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PAPER

Rooster sperm pellet cryopreservation protocols: effect of step variations on the qualitative parameters of post-thawed sperm

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ABSTRACT

The cryopreservation of sperm into pellets is not the preferred way to package avian semen but is quick and easy to do and does not require sophisticated technology. The aim of this study was to evaluate rooster sperm viability and mobility and the incidence of normal cells and sperm injuries in post-thawed pelleted sperm. The outcomes of different pelleting protocols were evaluated, which varied according to the parameter combinations used in each of the critical steps of the freezing process and in the thawing conditions which differed in methodology and temperature. The protocols employing 6% DMA showed the highest values of thawed sperm mobility. The most favourable thawing method in terms of sperm mobility was using the hot-plate at 60 °C, followed by the water-bath at 50 °C. The protocols resulting in the best sperm quality parameters employed a 1:2 dilution rate, a 30-min equilibration time at 4 °C, 6% DMA, and thawed 80 µL pellets using the water-bath at 50 °C or the hot-plate at 60 °C. According to the parameters evaluated, rooster sperm was highly susceptible to damage caused by the freezing-thawing methodology, although the survival rate of normal sperm cells still reached 39%, with 32% recovered mobility with respect to fresh sperm samples.

HIGHLIGHTS

- A feasible method for the ex-situ management of genetic resources in birds involves the use of cryopreserved sperm.
- The cryopreservation of domestic fowl semen into pellets constitutes a reasonably simple, quick and affordable technology.
- This technology may be extremely valid and practical for use on small farms.

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Fowl semen; freezing-thawing process; DMA; water-bath; hot-plate

Introduction

During the cryopreservation-thawing process, sperm suffer multiple stresses, including those caused by the temperature changes, the addition of cryoprotectant, the formation of ice and osmotic stress during the freezing (hyperosmotic) and thawing (hypoosmotic) processes (Isachenko et al. 2003; Kumar et al. 2019; Peris-Frau et al. 2020). Osmotic stress also derives from the addition of permeating cryoprotectants, which penetrate the cell, exchange with water and create a new osmotic equilibrium (Isachenko et al. 2003; Kumar et al. 2019; Peris-Frau et al. 2020). Moreover, the tolerance of poultry sperm to cryopreservation is known to vary between genotypic strains of chickens (Khan et al. 2021), rendering the use of a single freezing-thawing protocol for all strains impractical. Instead, the different components of each step of the freezing-thawing protocol need to be optimised not only for each species but also for specific breeds and strains.

Long-term bird conservation programs use sperm cryopreservation as a feasible method for the ex-situ management of genetic resources (Silyukova et al. 2020). According to the Food and Agricultural Organisation (FAO) of the United Nations, the global diversity of domestic animal species is under threat (Bélanger and Pilling 2019). Indeed, many domestic animal breeds worldwide have gained endangered status, are in a critical condition or already extinct. Many avian genetic stocks, in particular chicken lines,

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have been eliminated or are at risk of being eliminated by various institutions and companies, largely due to budget-related downsizing. This loss will adversely impact the future availability of genetic diversity, which might otherwise be of interest to academic scientific communities as well as industry (Bélanger and Pilling 2019). The decline in domestic animal diversity is recognised worldwide, as is the need to conserve genetic diversity.

In general, methods for the cryopreservation of semen involve a temperature reduction phase, cellular dehydration and freezing, followed by their thawing before further use (Kumar et al. 2019). Cryopreservation causes all cell activities to cease, with normal functions only restarting upon thawing (Isachenko et al. 2003). Importantly, in avian and mammalian species, the specific parameters defining the warming/thawing phase are just as critical as the freezing phase to obtain quality sperm (Isachenko et al. 2003; Jovičić et al. 2020). The preferred way to package poultry semen is in sperm straws, being more hygienic than the pellet method/being the method less likely to involve contaminations and facilitating easy sample identification. Until recently, the packaging of sperm into pellets with dimethylacetamide (DMA) as cryoprotectant was retained by some authors to be associated with higher fertility rates compared with straw packaging (Chalah et al. 1999; Tselutin et al. 1999). However, others have reported better results obtained with semen frozen in DMA straws (Tang et al. 2021).

As mentioned above, packaging poultry semen in straws is widely considered the method of choice for better hygiene and easier labelling (Miranda et al. 2018; Mosca et al. 2019; di Iorio et al. 2020; Mosca et al. 2020; Iaffaldano et al. 2021). This method generally uses the water-bath method for thawing. On the contrary, in the case of semen packed in pellets, the hot-plate seems to result in better fertility rates by permitting faster thawing (Tselutin et al. 1999). However, even though the straw packaging method is the one recommended by the FAO (Bélanger and Pilling 2019) and used in cryo-banking, the pellet packaging method may offer more advantages, and thus be more suitable, for small farm holders, who may wish, for example, to preserve the genetic heritage of a single male.

The aim of this study was to evaluate the effects of different variations of the cryopreservation protocol on sperm viability, the incidence of normal cells and sperm cell injuries, and sperm mobility in post-thawed pelleted rooster sperm. Different protocols were tested, which varied according to the combination of the parameters set at each critical step of the freezing process (sperm dilution, the equilibration time at 4 °C and the DMA concentration), the thawing modality (water-bath or hot-plate) and the thawing temperature.

Materials and methods

Experimental design

The model used for studying the variations in the freezing-thawing (F-T) protocol for rooster semen followed a $2 \times 2 \times 2 \times 4$ design: dilution (1:1 or 1:2), equilibration at 4 °C (Eq4 °C) (10 min or 30 min), dimethylacetamide (DMA) concentration (6% or 9%), thawing method (water-bath at 50 °C, 60 °C or 75 °C, or the hot-plate at 60 °C).

Reagents

All chemicals were purchased from Sigma Aldrich (Milano, Italy), except for Accudenz, a cell separation media that was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY, USA).

Birds

This study was approved by the Ethics Committee of Pisa University (Ref.: OPBA_33/2021), under article.2, paragraph.1, point b, of the Italian legislative decree n. 26/2014. Thirty Cobb broiler breeder males aged 42–47 weeks were housed individually in 1×2 m pens furnished with wooden perches. The environmental/ambient temperature remained between 18 °C and 20 °C, a 14 L:10D photoperiod was applied and the housing area was mechanically ventilated. Birds were fed according to the recommendations for this line (Cobb-Vantress.com 2015). Feed was administered in the morning or soon after semen collection. Birds had free access to fresh water. All males were trained for semen collection for one week prior to beginning of the trial (Castillo et al. 2021).

Fresh semen processing

Semen was collected every 3–4 days by means of the dorso-abdominal massage technique (Quinn and Burrows 1936) slightly modified (Castillo et al. 2021). Twenty to twenty-five clean ejaculates were collected during each sampling session (a total of 7 days of semen sampling were set aside for this study). The ejaculate from each bird was collected directly into a collection tube with 100 µL of pre-freezing Lake's diluent (Lake et al. 1981) plus 50 mM glycine. Only dense,

milky white ejaculates were chosen and subjected to analysis and processing within 40 min from collection. Samples with an uncharacteristic colour and/or fluidity were rejected. Ejaculate volume was assessed by weighing the tubes, before and after collection (Sartorius BL 150S ± 0.001 g). Semen was pooled (8 ejaculates/pool), and an aliquot was taken from each pool to assess the quality of the fresh semen. Sperm concentration was assessed in triplicate using a Bürker-Türk counting chamber (in a 5% formalin and 0.9% NaCl solution). The sperm viability percentage was evaluated in triplicate in samples of 500 cells using the eosin-nigrosin staining technique (Bakst and Cecil 1997). Viable cells did not stain at all, whereas cells considered dead appeared totally or partially stained pink. The sperm viability percentage was calculated relative to the total sperm count. Sperm mobility was assessed in triplicate using the Accudenz methodology (Froman et al. 1997), which measures the ability of these cells to swim through a dense layer of Accudenz at a temperature of 41 °C, the penetration of which was measured by spectrophotometry at an absorbance of 550 nm (Perkin Elmer Lambda).

Freezing into pellets methodology

Semen was frozen into pellets according to the standard methodology (Tselutin et al. 1999). The effect of 32 different freezing-thawing (F-T) protocol variation combinations on qualitative sperm parameters was evaluated. These F-T protocols varied according to differences in each of the three freezing steps (dilution, equilibration at 4 °C and DMA final concentration) and in the thawing process (water-bath [WB] at 50 °C, 60 °C or 75 °C or hot-plate [HP] at 60 °C). Pools were divided into two equal parts and diluted 1:1 or 1:2 (sperm:diluent, v:v) in pre-freezing Lake's diluent (Lake et al. 1981) supplemented with 50 mM glycine (dilution = D). Diluted pools were then divided into 400 µL aliquots and rapidly cooled inside a device set to −6 °C until the sample temperature indicated 4 °C. Samples were left to equilibrate at 4 °C (Eq4 °C) for 10 or 30 min in dimethylacetamide (DMA) cryoprotectant at 4 °C added to obtain a final concentration of 6 or 9%, mixed manually for one minute in an iced water-bath, left to stabilise for 4 min, and then 80 µL semen aliquots were dropped directly into liquid nitrogen. The resulting frozen semen pellets were collected and stored in liquid nitrogen, according to the protocol, in cryovials.

Thawing procedure

Thawing was performed via two different methods, the WB and the HP. In the first method (WB), pellets were melted employing a glass tube immersed in water heated to three different temperatures: 50 °C, 60 °C or 75 °C; one at a time, the pellets were placed inside the tube and the semen collected as soon as it melted. In the second method (HP), pellets were melted employing the hotplate set at 60 °C; one at a time, the pellets were placed on an aluminium dish and pushed gently using a micropipette tip to collect the semen as soon as it melted (Castillo et al. 2021) .

Thawed semen quality

Immediately after thawing, the semen's qualitative parameters were evaluated as per the same protocols described for fresh semen. The percentage of viable thawed cells was evaluated in triplicate and calculated relative to total thawed sperm count. The percentage of normal cells was calculated relative to the total number of F-T live sperm.

For live F-T sperm, cells were then grouped according to the presence of lesions of the head or the tail. The percentages of abnormal thawed heads and abnormal thawed tails were calculated relative to total number of live thawed sperm. The head injuries identified were classified as: bent, fractured, coiled, swollen-detached or knotted. The tail injuries identified were classified as: looping, coiled or head-less. Mobility of F-T sperm was assessed as absorbance units at 550 nm, and the recovered mobility was expressed as a percentage with respect to fresh sperm cell mobility.

Statistical analysis

The results are presented as means ± standard deviations (SD). All analyses considered a significance level of $p < 0.05$ and were performed using JMP-Statistical Discovery software (SAS-Institute Inc.v.5.0.1.). Percentage data were normalised through $\sqrt{x}$ Arcsine transformation. One-way ANOVA was used to compare the F-T sperm with the fresh sperm followed by the Duncan-test. The effects of the different steps of the F-T process on sperm parameters were analysed by four-way ANOVA followed by *t*-tests. Interactions between the sources of variation were not included in the model because they were not significant. Sperm qualitative parameters were evaluated considering the following sources of methodological variation: dilution, DMA % and thawing modality. The different protocols

were compared by one-way ANOVA followed by *t*-tests; seven replicates per protocol were applied. One-way ANOVA was again used to compare the different thawing conditions.

Results

The day of collection did not affect the qualitative parameters of fresh semen. The mean qualitative sperm parameters were the following: ejaculate volume 0.44 ± 0.08 mL, sperm concentration 4.75×10^9/mL, viability 73.4 ± 4.88%, normal sperm cells 91.90% and sperm mobility 0.341 ± 0.033 absorbance units. All F-T protocols brought about a reduction in sperm viability (range: 33–52%; $p < 0.01$), the percentage of normal cells (range: 13–26%; $p < 0.01$) and sperm mobility (range: 75–84%; $p < 0.01$) with respect to fresh semen.

Table 1 reports the qualitative sperm parameter values obtained according to the different combinations of F-T protocol step variations. A difference due to the dilution ratio was observed in sperm viability, mobility ($p < 0.05$) and the percentage of normal cells ($p < 0.01$), which were favoured by the 1:2 dilution ratio. Different Eq4 °C times had no effect, with no difference observed between the 10 min and 30 min equilibration times. The DMA concentration influenced the percentage of viable cells, with 9% DMA associated with higher values ($p < 0.01$). Finally, use of the hot-plate as thawing method was associated with higher values of sperm viability and mobility ($p < 0.01$), whereas the water-bath produced higher percentages of normal cells ($p < 0.05$).

The effects of F-T protocol step variations on the incidence of sperm injuries are reported in Table 2. The dilution rate affected the incidence of fractured cells, being higher with the 1:1 dilution ($p < 0.01$). No effect of Eq4 °C was observed. The DMA concentration affected the incidence of looping tails, being higher with 6% DMA ($p < 0.05$), and the incidence of headless cells, being higher with 9% DMA ($p < 0.01$). Thawing method had a clear effect, with the WB resulting in more fractured, knotted and headless cells ($p < 0.01$); whereas the HP produced more swollen-detached heads and a higher rate of coiled tails ($p < 0.01$).

The effects of variations in the thawing conditions on the qualitative parameters of the F-T sperm are reported in Table 3. The highest percentages of viable cells were observed with the HP and WB at 75 °C ($p < 0.01$), whereas the highest percentage of normal cells was obtained with the WB at 60 °C ($p < 0.01$). Sperm mobility was best preserved with the HP ($p < 0.01$). The highest rates of recovered mobility were in F-T sperm thawed at the lowest WB temperature (50 °C) and in those thawed with the HP ($p < 0.01$).

The incidences of head and tail injuries in F-T sperm thawed under different conditions are reported in Table 4. No differences were observed between the different thawing protocols in the incidence of cells with bent tails. Fewer fractured cells were observed when the HP method was applied ($p < 0.01$). The highest rates of coiled heads were observed at the higher two WB temperatures, 75 °C and 60 °C ($p < 0.01$). The highest rate of swollen-detached cells was observed with the HP ($p < 0.01$). Knotted sperm were more abundant with the WB at 50 °C and 60 °C ($p < 0.01$). The percentage of looping tails was highest for the WB set at 75 °C ($p < 0.01$). Coiled tails were most often observed with the WB at 75 °C and with the HP ($p = 0.0407$). The use of lower WB temperatures (50 °C and 60 °C) led to higher rates of head-less cells ($p < 0.01$).

Table 5 reports the effects of all 32 F-T protocols tested on the qualitative parameters of rooster sperm. Considering all the sperm parameters evaluated, protocols employing the 1:1 dilution (protocols 1–16)

Table 1. Effects of pellet freezing-thawing protocol step variations on rooster sperm qualitative parameters (mean ± *SD*).

		D[1]		Eq4°C[2]		DMA[3]		Th[4]
Viability (%)[7]	1:1	31.66 ± *6.90* $_{b}$	10	31.93 ± *6.27*	6	30.64 ± *6.90* $_{B}$	WB[5]	31.97 ± *5.76* $_{B}$
	1:2	33.29 ± *6.60* $_{a}$	30	33.05 ± *7.34*	9	34.24 ± *5.88* $_{A}$	HP[6]	34.02 ± *9.18* $_{A}$
		$p < 0.05$	*n.s.*			$p < 0.01$		$p < 0.01$
Normal sperm (%)[8]	1:1	70.32 ± *6.31* $_{B}$	10	71.77 ± *5.93*	6	70.73 ± *6.79*	WB	72.20 ± *6.72* $_{a}$
	1:2	72.81 ± *6.04* $_{A}$	30	71.36 ± *6.66*	9	72.37 ± *5.68*	HP	69.63 ± *4.26* $_{b}$
		$p < 0.01$	*n.s.*			*n.s.*		$p < 0.05$
Mobility$_{(Ass\ at\ 550\ nm)}$	1:1	0.067 ± *0.013* $_{b}$	10	0.067 ± *0.016*	6	0.072 ± *0.016*	WB	0.067 ± *0.014* $_{B}$
	1:2	0.072 ± *0.016* $_{a}$	30	0.071 ± *0.015*	9	0.066 ± *0.014*	HP	0.076 ± *0.011* $_{A}$
		$p < 0.05$	*n.s.*			*n.s.*		$p < 0.01$
Recovered mobility (%)	1:1	26.56 ± *3.92*	10	27.00 ± *3.60*	6	27.41 ± *3.73*	WB	26.76 ± *3.53*
	1:2	27.53 ± *3.61*	30	27.41 ± *4.01*	9	26.69 ± *3.87*	HP	27.91 ± *4.46*
		n.s.	*n.s.*			*n.s.*		*n.s.*

[1]Dilution (v:v); [2]equilibration at 4 °C (10 or 30 min.); [3]dimethylacetamide (DMA 6% or DMA 9% final concentration); [4]thawing method; [5]water-bath; [6]hot-plate (60 °C); [7]live F-T sperm rel. to counted F-T sperm; [8]normal F-T sperm rel. to live F-T sperm. $_{A–E}$ Means within a protocol step column with different subscripts are significantly different ($p < 0.01$). $_{a–d}$ Means within a protocol step column with different subscripts are significantly different ($p < 0.05$).

Table 2. Effect of pellet freezing-thawing protocol step variations on rooster sperm injuries (mean ± *SD*).

%[1]		D[2]	Eq4°C[3]		DMA[4]	Th[5]	
				Head			
Bent	1:1	0.60 ± *0.64*	10	0.64 ± *0.81*	6	0.74 ± *0.85*	WB[6] 0.69 ± *0.76*
	1:2	0.62 ± *0.80*	30	0.57 ± *0.63*	9	0.48 ± *0.55*	HP[7] 0.53 ± *0.59*
		n.s.		*n.s.*		*n.s.*	*n.s.*
Fractured	1:1	17.52 ± *5.13* $_{A}$	10	16.08 ± *4.69*	6	17.31 ± *5.67*	WB 18.47 ± *5.28* $_{A}$
	1:2	15.49 ± *5.08* $_{B}$	30	16.92 ± *5.66*	9	15.70 ± *4.60*	HP 14.50 ± *3.64* $_{B}$
		$p < 0.01$		*n.s.*		*n.s.*	$p < 0.01$
Coiled	1:1	0.23 ± *0.43*	10	0.23 ± *0.39*	6	0.24 ± *0.43*	WB 0.25 ± *0.42*
	1:2	0.19 ± *0.35*	30	0.20 ± *0.40*	9	0.17 ± *0.35*	HP 0.16 ± *0.29*
		n.s.		*n.s.*		*n.s.*	*n.s.*
Swo-det[8]	1:1	3.96 ± *3.11*	10	4.00 ± *3.07*	6	3.71 ± *2.97*	WB 2.59 ± *2.27* $_{B}$
	1:2	3.77 ± *3.22*	30	3.72 ± *3.26*	9	4.01 ± *3.35*	HP 5.55 ± *2.54* $_{A}$
		n.s.		*n.s.*		*n.s.*	$p < 0.01$
Knotted	1:1	0.43 ± *0.59*	10	0.45 ± *0.64*	6	0.52 ± *0.64*	WB 0.60 ± *0.68* $_{A}$
	1:2	0.58 ± *0.70*	30	0.57 ± *0.65*	9	0.50 ± *0.65*	HP 0.42 ± *0.51* $_{B}$
		n.s.		*n.s.*		*n.s.*	$p < 0.01$
				Tail			
Looping	1:1	2.61 ± *1.56*	10	2.66 ± *1.62*	6	2.83 ± *1.65* $_{a}$	WB 2.63 ± *1.58*
	1:2	2.41 ± *1.34*	30	2.35 ± *1.27*	9	2.19 ± *1.67* $_{b}$	HP 2.39 ± *1.03*
		n.s.		*n.s.*		$p < 0.05$	*n.s.*
Coiled	1:1	0.13 ± *0.31*	10	0.11 ± *0.30*	6	0.11 ± *0.31*	WB 0.07 ± *0.25* $_{B}$
	1:2	0.07 ± *0.15*	30	0.09 ± *0.17*	9	0.04 ± *0.14*	HP 0.13 ± *0.21* $_{A}$
		n.s.		*n.s.*		*n.s.*	$p < 0.01$
Headless	1:1	1.21 ± *1.28*	10	1.35 ± *1.34*	6	0.84 ± *1.00* $_{B}$	WB 1.36 ± *1.39* $_{A}$
	1:2	0.96 ± *1.23*	30	1.09 ± *1.16*	9	1.33 ± *1.42* $_{A}$	HP 0.82 ± *0.62* $_{B}$
		n.s.		*n.s.*		$p < 0.01$	$p < 0.01$

[1]Percentages relative to total live thawed sperm; [2]dilution (v:v); [3]equilibration at 4 °C (10 or 30-min.); [4]dimethylacetamide (6 or 9% final concentration); [5]thawing method; [6]water-bath; [7]hot-plate (60 °C); [8]swollen-detached. $_{A–E}$ Means within a protocol step column with different subscripts are significantly different ($p < 0.01$). $_{a–d}$ Means within a protocol step column with different subscripts are significantly different ($p < 0.05$).

Table 3. Qualitative parameters of rooster freezing-thawing (F-T) sperm thawed using a water-bath at three different temperatures (50 °C, 60 °C or 75 °C) or a hot-plate at 60 °C (mean ± *SD*).

Thawing-°C	Viability[3] (%)	Normal[4] (%)	Mobility $_{Ass}$[5]	Recovered mobility (%)
WB[1]-50	30.9 ± *6.11* $_{C}$	72.6 ± *5.74* $_{B}$	0.067 ± *0.016* $_{BC}$	28.3 ± *4.32* $_{A}$
WB-60	31.5 ± *6.21* $_{BC}$	74.9 ± *3.40* $_{A}$	0.070 ± *0.014* $_{B}$	26.7 ± *3.87* $_{BC}$
WB-75	33.6 ± *4.47* $_{AB}$	68.7 ± *2.33* $_{C}$	0.063 ± *0.013* $_{C}$	25.3 ± *2.67* $_{C}$
HP[2]-60	34.3 ± *4.52* $_{A}$	69.6 ± *2.66* $_{C}$	0.076 ± *0.016* $_{A}$	27.9 ± *4.46* $_{AB}$
	$p = 0.0102$	$p < 0.0001$	$p = 0.0008$	$p = 0.0002$

[1]Water-bath; [2]hot-plate; [3]live F-T sperm rel. to counted F-T sperm; [4]normal F-T sperm rel. to live F-T sperm; [5]mobility expressed as absorbance units at 550 nm. $_{A–C}$ Means within a column with different subscripts are significantly different ($p < 0.01$).

Table 4. Head and tail injuries (%) in rooster freezing-thawing (F-T) sperm thawed using a water-bath at three different temperatures (50 °C, 60 °C or 75 °C) or a hot-plate at 60 °C (mean ± *SD*).

	WB[1]-50	WB-60	WB-75	HP[2]-60	*p*
			Head		
Fracture	19.12 ± *6.98* $_{A}$	17.40 ± *4.67* $_{A}$	19.07 ± *3.60* $_{A}$	14.56 ± *3.64* $_{B}$	0.0001
Coiled	0.12 ± *0.32* $_{C}$	0.28 ± *0.49* $_{AB}$	0.35 ± *0.40* $_{A}$	0.17 ± *0.29* $_{BC}$	0.0035
Swo-det[3]	1.46 ± *1.07* $_{C}$	1.38 ± *1.40* $_{C}$	5.20 ± *1.78* $_{B}$	7.44 ± *2.54* $_{A}$	0.0001
Knotted	0.79 ± *0.81* $_{A}$	0.67 ± *0.71* $_{A}$	0.32 ± *0.36* $_{B}$	0.42 ± *0.51* $_{B}$	0.0006
			Tail		
Looping	2.17 ± *1.47* $_{B}$	2.39 ± *1.65* $_{B}$	3.32 ± *1.37* $_{A}$	2.42 ± *1.03* $_{B}$	0.0003
Coiled	0.07 ± *1.50* $_{b}$	0.06 ± *0.62* $_{b}$	0.07 ± *0.50* $_{ab}$	0.13 ± *0.64* $_{a}$	0.0407
Headless	1.72 ± *1.82* $_{a}$	1.45 ± *1.33* $_{ab}$	0.93 ± *0.63* $_{bc}$	0.79 ± *0.62* $_{c}$	0.0088

[1]Water-bath; [2]hot-plate; [3]swollen-detached. $_{A–C}$ Means within a row with different subscripts are significantly different ($p < 0.01$). $_{a–c}$ Means within a row with different subscripts are significantly different ($p < 0.05$).

were generally less suitable than the 1:2 dilution. Protocols employing the 1:2 dilution were more suitable, especially protocol 24 which involved the HP, and protocols 25 and 29 which involved the WB at 50 °C, even if the variability in the percentage of normal cells observed for these two protocols were both high. Similar outcomes were obtained with HP protocols 28 and 32 in three of the four evaluated parameters, although the variability observed in sperm viability was high for protocol 28. Protocols employing lower thawing temperatures with the 1:2 dilution and the WB tended to be less deleterious for sperm cells

Table 5. Qualitative parameters of cryopreserved rooster sperm following 32 freezing protocols, comprising all the combinations of two variations at each freezing step (dilution, Eq4°C, DMA concentration) and four different thawing conditions (mean ± *SD*).

n.[1]	D[2]	Eq4°C[3]	DMA[4]	Th[5]	Th temp.[8]	Viability[9] (%)	Normal10 (%)	Mobility $_{Ass.}$[11]	Recovered mobility (%)
1	1:1	10	6	WB[6]	50	27.44 ± *4.45* $_{G}$	73.36 ± *3.64* $_{ABCDEFGH}$	0.066 ± *0.013* $_{BCDEFGHI}$	27.91 ± *3.84* $_{bcde}$
2					60	29.59 ± *5.19* $_{DEFG}$	70.22 ± *7.94* $_{CDEFGHIJ}$	0.070 ± *0.016* $_{BCDEFGHI}$	26.07 ± *4.16* $_{bcdef}$
3					75	30.04 ± *3.87* $_{CDEFG}$	66.75 ± *4.94* $_{IJ}$	0.062 ± *0.011* $_{FGHI}$	24.92 ± *1.97* $_{cdef}$
4				HP[7]	60	30.58 ± *11.79* $_{G}$	66.16 ± *5.15* $_{J}$	0.062 ± *0.013* $_{EFGHI}$	24.86 ± *2.03* $_{cdef}$
5			9	WB	50	27.34 ± *2.62* $_{GHI}$	72.47 ± *4.30* $_{ABCDEFGHIJ}$	0.057 ± *0.012* $_{GHI}$	26.88 ± *5.10* $_{bcdef}$
6					60	34.68 ± *5.69* $_{ABCD}$	74.53 ± *5.49* $_{ABCDEF}$	0.072 ± *0.008* $_{ABCDEFGHI}$	27.80 ± *3.05* $_{bcde}$
7					75	34.08 ± *5.44* $_{ABCD}$	66.72 ± *4.37* $_{IJ}$	0.059 ± *0.013* $_{FGHI}$	24.47 ± *3.17* $_{ef}$
8				HP	60	33.51 ± *5.26* $_{BCDEF}$	71.07 ± *3.16* $_{BCDEFGHIJ}$	0.073 ± *0.009* $_{ABCDEFGH}$	27.80 ± *3.56* $_{bcde}$
9		30	6	WB	50	28.30 ± *5.34* $_{EFG}$	67.41 ± *12.98* $_{HIJ}$	0.065 ± *0.013* $_{CDEFGHI}$	27.61 ± *3.09* $_{bcdef}$
10					60	30.18 ± *3.84* $_{CDEFG}$	72.93 ± *5.35* $_{ABCDEFGHI}$	0.069 ± *0.011* $_{BCDEFGHI}$	25.92 ± *3.19* $_{bcdef}$
11					75	33.27 ± *3.88* $_{BCDEFG}$	68.94 ± *5.05* $_{EFGHIJ}$	0.070 ± *0.011* $_{BCDEFGHI}$	26.72 ± *2.56* $_{bcdef}$
12				HP	60	31.97 ± *12.09* $_{CDEFG}$	66.51 ± *4.84* $_{IJ}$	0.078 ± *0.011* $_{ABCDE}$	28.31 ± *8.96* $_{bcde}$
13			9	WB	50	33.56 ± *7.01* $_{BCDE}$	68.24 ± *6.60* $_{FGHIJ}$	0.060 ± *0.012* $_{FGHI}$	26.66 ± *4.40* $_{bcdef}$
14					60	31.58 ± *7.23* $_{CDEFG}$	78.68 ± *2.75* $_{A}$	0.069 ± *0.010* $_{BCDEFGHI}$	27.25 ± *3.99* $_{bcdef}$
15					75	35.25 ± *5.82* $_{ABC}$	68.84 ± *2.30* $_{EFGHIJ}$	0.060 ± *0.010* $_{FGHI}$	24.72 ± *3.26* $_{def}$
16				HP	60	34.67 ± *3.28* $_{ABCD}$	69.04 ± *2.72* $_{EFGHIJ}$	0.069 ± *0.012* $_{BCDEFGHI}$	26.66 ± *3.50* $_{bcdef}$
17	1:2	10	6	WB	50	28.01 ± *6.56* $_{EFG}$	74.37 ± *10.12* $_{ABCDEFG}$	0.073 ± *0.016* $_{ABCDEFGH}$	29.00 ± *2.90* $_{ab}$
18					60	30.82 ± *6.32* $_{CDEFG}$	74.81 ± *4.20* $_{ABCDEF}$	0.073 ± *0.022* $_{ABCDEFG}$	26.83 ± *4.89* $_{bcdef}$
19					75	30.58 ± *2.03* $_{CDEFG}$	67.95 ± *3.82* $_{GHIJ}$	0.062 ± *0.017* $_{EFGHI}$	24.97 ± *2.48* $_{cdef}$
20				HP	60	30.97 ± *2.74* $_{CDEFG}$	71.73 ± *3.86* $_{BCDEFGHIJ}$	0.081 ± *0.017* $_{ABC}$	29.13 ± *2.24* $_{ab}$
21			9	WB	50	33.06 ± *7.17* $_{BCDEFG}$	74.74 ± *8.98* $_{ABCDEF}$	0.057 ± *0.013* $_{HI}$	25.95 ± *4.57* $_{bcdef}$
22					60	34.70 ± *6.38* $_{ABCD}$	76.33 ± *3.96* $_{ABC}$	0.067 ± *0.010* $_{BCDEFGHI}$	27.16 ± *4.03* $_{bcdef}$
23					75	35.10 ± *3.83* $_{ABCD}$	71.01 ± *3.79* $_{BCDEFGHIJ}$	0.056 ± *0.013* $_{I}$	23.79 ± *3.13* $_{f}$
24				HP	60	37.62 ± *2.63* $_{AB}$	73.68 ± *2.94* $_{ABCDEFGH}$	0.080 ± *0.019* $_{ABCD}$	28.75 ± *4.33* $_{abc}$
25		30	6	WB	50	34.54 ± *4.28* $_{ABCD}$	77.18 ± *9.81* $_{AB}$	0.087 ± *0.015* $_{A}$	32.34 ± *3.64* $_{a}$
26					60	27.90 ± *5.50* $_{EFG}$	75.13 ± *4.44* $_{ABCDE}$	0.075 ± *0.017* $_{ABCDEF}$	26.92 ± *3.89* $_{bcdef}$
27					75	32.67 ± *2.68* $_{BCDEFG}$	68.99 ± *2.22* $_{EFGHIJ}$	0.075 ± *0.015* $_{ABCDEF}$	27.51 ± *2.33* $_{bcdef}$
28				HP	60	34.86 ± *13.48* $_{ABCD}$	69.38 ± *3.10* $_{DEFGHIJ}$	0.082 ± *0.015* $_{AB}$	29.08 ± *3.16* $_{ab}$
29			9	WB	50	34.38 ± *6.23* $_{ABCD}$	73.01 ± *10.06* $_{ABCDEFGHI}$	0.073 ± *0.017* $_{ABCDEFGH}$	29.46 ± *5.04* $_{ab}$
30					60	30.06 ± *8.14* $_{CDEFG}$	75.69 ± *4.41* $_{ABCD}$	0.061 ± *0.017* $_{FGHI}$	24.77 ± *4.88* $_{def}$
31					75	38.37 ± *2.92* $_{AB}$	70.69 ± *2.37* $_{BCDEFGHIJ}$	0.063 ± *0.009* $_{DEFGHI}$	25.74 ± *2.40* $_{bcdef}$
32				HP	60	39.43 ± *4.62* $_{A}$	69.35 ± *3.76* $_{DEFGHIJ}$	0.080 ± *0.016* $_{ABCD}$	28.50 ± *2.14* $_{abcd}$
						$p = 0.0003$	$p = 0.0005$	$p = 0.0058$	$p = 0.0223$

[1]Protocol number; [2]dilution (v:v); [3]equilibration at 4 °C (min); [4]dimethylacetamide (% final concentration); [5]thawing; [6]water-bath; [7]hot-plate; [8]thawing temperature °C; [9]live F-T sperm rel. to counted F-T sperm; [10]normal F-T sperm rel. to live F-T sperm; [11]mobility expressed as absorbance units at 550 nm. $_{A–J}$ Means within a column with different subscripts are significantly different ($p < 0.01$). $_{a–f}$ Means within a column with different subscripts are significantly different ($p < 0.05$).

in terms of the percentages of normal cells, which remained high. Higher viability was observed with the HP, particularly with the 1:2 dilution. Considering the mobility parameters, the results tended to be higher with the 1:2 dilution, particularly when combined with the HP or WB at 50 °C. The top five mean mobility values obtained using the 1:2 dilution were protocols 25 (6%-WB-50 °C), 28 (6%-HP), 20 (6%-HP), 24 (9%-HP) and 32 (9%-HP). And the top five protocols (with 1:2 dilution) obtaining the highest percentages of recovered mobility were 25 (6%-WB-50 °C), 29 (9%-WB-50 °C), 20 (6%-HP), 28 (6%-HP) and 17 (6%-WB-50 °C).

Table 6 reports the 32 F-T protocols tested and their effects on the incidence of head and tail injuries in rooster sperm. Fractured cells were mainly observed in protocols employing a higher WB temperature, especially with 1:1 dilution ($p < 0.01$). Protocol number 24 (using the HP) produced the lowest number of fractured cells ($p < 0.01$). Swollen-detached cells were particularly common in HP protocols, especially compared with the values obtained using the WB at either 50 °C or 60 °C ($p < 0.01$). The incidence of knotted cells generally remained below 1% and was highly variable ($p < 0.05$). Most of the headless sperm were observed in protocols employing the 1:1 dilution and the WB at 50 °C or 60 °C. No differences were observed between the protocols in the frequency of bent or coiled heads or looping or coiling tails.

Discussion

A key parameter influencing the success of avian sperm cryopreservation is the quality of the fresh material (Blesbois 2011; Castillo et al. 2021). In the present study, the mean ejaculate volume (0.44 mL) and sperm concentration (4.75×10^9/mL) of fresh semen obtained from 42 to 47-week-old Cobb broiler breeders were higher than 0.35 mL and 3.52×10^9/mL reported for the same breed (24–54-week-old birds) (Cerolini et al. 2006) and for 45-week-old Ross line broiler breeders too (Teymouri zadeh et al. 2020). One of the key factors influencing the quality of collected ejaculates is the collection technique (Łukaszewicz et al. 2015; Krohn et al. 2019; Castillo et al. 2021); thus, use of the cooperative method in the present study, versus the conventional method in the above-

Table 6. Head and tail injuries (%) in cryopreserved rooster sperm following 32 freezing protocols, comprising all the combinations of two variations at each freezing step (dilution, Eq4°C, DMA concentration) and four different thawing conditions (mean ± *SD*).

						Head			Tail
n.[1]	D[2]	Eq4°C[3]	DMA[4]	Th[5]	t° Th[8]	Fractured	Swo-det[9]	Knotted	Head-less
1	1:1	10	6	WB[6]	50°	18.33 ± *1.25* $_{BCDEF}$	1.93 ± *1.47* $_{E}$	0.86 ± *1.30* $_{abcdefgh}$	1.03 ± *0.57* $_{CDEFGH}$
2					60°	21.38 ± *4.92* $_{AB}$	1.46 ± *0.84* $_{EF}$	0.39 ± *0.37* $_{bcdefgh}$	1.38 ± *0.78* $_{ABCDEFG}$
3					75°	20.29 ± *3.51* $_{ABC}$	4.74 ± *1.38* $_{D}$	0.27 ± *0.45* $_{efgh}$	0.77 ± *0.79* $_{EFGH}$
4				HP[7]	60°	14.85 ± *4.43* $_{EFGH}$	7.07 ± *1.99* $_{ABCD}$	0.32 ± *0.64* $_{fgh}$	0.57 ± *0.47* $_{GH}$
5			9	WB	50°	18.37 ± *3.26* $_{BCDEFG}$	1.50 ± *1.09* $_{EF}$	0.60 ± *0.35* $_{abcdefg}$	3.34 ± *2.11* $_{A}$
6					60°	17.62 ± *3.13* $_{BCDEFG}$	2.06 ± *2.41* $_{EF}$	0.42 ± *0.42* $_{bcdefgh}$	1.94 ± *0.92* $_{ABCD}$
7					75°	19.75 ± *3.21* $_{ABCD}$	6.39 ± *1.85* $_{ABCD}$	0.28 ± *0.32* $_{cdefgh}$	1.51 ± *0.28* $_{ABCDEF}$
8				HP	60°	13.77 ± *2.34* $_{FGH}$	7.36 ± *3.18* $_{ABCD}$	0.30 ± *0.36* $_{cdefgh}$	1.21 ± *1.03* $_{BCDEFGH}$
9		30	6	WB	50°	25.54 ± *11.05* $_{A}$	1.15 ± *1.30* $_{EF}$	0.51 ± *0.41* $_{abcdefgh}$	0.91 ± *0.45* $_{BCDEFGH}$
10					60°	18.93 ± *4.43* $_{BCDEF}$	0.89 ± *0.73* $_{EF}$	1.05 ± *0.64* $_{ab}$	2.19 ± *1.96* $_{ABCDE}$
11					75°	19.65 ± *5.20* $_{ABCDE}$	4.80 ± *1.98* $_{CD}$	0.33 ± *0.37* $_{bcdefgh}$	1.00 ± *0.77* $_{CDEFGH}$
12				HP	60°	17.97 ± *4.00* $_{BCDEFG}$	7.66 ± *2.28* $_{ABC}$	0.37 ± *0.64* $_{efgh}$	0.41 ± *0.43* $_{H}$
13			9	WB	50°	21.72 ± *4.11* $_{AB}$	1.51 ± *0.98* $_{EF}$	0.72 ± *0.45* $_{abcdefg}$	2.76 ± *2.45* $_{AB}$
14					60°	15.13 ± *4.20* $_{DEFGH}$	1.74 ± *1.56* $_{EF}$	0.80 ± *0.74* $_{abcdefg}$	0.76 ± *1.16* $_{FGH}$
15					75°	19.71 ± *4.12* $_{ABCD}$	5.47 ± *2.41* $_{BCD}$	0.20 ± *0.24* $_{efgh}$	0.93 ± *0.40* $_{BCDEFGH}$
16				HP	60°	14.11 ± *2.27* $_{FGH}$	7.54 ± *2.00* $_{ABCD}$	0.20 ± *0.32* $_{efgh}$	1.23 ± *0.81* $_{BCDEFGH}$
17	1:2	10	6	WB	50°	17.78 ± *9.89* $_{BCDEFG}$	1.17 ± *1.34* $_{EF}$	0.27 ± *0.34* $_{defgh}$	0.55 ± *0.51* $_{GH}$
18					60°	16.84 ± *2.94* $_{BCDEFG}$	1.65 ± *1.62* $_{EF}$	0.33 ± *0.37* $_{bcdefgh}$	1.93 ± *2.21* $_{ABCDEFG}$
19					75°	19.73 ± *4.15* $_{ABCD}$	4.86 ± *1.49* $_{CD}$	0.09 ± *0.23* $_{h}$	1.00 ± *0.77* $_{CDEFGH}$
20				HP	60°	13.56 ± *3.78* $_{GH}$	6.94 ± *2.35* $_{ABCD}$	0.63 ± *0.72* $_{abcdefgh}$	0.75 ± *0.46* $_{EFGH}$
21			9	WB	50°	16.70 ± *5.30* $_{BCDEFGH}$	1.62 ± *0.59* $_{EF}$	0.85 ± *0.60* $_{abcde}$	3.07 ± *2.90* $_{ABC}$
22					60°	16.15 ± *3.67* $_{CDEFGH}$	1.39 ± *0.99* $_{EF}$	1.11 ± *1.19* $_{abc}$	1.37 ± *1.07* $_{BCDEFG}$
23					75°	16.79 ± *3.83* $_{BCDEFG}$	5.65 ± *1.97* $_{BCD}$	0.59 ± *0.40* $_{abcdefg}$	0.75 ± *0.60* $_{DEFGH}$
24				HP	60°	11.85 ± *3.04* $_{H}$	8.22 ± *2.39* $_{AB}$	0.37 ± *0.34* $_{bcdefgh}$	0.75 ± *0.54* $_{EFGH}$
25		30	6	WB	50°	15.98 ± *7.47* $_{CDEFGH}$	1.50 ± *1.33* $_{EF}$	1.24 ± *0.75* $_{a}$	0.65 ± *0.57* $_{FGH}$
26					60°	17.86 ± *3.42* $_{BCDEFG}$	0.93 ± *0.89* $_{EF}$	0.94 ± *0.70* $_{abcd}$	1.41 ± *1.57* $_{BCDEFGH}$
27					75°	18.37 ± *3.20* $_{BCDEF}$	4.63 ± *1.62* $_{D}$	0.46 ± *0.42* $_{abcdefgh}$	0.55 ± *0.59* $_{GH}$
28				HP	60°	15.48 ± *3.13* $_{CDEFGH}$	5.72 ± *3.45* $_{BCD}$	0.74 ± *0.44* $_{abcdef}$	0.59 ± *0.24* $_{EFGH}$
29			9	WB	50°	18.40 ± *6.06* $_{BCDEFG}$	1.30 ± *0.54* $_{EF}$	1.23 ± *1.33* $_{ab}$	1.69 ± *1.07* $_{ABCDEF}$
30					60°	16.04 ± *8.08* $_{BCDEF}$	0.64 ± *0.65* $_{F}$	0.28 ± *0.29* $_{bcdefgh}$	0.73 ± *0.77* $_{EFGH}$
31					75°	18.17 ± *1.44* $_{BCDEFG}$	4.93 ± *1.70* $_{CD}$	0.32 ± *0.33* $_{bcdefgh}$	0.93 ± *0.54* $_{BCDEFGH}$
32				HP	60°	14.27 ± *3.80* $_{EFGH}$	9.35 ± *2.03* $_{A}$	0.29 ± *0.34* $_{cdefgh}$	0.97 ± *0.57* $_{BCDEFGH}$
						$p = 0.0009$	$p < 0.0001$	$p = 0.0333$	$p = 0.0033$

[1]Protocol number; [2]dilution (v:v); [3]equilibration at 4 °C (min.); [4]dimethylacetamide (% final concentration); [5]thawing; [6]water-bath; [7]hot-plate; [8]thawing temperature °C; [9]swollen-detached. $_{A–H}$ Means within a column with different subscripts are significantly different ($p < 0.01$). $_{a–h}$ Means within a column with different subscripts are significantly different ($p < 0.05$).

cited studies on broiler breeders, may have positively influenced these parameters. The sperm viability in this trial was lower (73% vs. 82%) than a previous study of breeders of the same type (Castillo et al. 2010), and of Ross line breeders too (Teymouri zadeh et al. 2020). The mean sperm mobility (0.340) of fresh sperm was higher than recorded in our previous trial (0.250) (Castillo et al. 2010). In breeder turkey toms, sperm mobility ranged from 0.147 to 0.366 (Donoghue et al. 2003), indicating that mobility can vary greatly between facilities, among species and breeds (Manier et al. 2019).

Effects of dilution rate were observed on spermatozoa viability, the incidence of normal cells, and sperm mobility in F-T sperm. Comparing all 32 freezing protocols, the more favourable results in terms of normal cells and sperm mobility were obtained at the higher dilution rate (1:2). Additionally, a lower incidence of fractured cells emerged in the more diluted sperm. In accordance, higher fertility rates were reported for a higher dilution rate of cryopreserved sperm from commercial pedigree chickens (1:3 vs. 1:4) (Thelie et al. 2019). Indeed, high dilution is known to be favourable for chicken sperm cell survival (Woelders 2021). As it is highly sensitive to products of cell metabolism in its environment, higher dilution rates may act favourably by diluting out these products (Thelie et al. 2019). Moreover, a higher dilution rate increases the available space for each cell, with consequent higher nutrient accessibility (Woelders 2021) and a higher availability of cryoprotectant (Thelie et al. 2019). The effect of dilution also depends greatly on the kind of diluent used (Parker and McDaniel 2006). For instance, the performance of F-T sperm from turkeys was positively impacted by a higher cryopreservation dilution in association with diluent type and thawing temperature (Iaffaldano et al. 2011).

No effect of Eq4 °C time was observed on the qualitative sperm parameters or on the incidence of sperm injuries in this study, confirming the findings of previous studies on rooster sperm (which considered 20 vs. 40 min) (Gliozzi et al. 2017). In the Eq4 °C phase of the

cryopreservation protocol, cell metabolism begins to slow, and events occurring throughout the cooling process have the potential to influence post-thaw sperm survival (Dias et al. 2018). The optimal cooling rate depends greatly on the characteristics of cell membrane permeability, which are associated with membrane composition and the type and concentration of the cryoprotectant (Sieme et al. 2015). In chickens, turkeys and guinea fowls, membrane fluidity can be used as an indicator of the sperm cells' ability to survive the F-T process and recover their physiological state (Blesbois 2011).

Cryoprotectant concentration (6% vs. 9%) had no effect on the percentage of normal cells or on the sperm mobility parameters, as similarly reported by Gliozzi and colleagues (Gliozzi et al. 2017). However, the higher DMA concentration did protect a higher percentage of cells from death, again confirming the above-cited study by Gliozzi et al. and was associated with a lower incidence of looping tails. By contrast, a higher incidence of headless cells was associated with the higher DMA concentration. Other authors, using the straw method as a packaging system, also reported better sperm cell viability with 9% DMA, but this was observed in association with a higher rate of sperm motile function as well (Mosca et al. 2019). That said, in another study of pelleted sperm, thawed using the WB, the viability was not affected by DMA concentration, although higher numbers of motile cells were obtained with DMA at 6% vs. 9% (Zaniboni et al. 2014). Authors have suggested that the toxic effect of directly adding sperm to a high DMA concentration can be reduced by adding the cryoprotectant to the stock solution instead of directly to the diluted sperm, thereby improving sperm motility (Tang et al. 2021). In our study, considering all 32 protocols, no clear advantage of either DMA concentration was evident, although the protocols associated with the highest mobility parameters were all achieved using 6% DMA and independently of the thawing method adopted (WB and HP).

The most favourable method for thawing 80 μL pellets in terms of sperm mobility was the HP, followed by the WB at 50 °C. Although the HP resulted in higher numbers of swollen-detached cells, the percentage of fractured cells was greater with the WB. Better fertility rates have previously been observed for pelleted sperm thawed using the HP compared with the WB, as well as compared with straw packaged sperm thawed in a WB (Tselutin et al. 1999). The thawing of pelleted sperm on a hot-plate permits a faster melting rate, which is less detrimental to the cell (Tselutin et al. 1999). When sperm pellets are thawed slowly at temperatures above their critical freezing temperature, which ranges from −15 °C to −60 °C, the sperm are subjected to physical damage due to ice recrystallization (Gao and Critser 2000; Kumar et al. 2019). By contrast, thawing at faster rates leads to osmotic damage due to the cryoprotectant not being able to leave the cell fast enough, resulting in the osmotic entry of extracellular water into the cell, provoking cell swelling (Kumar et al. 2019). In the present study, the higher percentage of swollen-detached cells observed when pellets were thawed using the HP suggests that this may well have happened. Other authors observed evidence of osmotic shock in sperm being cooled at high rates, and thereafter thawed at low temperature rates, and they attributed the osmotic imbalance generated during the thawing process to diffusion limited ice crystallisation in the extracellular fluid occurring during the freezing process (John Morris et al. 2012). On the contrary, no evidence of intracellular ice in the sperm head was found with either ultra-rapid or slow cooling rates for mammalian species (Bóveda et al. 2020). Contrary to previous reports which ascribe the damage incurred to sperm by rapid cooling rates to the formation of ice inside the cell (Mazur et al. 1972; Blesbois 2011), it seems that sperm intracellular vitrification is quite easy to achieve due to their small size, even under slow-cooling rates (Isachenko et al. 2003; Bóveda et al. 2020). That said, extracellular ice crystals continue to be formed (Bóveda et al. 2020).

Among the parameters used to evaluate sperm quality, mobility values are among the most reliable for predicting sperm quality (Froman et al. 1997; Birkhead et al. 1999). This parameter is a quantifiable and heritable trait and considered to be a primary determinant of overall rooster fertility, as demonstrated in previous reports in which male selection according to sperm mobility led to an increase in the number of fertilised eggs (Froman et al. 1997; Birkhead et al. 1999; Jarrell et al. 2020). Sperm with suitable mobility are preferentially selected within the female bird's oviduct (Bakst et al. 1994). In tests of sperm mobility, sperm must make a forward progression against a resistant dense layer, simulating the pathway within the female oviduct tract (Froman et al. 1997). Contrarily, this penetrating effort is not considered in conventional assessment for progressive sperm motility, where saline aqueous solution is used to record sperm kinetics. One report showed that greater numbers of low-mobility sperm are associated with a higher number of early embryo deaths (Manier et al. 2019), although the same authors

also showed that high-mobility sperm are not necessarily more fertile, and differences among species and breeds may exist (Manier et al. 2019). In a previous study, we reported mobility values of 0.250 absorbance units for fresh sperm and recovered mobility rates of 30% post thawing, resulting in a 33% fertility capacity of F-T sperm, with approx. 50% of fertilised eggs hatching (Castillo et al. 2010). Similarly, in pheasants we recorded a mean mobility value of 0.270 absorbance units for fresh sperm, and a fertility rate of 30% after the F-T process, with 29% of eggs hatching (Castillo et al. 2021). Additionally, these data were obtained with inseminating doses of approx. 42×10^6 and 35×10^6 viable normal cells in chickens and pheasants, respectively (Castillo et al. 2010, 2021). Therefore, considering the high mobility of the fresh sperm obtained in this study and the data for all the protocols investigated, the main parameter of choice for assessing F-T protocols was sperm mobility. Consequently, the best combination of protocol parameters was 1:2 dilution, Eq4 °C for 30 min, 6% DMA, and either the WB at 50 °C or HP as thawing method (protocols 25 and 28, respectively); followed by 1:2 dilution, Eq4 °C for 10 min, 6% or 9% DMA, and the HP thawing method (protocols 20 and 24, respectively).

Conclusions

Broiler rooster sperm exhibited high susceptibility to damage caused by the freezing-thawing processing of sperm pellets, with DMA as cryoprotectant; however, the survival of normal sperm cells reached a rate of 39%, and the recovered mobility of these cells stood at 32%. The thawing method is just as important as the freezing process, and considering the parameter used to predict fertilising capacity (i.e. sperm mobility), the hot-plate and the water-bath at 50 °C seem to be the best methods for sperm cells packed in 80 µL pellets. However, as observed in this study, the combined effect of all the protocol steps must be considered when tailoring the cryopreservation protocol to a specific species/breed, rather than the single variables at each critical step.

For the purposes of gene banking, in which high safety standards and easy sample identification are required, the straw method is undoubtedly the better choice; however, cryopreserved sperm pellets may be more suitable in other contexts, such as small farms, and still achieve good results. Moreover, the pelleting technique does not involve any sophisticated equipment and the entire process can be completed very quickly.

Ethical approval

This study was approved by the Ethics Committee of Pisa University (Ref.: OPBA_33/2021), under article.2, paragraph.1, point b, of the Italian legislative decree n. 26/2014.

Disclosure statement

No potential conflicts of interest are reported by the author(s).

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Data availability statement

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

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