{ab}	43.22 ± 2.38^{bcd}	$64.52 \pm 2.12^{a–d}$	66.65 ± 1.54^{ab}	3.68 ± 0.22^{ab}	7.69 ± 0.24^{a}
MUG	51.43 ± 11.07^{de}	32.41 ± 7.64^{abc}	19.23 ± 5.19^{e}	57.35 ± 2.88^{a}	36.26 ± 3.16^{e}	62.90 ± 3.09^{abc}	2.84 ± 0.50^{c}	4.43 ± 0.89^{e}
PEP	58.95 ± 2.84^{cde}	23.72 ± 1.60^{cde}	37.18 ± 2.16^{c}	$39.98 \pm 1.11^{b–e}$	$63.26 \pm 0.92^{a–d}$	62.71 ± 0.89^{abc}	3.31 ± 0.06^{bc}	7.25 ± 0.13^{ab}
RBM	82.60 ± 6.54^{a}	36.94 ± 2.97^{a}	55.01 ± 4.11^{a}	46.98 ± 2.50^{b}	68.30 ± 1.91^{a}	67.77 ± 1.70^{a}	3.48 ± 0.24^{abc}	6.94 ± 0.25^{abc}
SIC	$68.14 \pm 1.25^{a–d}$	$29.87 \pm 0.86^{a–d}$	44.62 ± 1.30^{abc}	44.00 ± 0.99^{bc}	67.64 ± 1.24^{ab}	65.32 ± 1.15^{ab}	2.97 ± 0.07^{bc}	6.23 ± 0.14^{bcd}
VLB	47.25 ± 8.42^{e}	19.84 ± 0.70^{e}	34.06 ± 8.63^{c}	$39.54 \pm 3.32^{b–e}$	59.97 ± 2.46^{bcd}	62.80 ± 2.89^{abc}	2.90 ± 0.96^{bc}	6.44 ± 0.38^{bcd}
p-Value	0.0109	0.0006	0.0001	0.0001	0.0001	0.0001	0.0035	0.0001

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN (VSL/VCL × 100): linearity; STR (VSL/VAP × 100): straightness; WOB (VAP/VCL × 100): wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; ANC: Ancona; BPM: Bionda piemontese; BSL: Bianca di Saluzzo; LVA: Livorno argento; LVB: Livorno bianca; LVN: Livorno nera; LVO: Livorno collo oro; MBZ: Mericanel della Brianza; MUG: Mugellese; PEP: Pepoi; RBM: Robusta maculata; SIC: Siciliana; VLB: Valdarnese bianca.
[a–e]Different superscripts indicate significant differences among breeds.

recorded in ANC (1.80×10^9/mL) and RBM (1.52×10^9/mL), while the other breeds showed similar values, ranging from 2.04 to 3.28×10^9/mL (Table 3). Therefore, as a result, the PEP breed showed significantly higher TSO compared to the other breeds. The higher values of SMI were observed in the MBZ breed (96%), and it was significantly higher compared to the SMI mean values (range 79–88%) recorded in LVN, LVB, LVO, ANC, MUG, BPM, and BSL breeds, whereas intermediate values were recorded in the other breeds. The highest value of PM (33%) was recorded in the RBM breed, and it was significantly higher compared to the PM mean values (range 17–22%) recorded in BPM, BSL, MUG, PEP, and VLB breeds (Table 3).

The results of ANOVA showed that all sperm kinetic variables in fresh ejaculates were affected by the breed ($p < 0.05$). Mean values of the kinetic parameters per breed recorded with CASA analysis are reported in Table 4. The RBM breed exhibited generally higher values than the other breeds for VCL, VSL, VAP, STR, and WOB. However, it is important to note that significant differences varied among the various parameters. Specifically, VCL was higher in the RBM than in the BSL, MUG, PEP, and VLB breeds ($p < 0.05$). The VSL and VAP were higher in RBM when compared to ANC, BPM, BSL, LVA, LVO, PEP, and VLB ($p < 0.05$). The STR and WOB values were significantly higher in the RBM compared to LVO, MUG, VLB and BSL, LVA, and LVO, respectively. Considering the overall qualitative sperm parameters, the RBM, SIC, and MBZ breeds produced ejaculates with better sperm quality, corresponding to higher SMI, TM, PM, LVN, and STR, compared to other Italian breeds.

Table 5. Pearson correlation coefficients between body weight of roosters, quantitative and qualitative variables measured in fresh semen.

		VO	CO	TSO	SMI	TM	PM	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Weight		0.142	**−0.280****	0.016	**0.200***	0.150	0.054	**0.229****	0.129	**0.287****	−0.013	**0.187***	0.120	0.134	0.119
	p-Value	*0.108*	***0.001***	*0.860*	***0.023***	*0.089*	*0.542*	***0.009***	*0.144*	***0.001***	*0.886*	***0.033***	*0.174*	*0.128*	*0.178*
VO			**0.493****	**0.917****	0.151	**0.211***	−0.087	−0.011	−0.121	−0.026	−0.145	−0.009	−0.099	0.096	0.117
	p-Value		***0.000***	***0.000***	*0.073*	***0.011***	*0.301*	*0.894*	*0.148*	*0.756*	*0.082*	*0.911*	*0.236*	*0.253*	*0.161*
CO				**0.738****	**0.269****	**0.327****	0.019	0.040	−0.094	−0.002	**−0.208***	−0.013	−0.177*	0.116	−0.034
	p-Value			***0.000***	***0.001***	***0.000***	*0.820*	*0.635*	*0.264*	*0.981*	***0.012***	*0.879*	*0.033*	*0.166*	*0.686*
TSO					**0.170***	**0.236****	−0.084	0.005	−0.118	−0.019	**−0.179***	−0.023	−0.132	0.123	0.106
	p-Value				***0.045***	***0.005***	*0.320*	*0.956*	*0.162*	*0.823*	***0.033***	*0.783*	*0.115*	*0.144*	*0.210*
SMI						**0.684****	**0.512****	**0.386****	**0.373****	**0.419****	0.146	0.135	**0.245****	**0.171***	0.135
	p-Value					***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	*0.083*	*0.110*	***0.003***	***0.042***	*0.109*
TM							**0.538****	**0.535****	**0.402****	**0.534****	−0.025	0.024	**0.164***	**0.379****	0.066
	p-Value						***0.000***	***0.000***	***0.000***	***0.000***	*0.763*	*0.775*	***0.048***	***0.000***	*0.430*
PM								**0.660****	**0.763****	**0.706****	**0.305****	**0.290****	**0.370****	**0.315****	**0.309****
	p-Value							***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
VCL									**0.766****	**0.917****	−0.065	0.067	0.070	**0.734****	**0.200***
	p-Value								***0.000***	***0.000***	*0.441*	*0.424*	*0.405*	***0.000***	***0.016***
VSL										**0.818****	**0.563****	**0.308****	**0.563****	**0.364****	**0.340****
	p-Value									***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
VAP											0.109	**0.310****	**0.374****	**0.575****	**0.363****
	p-Value										*0.192*	***0.000***	***0.000***	***0.000***	***0.000***
LIN												**0.407****	**0.796****	**−0.390****	**0.249****
	p-Value											***0.000***	***0.000***	***0.000***	***0.003***
STR													**0.476****	**−0.213****	**0.501****
	p-Value												***0.000***	***0.010***	***0.000***
WOB														**−0.240****	**0.409****
	p-Value													***0.004***	***0.000***
ALH															**0.187***
	p-Value														***0.024***

VO: volume (mL); CO: sperm concentration ($\times 10^9$/mL); TSO: total sperm output ($\times 10^9$/mL); SMI: sperm membrane integrity (%); TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN (VSL/VCL × 100): linearity (%); STR (VSL/VAP × 100): straightness (%); WOB (VAP/VCL × 100): wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz).

*Correlation is significant at the 0.05 level (two-tailed).

**Correlation is significant at the 0.01 level (two-tailed).

In bold are expressed the significant correlation and in italic the *p*-value of significant correlation.

To assess the correlations between body weight and various parameters reflecting the quality of fresh sperm, as well as to explore correlations among individual sperm variables, Pcc were computed (Table 5). The body weight was negatively correlated with sperm concentration ($p < 0.01$) and positively correlated with SMI, STR ($p < 0.05$), VCL, and VAP ($p < 0.01$). Semen volume was found to be positively correlated with CO, TSO ($p < 0.01$), and TM ($p < 0.05$). Sperm concentration was positively correlated with volume, TSO, and TM ($p < 0.01$), and negatively correlated with LIN. TSO exhibited a positive correlation with VO, CO, TM ($p < 0.01$), and SMI ($p < 0.05$) but showed a negative correlation with LIN. TM and PM were positively correlated with SMI and most kinetic parameters ($p < 0.01$) and were additionally correlated amongst themselves ($p < 0.01$). Furthermore, the general trend was that all kinetic parameters displayed a positive correlation when compared with each other, with the exception of ALH, which showed a negative correlation with LIN, STR, and WOB ($p < 0.01$).

Table 6. Descriptive statistics of semen variables measured in frozen/thawed ejaculates of Italian chicken breeds.

Parameters	*n*	Mean	Minimum	Maximum	*SE*	CV (%)
SMI, %	58	31.03	15.30	45.74	0.892	21.89
TM, %	58	25.92	15.33	41.37	0.825	24.23
PM, %	58	2.53	0.30	6.50	0.141	42.35
VCL, μm/s	58	33.28	20.94	40.49	0.619	14.18
VSL, μm/s	58	11.26	4.97	16.85	0.342	23.16
VAP, μm/s	58	18.95	10.57	26.19	0.479	19.24
LIN, %	58	32.98	19.19	41.17	0.569	13.13
STR, %	58	57.92	47.05	66.18	0.560	7.36
WOB, %	58	55.91	40.72	64.95	0.620	8.44
ALH, μm	58	2.23	1.34	3.34	0.065	22.01
BCF, Hz	58	4.64	2.01	8.08	0.206	33.73

SMI: sperm membrane integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN (VSL/VCL × 100): linearity; STR (VSL/VAP × 100): straightness; WOB (VAP/VCL × 100): wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

Post-thaw semen quality: descriptive statistics, analysis of variance, and correlations

The descriptive statistics of semen variables in frozen/thawed ejaculates are reported in Table 6. Variability increased in SMI, TM, and PM compared to the fresh

Table 7. Mean ± *SE* and *p*-values of quality parameters measured in frozen/thawed ejaculates of different Italian chicken breeds.

	Semen parameters										
Breed	SMI, %	TM, %	PM, %	VCL, μm/s	VSL, μm/s	VAP, μm/s	LIN, %	STR, %	WOB, %	ALH, μm	BCF, Hz
ANC	28.93 ± 1.53^{abc}	17.32 ± 0.63^{d}	1.50 ± 0.13^{b}	25.81 ± 0.92^{d}	7.75 ± 0.47^{d}	13.92 ± 0.33^{d}	30.00 ± 1.58^{c}	55.56 ± 2.92^{ab}	54.03 ± 0.79^{ab}	1.65 ± 0.05^{d}	3.84 ± 0.46^{c}
BPM	37.81 ± 1.37^{a}	29.62 ± 2.16^{ab}	2.72 ± 0.14^{ab}	36.45 ± 0.94^{ab}	11.48 ± 0.31^{bc}	19.93 ± 0.43^{bc}	31.37 ± 0.54^{bc}	57.28 ± 0.63^{ab}	54.57 ± 0.49^{ab}	3.16 ± 0.09^{a}	6.53 ± 0.26^{ab}
BSL	31.33 ± 5.18^{ab}	29.23 ± 6.87^{ab}	3.00 ± 1.22^{ab}	36.73 ± 2.28^{ab}	11.57 ± 1.43^{bc}	20.15 ± 1.97^{bc}	31.00 ± 2.19^{bc}	56.65 ± 1.97^{ab}	54.28 ± 2.23^{ab}	2.82 ± 0.26^{a}	6.15 ± 0.40^{ab}
LVA	24.75 ± 4.74^{bc}	23.59 ± 0.77$^{a–d}$	2.44 ± 0.28^{ab}	34.08 ± 1.14^{abc}	14.36 ± 0.63^{ab}	22.08 ± 1.28^{ab}	38.31 ± 2.01^{a}	58.03 ± 3.39^{ab}	59.68 ± 1.15^{a}	1.91 ± 0.07^{cd}	3.16 ± 0.11^{c}
LVB	33.51 ± 2.16^{ab}	29.95 ± 1.70^{ab}	2.36 ± 0.27^{ab}	29.80 ± 2.01^{cd}	10.36 ± 0.87^{cd}	16.74 ± 1.17^{cd}	34.84 ± 1.21^{abc}	61.13 ± 1.55^{a}	56.63 ± 1.48^{ab}	1.75 ± 0.13^{d}	3.89 ± 0.22^{c}
LVN	29.19 ± 1.13^{abc}	22.30 ± 1.42$^{a–d}$	1.97 ± 0.22^{ab}	31.20 ± 1.01^{bcd}	9.44 ± 0.46^{cd}	16.38 ± 0.50^{cd}	30.51 ± 1.41^{c}	57.54 ± 1.83^{ab}	52.62 ± 1.47^{b}	2.11 ± 0.11^{cd}	3.41 ± 0.33^{c}
LVO	24.98 ± 3.33^{bc}	21.94 ± 2.35$^{a–d}$	3.07 ± 0.40^{ab}	36.60 ± 1.54^{ab}	15.54 ± 0.51^{a}	24.74 ± 0.49^{a}	36.58 ± 1.50^{ab}	54.98 ± 1.48^{ab}	59.99 ± 1.52^{a}	2.05 ± 0.06^{cd}	3.24 ± 0.13^{c}
MBZ	34.08 ± 4.90^{a}	21.28 ± 2.36^{abc}	2.65 ± 0.50^{ab}	38.79 ± 0.16^{a}	13.70 ± 0.64^{ab}	22.29 ± 0.64^{ab}	35.30 ± 1.75^{abc}	61.28 ± 0.98^{a}	57.45 ± 1.86^{ab}	2.95 ± 0.10^{a}	7.32 ± 0.62^{a}
MUG	21.82 ± 0.44^{bc}	20.58 ± 1.58^{cd}	1.73 ± 0.05^{b}	30.29 ± 0.07^{cd}	10.49 ± 0.45^{cd}	17.35 ± 0.42^{cd}	34.64 ± 1.35^{abc}	60.42 ± 1.18^{a}	57.48 ± 1.10^{ab}	2.33 ± 0.28^{bc}	3.86 ± 0.24^{c}
PEP	31.03 ± 1.94^{ab}	28.25 ± 2.43^{abc}	3.52 ± 0.65^{a}	36.59 ± 1.08^{ab}	11.98 ± 0.92^{bc}	20.36 ± 1.14^{bc}	31.91 ± 1.69^{bc}	57.32 ± 1.40^{ab}	54.79 ± 1.75^{ab}	2.54 ± 0.09^{bc}	6.70 ± 0.37^{ab}
RBM	21.85 ± 1.21^{bc}	22.95 ± 4.53$^{a–d}$	1.50 ± 0.83^{b}	32.06 ± 3.29^{bc}	8.04 ± 1.75^{d}	14.83 ± 2.55^{d}	24.54 ± 3.00^{d}	53.29 ± 3.13^{b}	45.72 ± 3.29^{c}	2.42 ± 0.40^{bc}	5.95 ± 1.01^{b}
SIC	36.79 ± 1.41^{a}	30.20 ± 1.09^{a}	2.84 ± 0.16^{ab}	34.33 ± 0.83^{abc}	11.82 ± 0.30^{bc}	20.41 ± 0.55^{bc}	34.54 ± 0.67^{abc}	58.32 ± 0.95^{ab}	59.54 ± 0.74^{a}	2.23 ± 0.05^{bc}	4.01 ± 0.12^{c}
p-Value	0.0004	0.0008	0.0253	0.0001	0.0001	0.0003	0.046	0.0001	0.0001	0.0001	0.0001

SMI: sperm membrane integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN (VSL/VCL × 100); linearity; STR (VSL/VAP × 100); straightness; WOB (VAP/VCL × 100); wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; ANC: Ancona; BPM: Bionda piemontese; BSL: Bianca di Saluzzo; LVA: Livorno argento; LVB: Livorno bianca; LVN: Livorno nera; LVO: Livorno collo oro; MBZ: Mericanel della Brianza; MUG: Mugellese; PEP: Pepoi; RBM: Robusta maculata; SIC: Siciliana.
$^{a–e}$Different superscripts indicate significant differences among breeds.

ejaculates, whereas it decreased in kinetic parameters, except for BCF.

Semen quality parameters assessed after cryopreservation showed a significant effect on the breed, and the mean and *p*-values are shown in Table 7. The highest post-thaw SMI values were recorded in BPM, SIC, and MBZ, which were significantly different from LVA, LVO, MUG, and RBM breeds. TM recorded in the post-thaw ejaculates of SIC, BPM, BSL, LVB, and PEP breeds showed high comparable values (range 28–30%), which were significantly different from those recorded in the ANC and MUG breeds, being 17 and 21%, respectively. The PEP breed showed significantly higher values of PM (3.5%) in thawed ejaculates compared to the ANC, MUG, and RBM breeds (range 1.5–1.7%) (Table 7).

In terms of kinetic parameters, the MBZ breed exhibited a significantly higher VCL compared to ANC, LVB, LVN, MUG, and RBM in post-thaw ejaculates. Furthermore, LVO had higher VSL and VAP values compared to all other breeds, except for LVA and MBZ. LIN in cryopreserved semen showed the highest value in the LVA breed (38%) that was significantly higher compared to the mean values recorded in ANC, BPM, BSL, LVN, PEP (30–32%), and RBM breeds, the latter having the significant lowest value (24%). The STR values were similar across all breeds and significant differences were found only between LVB, MBZ, and MUG (range 60–61%) and RBM (53%). Significant higher values of ALH were recorded in post-thaw semen of BPM, BSL, and MBZ (range 2.8–3.2 μm) compared to all other breeds (range 1.6–2.5 μm). Finally, BCF ranged from 3.1 to 6.7 in post-thaw ejaculates and the significant higher mean values were measured in the BPM, BSL, MBZ, MBZ, PEP, and RBM breeds.

Pearson correlation coefficients were calculated to investigate the relationships among the variables assessed in frozen/thawed semen (Table 8). SMI demonstrated positive correlations with TM, PM ($p < 0.01$), VCL, VAP, STR, WOB, and ALH ($p < 0.05$). TM and PM were positively correlated ($p < 0.01$) and displayed a positive correlation with all kinetic parameters, with the majority being significant at $p < 0.01$. On the other hand, when exploring the correlations among the kinetic parameters, it was observed that VCL, VAP, and VSL were positively correlated with each other ($p < 0.01$). Additionally, they were also correlated with LIN and ALH ($p < 0.01$). Furthermore, VCL was positively correlated with BCF ($p < 0.01$), VSL with STR and WOB, and finally, VAP with WOB ($p < 0.01$). Moreover, the general trend suggested a positive correlation among all

Table 8. Pearson's correlation coefficients between quality variables measured in frozen/thawed chicken semen.

		TM	PM	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
SMI		**0.663****	**0.568***	**0.328***	0.236	**0.309***	0.215	**0.270***	**0.277***	0.280*	0.191
	p-Value	***0.000***	***0.000***	***0.012***	*0.075*	***0.018***	*0.106*	***0.040***	***0.035***	*0.033*	*0.150*
TM			**0.742****	**0.436****	**0.390****	**0.406****	**0.346****	**0.410****	**0.296***	**0.284***	**0.308***
	p-Value		***0.000***	***0.001***	***0.002***	***0.002***	***0.008***	***0.001***	***0.024***	***0.031***	***0.019***
PM				**0.691****	**0.701***	**0.728****	**0.441****	**0.305***	**0.405****	**0.474****	**0.410****
	p-Value			***0.000***	***0.000***	***0.000***	***0.001***	***0.020***	***0.002***	***0.000***	***0.001***
VCL					**0.795****	**0.856****	**0.302***	0.183	0.238	**0.712****	**0.495****
	p-Value				***0.000***	***0.000***	***0.021***	*0.168*	*0.073*	***0.000***	***0.000***
VSL						**0.958****	**0.754****	**0.394****	**0.646****	**0.389****	0.162
	p-Value					***0.000***	***0.000***	***0.002***	***0.000***	***0.003***	*0.225*
VAP							**0.607****	0.187	**0.626****	**0.495****	0.235
	p-Value						***0.000***	*0.159*	***0.000***	***0.000***	*0.075*
LIN								**0.701****	**0.861****	−0.006	−0.152
	p-Value							***0.000***	***0.000***	*0.963*	*0.253*
STR									**0.339****	0.068	0.045
	p-Value								***0.009***	*0.614*	*0.736*
WOB										0.020	−0.160
	p-Value									*0.880*	*0.231*
ALH											**0.728****
	p-Value										***0.000***

SMI: sperm membrane integrity (%); TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN (VSL/VCL × 100): linearity (%); STR (VSL/VAP × 100): straightness (%); WOB (VAP/VCL × 100): wobble (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz).
*Correlation is significant at the 0.05 level (two-tailed).
**Correlation is significant at the 0.01 level (two-tailed).
In bold are expressed the significant correlation and in italic the *p*-value of significant correlation.

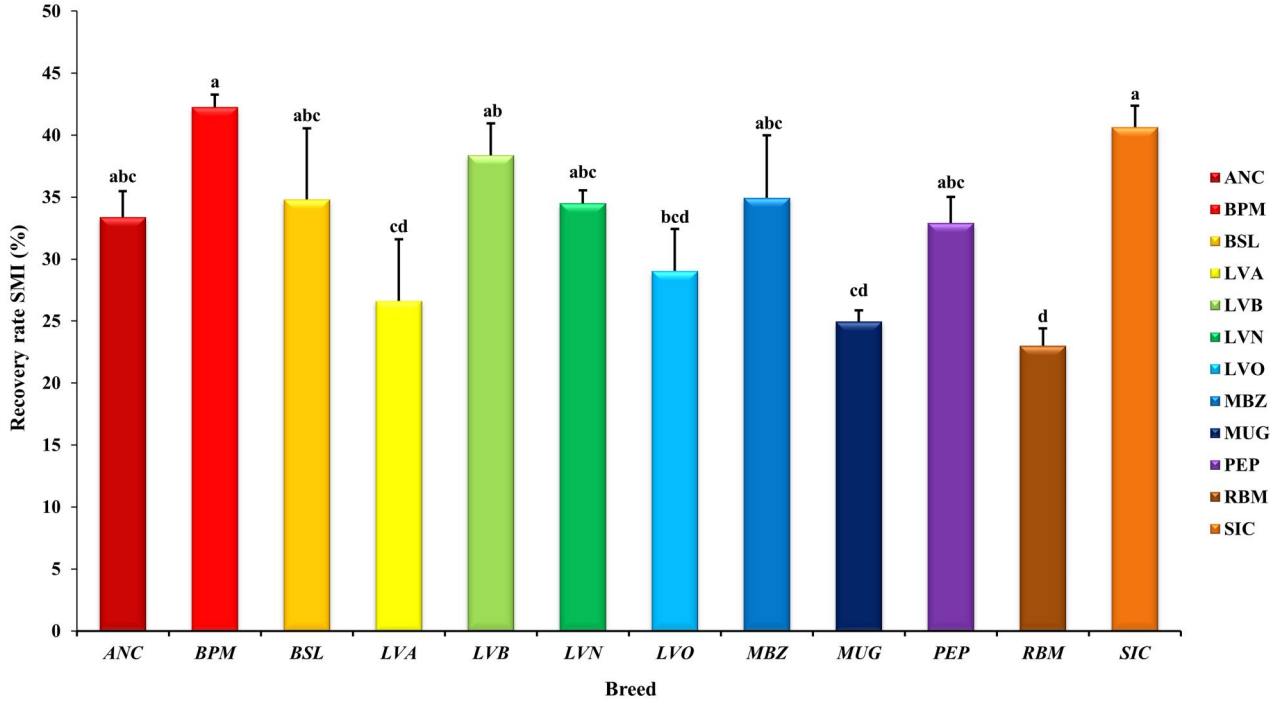

Figure 1. Recovery rate (mean ± *SE*) of sperm membrane integrity (SMI) after cryopreservation recorded in different Italian chicken breeds. ANC: Ancona; BPM: Bionda piemontese; BSL: Bianca di Saluzzo; LVA: Livorno argento; LVB: Livorno bianca; LVN: Livorno nera; LVO: Livorno collo oro; MBZ: Mericanel della Brianza; MUG: Mugellese; PEP: Pepoi; RBM: Robusta maculata; SIC: Siciliana.

kinetic parameters when compared with each other, apart from ALH and BCF. Although ALH and BCF were positively correlated, they showed a positive correlation with only few parameters, specifically, ALH with VSL, VAP, and VCL, while BCF with VCL.

Recovery rate

The recovery values in SMI and TM were significantly affected by the breed ($p < 0.05$) and the mean values per breed are shown in Figures 1 and 2, respectively. In contrast, the recovery in PM was not significantly

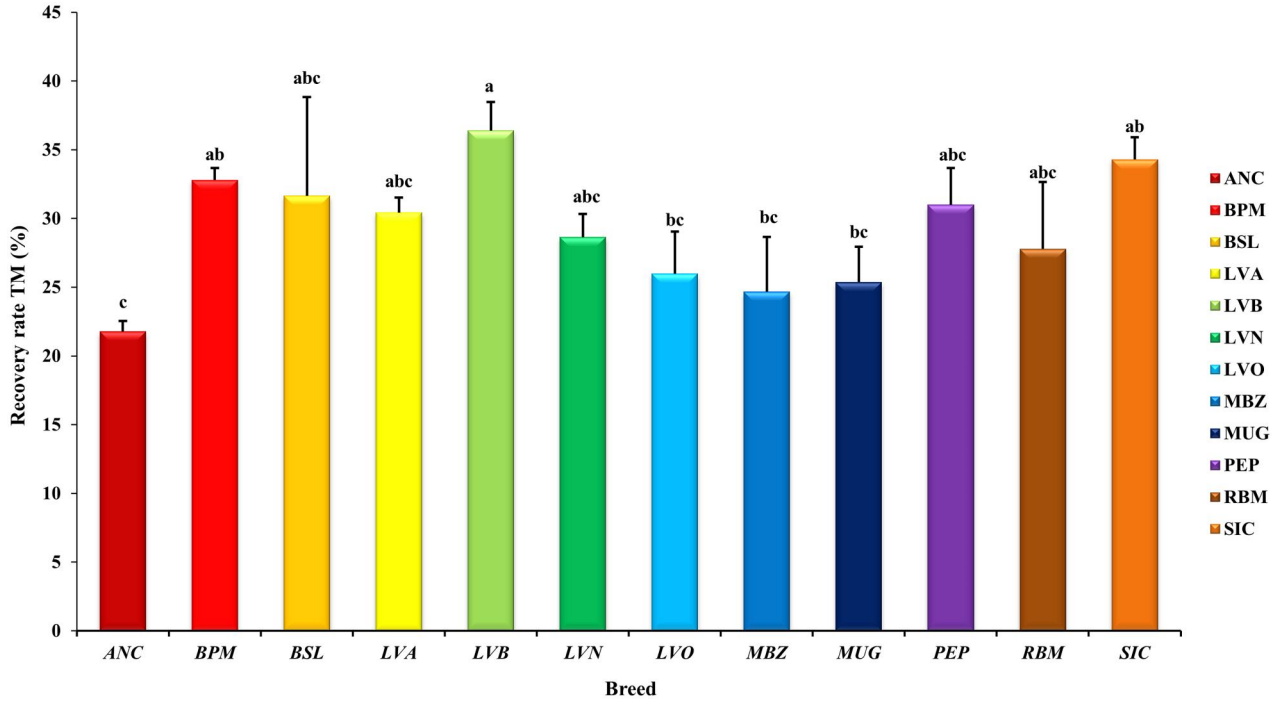

Figure 2. Recovery rate (mean ± *SE*) of total sperm motility (TM) after cryopreservation recorded in different Italian chicken breeds.

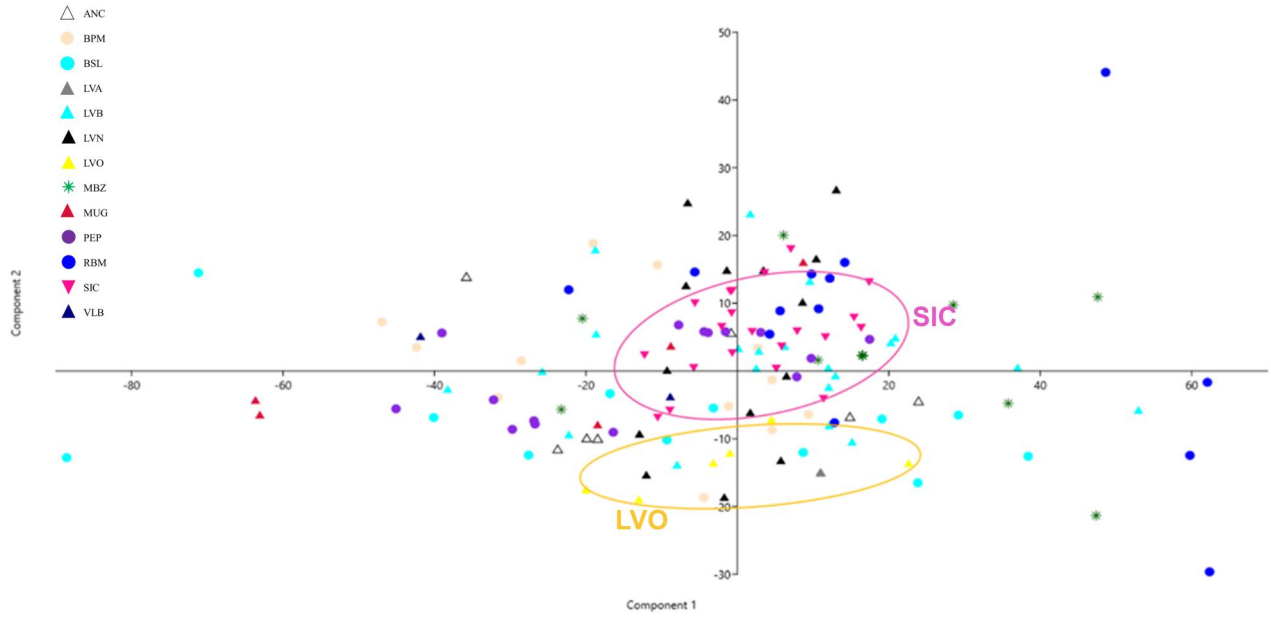

Figure 3. Principal component analysis with quantitative and qualitative parameters; component 1 (horizontal axis) and 2 (vertical axis). Scatter Plot of principal component analysis (PCA) of fresh semen within the thirteen local chicken breeds. Every sign represents a donor, every coloured symbol a breed.

affected by the breed, exhibiting a general decline, with a mean value of only 9.3%. Across all breeds, the average recovery rate was 34.6% for SMI and 30.5% for TM. The BPM and SIC breeds achieved the highest recovery rates for SMI, ~42%, and similar lower values were recorded in several breeds: ANC, BSL, LVB, LVN, MBZ, and PEP. Conversely, LVA, LVO, MUG, and RBM showed the significant lowest recovery rate of SMI after freezing/thawing, averaging nearly 24%. A similar result was also found for the recovery rate of motile sperm, the breed exhibiting the highest recovery rate was LVB (37.9%) and similar lower values were recorded in several breeds: BPM, BSL, LVA, LVN, PEP, RBM, and SIC. In contrast, ANC breed recovered the

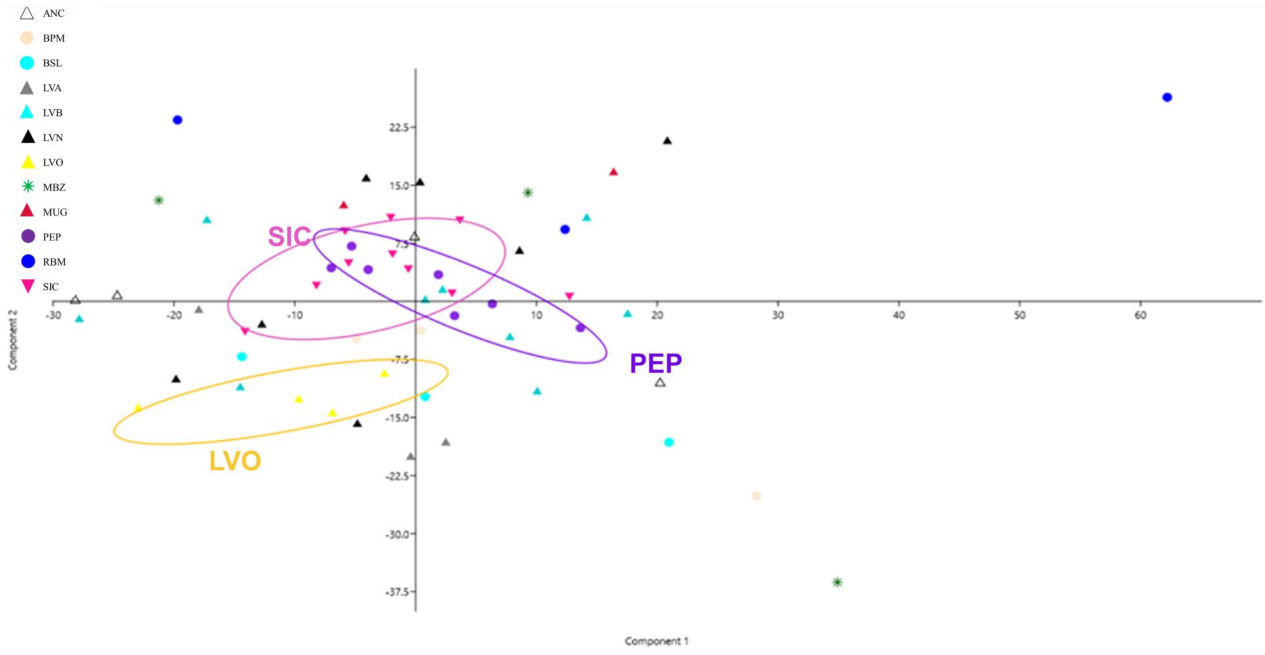

Figure 4. Principal component analysis with quantitative and qualitative parameters; component 1 (horizontal axis) and 2 (vertical axis). Scatter Plot of principal component analysis (PCA) of frozen thawed semen within the 12 local chicken breeds. Every sign represents a donor, every coloured symbol a breed.

lowest proportion of motile sperm, only 22%, significantly different from the value of LVB breed.

It is of interest to underline that the sperm damage occurring during freezing/thawing is not constantly related to sperm quality of fresh ejaculates. The SIC breed showed high recovery rates in SMI and TM, in agreement with the high sperm quality recorded before cryopreservation; whereas RBM ejaculates showed a low recovery of sperm with membrane integrity after cryopreservation despite its high quality before *in vitro* processing.

Principal component analysis (PCA)

In fresh semen PCA (Figure 3), the first three components describe the 86.20% of the total variation. The reported scatterplot describes a good clustering ability of SIC and LVO samples. Semen of other breeds shows high variability in the analysed parameters. The parameters which mainly influence fresh semen traits are VCL for the first component, LIN for the second component, and for the third component TM, SIC, and LVO breeds are clearly separated on component 2.

In frozen semen PCA, the first three components describe the 69.59% of the total variation of the samples' set. Fresh semen VCL is the variable which mainly influence both PCs (PC1 and PC2). PC2 variance depends on fresh semen LIN too. The PCA scatter plot in thawed semen too defines an evident clustering ability in SIC and LVO samples. Thawed PEP semen shows a good clustering ability too. As in fresh condition, SIC and LVO mainly separate on component 2, PEP samples show overlapping areas with SIC samples on component one and are separated from LVO samples on component 2 (Figure 4).

Discussion

The present study provides, for the first time, the phenotypic characterisation of semen and its sensitivity to the cryopreservation process in several Italian native chicken breeds. Our findings offer valuable insights into the fresh semen quality of each breed, aiding breeders and conservationists in selecting superior breeding males and planning effective strategies to preserve genetic diversity. Additionally, the sensitivity to sperm cryopreservation of different breeds, an essential method for safeguarding genetic material over the long term, was studied. By discerning the responses of different breeds to cryopreservation, the study highlighted the possibility of developing specific protocol to ensure optimal semen quality after thawing across different genetic backgrounds. The present results revealed a wide variability in all parameters considered across different breeds, particularly in quantitative variables of fresh semen, such as volume, concentration, and TSO. In terms of qualitative traits (SMI and motility parameters), variability across breeds was more pronounced in frozen than in fresh semen. Interestingly, except for

total motility of fresh semen, the breed significantly affected all semen quality parameters in both fresh and thawed ejaculates.

Phenotypic characterisation of fresh semen

The evaluation of sperm quality characteristics of chickens provides a valuable indicator of their reproductive potential and has been reported to be an important predictor of fertility and subsequent egg hatchability (Peters et al. 2004). Specifically, sperm motility and their relative kinetic parameters are crucial determinants of rooster fertility in domestic chickens, and choosing semen donors based on these traits enhances fertilisation success (Froman et al. 1997, 1999; Birkhead et al. 1999; Blesbois et al. 2008; Jarrell et al. 2020). In this regard, recently, Tesfay et al. (2020) reported that the fertility rate of Rhode Island Red and White Leghorn was positively correlated with SMI, sperm concentration, total motility, and the majority of kinetic traits. Therefore, the knowledge acquired in our study represents a valuable potential tool in predicting the fertilising ability of each donor. This capability facilitates the selection of males with superior semen quality for breeding purposes. Furthermore, when combined with genetic analysis, it allows for the identification of males exhibiting not only high genetic variability and low inbreeding but also optimal semen quality. This comprehensive approach ensures the selection of the most suitable males for breeding purposes, ultimately enhancing the genetic diversity and reproductive efficiency of the breeding population.

In this research, the production and quality of fresh semen were evaluated across several chicken breeds. A review of existing literature uncovered numerous studies exploring different aspects of fertility, semen quality, and other parameters pertinent to poultry reproduction (Peters et al. 2008; Mavi et al. 2018; Mussa et al. 2023). A consistent finding across most of these studies, including the present one, is the significant inter-breed variability observed in male reproductive traits.

Variations in fresh semen characteristics among different chicken breeds and lines can be attributed to several factors, including genetic differences, individual performance, management practices, collection methods, and age (Tabatabaei et al. 2009, 2010; Mussa et al. 2023; Ayeneshet et al. 2024). Peters et al. (2008) conducted a comparative analysis of fresh semen quality among seven chicken breeds, revealing substantial variability in semen volume, sperm concentration, and total motility. Likewise, Ameen et al. (2014) documented significant differences in ejaculate volume, sperm concentration, motility, and viability among five distinct breeds of Nigerian chickens. The Authors found that body weight positively influenced semen volume; in fact, the Hubbard breed had the heaviest body weight (5.06 kg) and the higher ejaculate volume (0.59 mL) in comparison to the Yoruba Ecotype, having the lightest body weight (1.78 kg) and the lowest ejaculate volume (0.24 mL). Body weight has been identified as a potential indicator of semen volume and concentration in certain cockerel breeds. Poultry breeds with greater body weight tend to have larger testes and higher sperm production rates during spermatogenesis (Adeyemo et al. 2007), resulting in a higher number of spermatozoa. However, it has been also noted that cockerels with higher body weights are inclined to produce ejaculates with greater volume but lower sperm concentration (Adeyemo et al. 2007).

In our study, we found a negative correlation between body weight and sperm concentration. Specifically, the breed with the highest weight (4.22 kg), RBM, exhibited the lowest sperm concentration (1.52×10^9/mL), while the highest concentration (4.28×10^9/mL) was measured in PEP, one of the lightest breeds (1.96 kg). These findings align with those from Göger et al. (2018), who observed that heavier cockerels from four distinct lines exhibited lower sperm concentrations.

Moreover, we found a positive correlation between semen volume and sperm concentration.

Sensitivity to cryopreservation process

The cryopreservation protocol employed in this study was developed through prior research efforts (Madeddu et al. 2016, Mosca et al. 2016, 2019; Zaniboni et al. 2022). Subsequently, it was officially established as the reference freezing method for the implementation of Italian Semen Cryobank of Autochthonous Chicken and Turkey Breeds (Iaffaldano et al. 2021). Consistent with previous findings documented in both commercial lines and native breeds (Zaniboni et al. 2022; Madeddu et al. 2024), semen quality in frozen/thawed samples showed a significant deterioration. SMI decreased from 89.0 to 31.0%, whilst TM and PM were reduced from 83.5 and 26.4 to 25.9% and 2.5%, respectively. Although a decline in the kinetic parameters was also observed, it was less pronounced, particularly in the case of LIN, STR, WOB, ALH, and BCF.

It is widely acknowledged that the ability of spermatozoa to endure cryopreservation varies among avian species (Blanco et al. 2008, 2012) as well as among different breeds or strains (Siudzińska and Łukaszewicz 2008; Purdy et al. 2009; Long et al. 2010; Makhafola et al. 2010; Woelders 2021; Zong et al. 2023). Additionally, intra-species differences in freezability have been documented (Blesbois et al. 2007; Kowalczyk and Łukaszewicz 2015). In this context, several studies have explored the impact of breed on the success of cryopreserving domestic fowl semen. Siudzińska and Łukaszewicz (2008) observed significant variations in post-thaw semen quality among four fancy breeds, namely White Crested Black Polish, Black Minorca, Greenleg Partridge, and Italian Partridge. They found that the Black Minorca breed exhibited the highest resistance to the freezing-thawing process, corresponding to 33.6% post-thaw sperm viability. Similarly, Blesbois et al. (2007) and Wishart (2009) highlighted breed-related disparities in frozen semen quality in the chicken. Long et al. (2010) also documented significant variability in frozen semen quality among eight poultry lines.

According to previous studies (Siudzińska and Łukaszewicz 2008; Purdy et al. 2009; Long et al. 2010; Makhafola et al. 2010), the responsiveness of spermatozoa to withstand the cryopreservation process was found largely variable in the 12 chicken populations belonging to nine Italian breeds. The breeds that displayed better quality after cryopreservation process were BPM, BSL, LVB, PEP, and SIC, having 35–42% undamaged membrane sperm and 32–37% motile sperm after thawing. The same breeds also showed higher recovery values in terms of SMI and TM, thus confirming their higher resistance to cryopreservation processing compared to the other Italian chicken breeds.

Focusing the analysis through PCA on breed distribution of semen traits both in fresh and thawed samples, LVO and SIC showed in fresh semen the highest clustering ability presenting a more compact distribution in the four quadrants. This distribution of fresh samples is strictly related to VCL and LIN parameters, which determine breed specific homogeneity. Similar results have been reported in thawed samples too. Breeds specific clustering ability has been described in SIC and LVO thawed samples as in fresh ones, in addition, thawed PEP samples showed high levels of homogeneity in sample characteristics too.

The differences detected among the breeds could be attributed to genetic factors, breed-specific physiological characteristics, or variations in sperm membrane composition. Such features underscore the relevance of customised approaches in semen cryopreservation, wherein specific protocols may need to be developed or adjusted for individual breeds to maximise post-thaw semen quality and fertility potential. Population structure is a fundamental parameter influencing animal productions and conservation strategies, our results about semen traits variability are linked to those reported by Cendron et al. (2020). In their research about genome-wide SNPs analysis in local Italian chicken breeds they reported high genetic homogeneity in SIC and PEP birds (inbreeding coefficient F_{HOM}), a possible birds' genotype/semen traits phenotype correspondence could be investigated. As documented in the literature, avian sperm is less resilient to freezing compared to mammalian sperm, primarily due to differences in the lipid composition of their plasma membranes (Blesbois et al. 2005; Long 2006; Di Iorio, Rusco, Iampietro, Colonna, et al. 2020; Di Iorio, Rusco, Iampietro, Maiuro, et al. 2020). This sensitivity affects their ability to withstand the freezing process and impacts post-thaw fertility (Chuaychu-noo et al. 2017; Iaffaldano et al. 2018; Sun et al. 2022).

Recent advancements in OMICS technologies have provided insights into the molecular aspects of how cryopreservation affects avian sperm. Key findings include changes in seminal plasma, such as fructose, malondialdehyde (MDA), and superoxide dismutase (SOD), which suggest that oxidative stress and antioxidant defence mechanisms are critical for sperm survival during freezing (Partyka et al. 2012). Studies using proteomic and genomic approaches have identified specific biomarkers associated with improved sperm freezability and overall fertility (Khan et al. 2021). These biomarkers include proteins involved in energy metabolism, hydrolase activity, signal transduction, and sperm motility (Labas et al. 2015; Bastan and Akcay 2021; Xu et al. 2021; Ann et al. 2022), as well as stress-response genes, such as HSP90, HSP70, CIRBP, and RHOA (Labas et al. 2015). Recently, the decrease in quality of turkey semen after freezing/thawing was correlated with alterations in the levels of metabolites found in both the aqueous (amino acids, organic acids) and lipid extracts of sperm, as determined through NMR analysis (Paventi et al. 2022). Understanding these molecular markers is crucial for the improvement of the cryopreservation process. To better comprehend the biological mechanisms underlying the variability observed among the different Italian chicken breeds, future research should focus on molecular studies, which will allow for targeted improvements in post-thawing results.

Conclusions

The results of this research provide invaluable insights into the reproductive capabilities and conservation potential of Italian chicken breeds. We identified significant inter-breed variability in both fresh and frozen semen parameters. Some breeds, namely BPM, BSL, LVB, PEP, and SIC, are more resilient to the freezing and thawing process, and others are particularly susceptible and likely require specific cryopreservation protocols to improve semen quality after thawing. Our findings pave the way for further research, particularly in the application of OMICS technologies, to understand the molecular mechanisms underlying breed-specific differences in semen cryopreservation. Overall, this work enriches the field of poultry reproduction by offering new insights that can be applied globally to protect and enhance poultry genetic resources and ensure their sustainability and diversity.

Ethical approval

The handling of animals and semen collection was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

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

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