Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time

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ABSTRACT A sperm cryopreservation protocol requiring dimethylacetamide (DMA, 6%) as a cryoprotectant was optimized via assays involving different prefreezing equilibration times (1, 10, 30, 60, and 120 min at 5°C) and different freezing rates achieved by the following: 1) using nitrogen vapor to reduce the temperature from 5°C to −85°C at 10°C/min (slow freezing rate); 2) using a biological freezer unit in a 2-step method to reduce the temperature from 5°C to −35°C at 7°C/min and then from −35°C to −140°C at 60°C/min (medium freezing rate); or 3) using a biological freezer unit in a 1-step freezing method to reduce the temperature from 5°C to −180°C at 60°C/min (rapid freezing rate). Heterospermic semen samples from chicken breeds raised as part of a Spanish genetic resource conservation program were used in all assays. The 1-min equilibration treatment was associated with a lower percentage of viable thawed spermatozoa than the 30-min treatment (P < 0.05). The remaining sperm variables studied were not affected by equilibration time. The medium-rate 2-step freezing method was associated with a higher percentage of motile spermatozoa after thawing and with greater acrosome integrity (P < 0.05) than the slow nitrogen vapor or rapid 1-step methods. Thawed sperm movement quality and plasma membrane integrity (as assessed by the hypoosmotic swelling test) were better (P < 0.05) in samples frozen by the medium-rate 2-step freezing method than in those subjected to the slow nitrogen vapor method. Fertility was not influenced by freezing method, although that achieved with the medium rate 2-step freezing method showed a trend toward being greater than that achieved with the rapid 1-step method (P = 0.07). Together, the present results suggest that slow cooling rates are not recommendable when using dimethylacetamide. The 2-step freezing method may be useful in the establishment of a germplasm bank for Spanish chicken breeds.

Key words: cryobank, equilibration time, freezing rate, Spanish breed, spermatozoa

INTRODUCTION

Spermatozoa are the most accessible sex cells and are currently the main type preserved in the majority of genetic resource banks (Blesbois et al., 2007). Despite the recognized importance of poultry genetic resources, few countries have taken serious steps to protect them. However, several national programs (e.g., in France, the United States, and the Netherlands) are currently underway to cryopreserve chicken semen (Blesbois and Brillard, 2007). The French Avian Cryobank came about by making use of semen cryopreserved as part of the ex situ management of chicken genetic diversity (Blesbois et al., 2007). In the United States, the USDA established the National Animal Germplasm Program in 1999, and the semen of many chicken breeds has been cryopreserved (Blackburn, 2006). In the Netherlands, the Center for Genetic Resources has undertaken several studies designed to optimize the cryopreservation of poultry semen (Woelders et al., 2006).

Different Spanish chicken breeds have been raised as part of a genetic resources conservation program since 1975 (Campo and Orozco, 1982; Campo, 1998). Given the risks of epidemics such as avian influenza, the Spanish Ministry for Science and Innovation, via the Spanish National Institute for Agricultural and Food Research, recently supported the establishment of a germplasm bank for Spanish chicken breeds.

Many cryoprotectants and freezing protocols have been used in the production of poultry breed cryobanks (Tselutin et al., 1999). Although glycerol is usually regarded as a suitable cryoprotectant for fowl semen...
(Seigneurin and Blesbois, 1995), it has biological limitations (e.g., a contraceptive effect; Hammerstedt and Graham, 1992). It must therefore be removed before the use of semen in artificial insemination, which is an important drawback. Further, the necessary postthawing centrifugation of glycerol-treated spermatozoa leads to their damage. Dimethylacetamide (DMA) may offer an alternative because semen samples can be thawed without further processing, and high levels of fertility have been obtained with this cryoprotectant (Chalah et al., 1999; Tselutin et al., 1999). The best fertility rates are obtained with DMA when the spermatozoa are frozen in pellets (Blesbois et al., 2007), but this method does not allow for the adequate identification of semen samples. Further, cross-contamination can occur (Wishart, 2009). Optimizing cryopreservation involving DMA as the cryoprotectant and the use of straws for semen packaging should therefore be a goal.

Because the highest fertility rates are achieved with DMA when semen is frozen rapidly (Chalah et al., 1999), it might be hypothesized that the cryoprotective capacity of DMA is reduced at lower freezing rates (the opposite of that seen with glycerol). The aims of the present work were 1) to identify the minimum equilibration time required for DMA to exert its cryoprotective effects on semen samples of chicken breeds involved in the Spanish genetic resources conservation program, and 2) to evaluate the cryoprotective capacity of DMA at different freezing rates, comparing a slow freezing rate involving nitrogen vapor, a medium rate involving a 2-step freezing procedure described previously for use with glycerol (Blesbois and Brillard, 2007; Blesbois et al., 2008), and a rapid rate involving a 1-step freezing method similar to that reported by Blesbois et al. (2007) and designed for use with DMA.

Because the tolerance of poultry sperm to cryopreservation varies among chicken breeds (Long, 2006), a breed-dependent response to the freezing process might be expected. However, designing specific protocols for each breed would be costly and require a great deal of time. The present work therefore proposed to make use of pooled samples of semen from each breed in an attempt to develop a single freezing protocol for use with all the breeds in the planned conservation program.

**MATERIALS AND METHODS**

**Experimental Birds**

The birds used in this study were 12 Spanish chicken breeds (Black-Barred Andaluza, Black-Red Andaluza, Blue Andaluza, Black Castellana, Buff Prat, White Prat, Red-Barred Vasca, Red Villafranquina, Birchen Leonesa, Blue Leonesa, White-Faced Spanish, and Black Menorca), 2 synthetic Spanish breeds (Quail Castellana and Quail Silver Castellana), a selected White Leghorn population (originated by crossing 3 strains selected for egg number and egg weight; Campo and Jurado, 1982), and the eV tester line (Smyth, 1976). The synthetic breeds Quail Castellana and Quail Silver Castellana originated from an F2 cross between Black Castellana and Buff Prat birds (Campo and Orozco, 1986; Campo, 1991).

A total of 32 selected roosters (2 of each breed), all of which were 1 yr old at the beginning of the experiment, were housed under natural photoperiod and temperature conditions in 2 sand-floor pens (12 m²) with partial roof cover at the El Encín Research Station (Madrid, Spain, 40°31'N). These birds were raised as part of the genetic resources conservation program of the Spanish National Institute for Agricultural and Food Research (Campo and Orozco, 1982; Campo, 1998). These semen donors were selected via the preliminary assay of sperm variables predictive of fertility (Santiago-Moreno et al., 2009a). All birds were fed a commercial feed containing 16% CP, 2,700 kcal of ME/kg, 3.5% Ca, and 0.5% available P over the entire experimental period.

**Semen Collection and Prefreezing and Postthawing Assessment of Sperm Variables**

Semen was collected once weekly over the study period in 15-mL graduated centrifuge tubes (Sterilin, Sterilin Ltd., Newport, UK) using the massage technique described by Burrows and Quinn (1937). Pools of semen from the 32 roosters were made on each occasion. Each pool was immediately diluted 1:1 (vol/vol) at field temperature using a medium (hereinafter referred to as Lake and Ravie medium; Lake and Ravie, 1984) comprising sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate 4H₂O (0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone [relative molecular mass (M_r) = 10,000; 0.3 g], and H₂O (100 mL; final pH 7.08, final osmolality 343 mOsm/kg). This diluted, pooled semen was then immediately cooled to 5°C and transported to the laboratory, and sperm concentration and sperm variables (sperm motility, plasma membrane integrity, acrosome integrity) were examined (within 45 min of collection) before freezing. Sperm concentrations were calculated by determining the light absorption of the semen (Bakst and Cecil, 1997) at a wavelength of 540 nm using a spectrophotometer (Spectronic 20, Bausch and Lomb Co., Rochester, NY). All pools were then further diluted as required with Lake and Ravie medium to a concentration of 1,200 × 10⁶ sperm/mL for freezing via the methods described below. All sperm variables were measured again for each pool after their eventual thawing.

Sperm motility was assessed by placing a small droplet of each sample, previously diluted 1:21 (vol/vol) in Lake and Ravie medium, on a warmed (37°C) glass slide. The percentage of motile spermatozoa was evaluated subjectively using a phase-contrast microscope (Zeiss, Oberkochen, Germany) at 100×. The quality of motility was scored on a scale of 0 (lowest) to 5 (highest): 0 = no movement, 1 = tail movements but
no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement, and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement. Plasma membrane integrity was assessed by means of the hypoosmotic swelling test according to the procedure described by Santiago-Moreno et al. (2009b). This test enables the determination of the functional intactness of sperm membrane as spermatozoa swell (positive endosmosis) under hypoosmotic conditions due to the influx of water, and the expansion of the membranes causes coiled mid pieces and tail. Propidium iodide (PI) was used as a fluorochrome for the fluorescent examination of membrane integrity. Propidium iodide is a DNA-specific stain that cannot cross the intact plasma membrane; it therefore allows the identification of viable spermatozoa, which exclude the dye. Stock solutions of PI (1 mg of PI and 1 mL of PBS) were stored frozen in 50-μL aliquots. Spermatozoa (5 μL) were diluted with 500 μL of HEPES medium (20 mM HEPES, 197 mM NaCl, 2.5 mM KOH, and 10 mM glucose). The medium had a pH of 6.7 at room temperature and an osmolality of 350 mOsm/kg. Five microliters of the PI stock solution was added to the diluted sperm suspension and incubated at room temperature for 10 s. Samples were evaluated using an epifluorescence microscope at 1,000× (wavelength: 450–490 nm). Spermatozoa with no PI staining were deemed live.

The percentage of spermatozoa with an intact acrosome was determined by examining 200 aniline blue-stained cells by phase-contrast microscopy (magnification 1,000×), following the procedure of Santiago-Moreno et al. (2009b). Because extender containing DMA prevents the adequate staining of sperm cells, frozen–thawed samples were previously washed with DMA, thus leaving a final 6% DMA concentration (vol/vol) with Lake and Ravie medium containing 18% DMA, and kept for equilibration at 5°C for 1, 10, 30, 60, or 120 min. After equilibration, these samples were loaded into 0.25-mL French straws (Minitüb, Landshut, Germany) and cooled semen (see above) was further diluted 1:0.5 (vol/vol) with Lake and Ravie medium containing 18% DMA, thus leaving a final 6% DMA concentration (1,585 mOsm/kg, pH 6.90). Five aliquots were prepared and kept for equilibration at 5°C for 1, 10, 30, 60, or 120 min. After equilibration, these samples were loaded into 0.25-mL French straws (Minitüb, Landshut, Germany) and frozen by placing them directly into the nitrogen vapor 5 cm above the surface of a liquid nitrogen bath (a 1.1-L expanded polythene box with a top surface area of 560 cm²) for 10 min before plunging them into the liquid nitrogen itself. Because the freezing rate obtained with nitrogen vapor may vary depending on the size of the bath and on the time that elapses between when the bath is first filled and when the straws are suspended in the nitrogen vapor, the method was standardized using a thermometer equipped with a probe resistant to freezing (Ventix K/J/T thermometer; Ventix, China). The time elapsed between the filling of the bath and the straws being suspended in the nitrogen vapor was 30 min. The straws were thawed after 15 d, for 30 s at 37°C in a water bath. The content of 1 straw per sample was poured into a polystyrene tube and assessed for sperm quality as above.

**Experiment 2: Effect of Freezing Rate on Frozen–Thawed Spermatozoa**

The semen collection period for this experiment lasted 7 mo (31 wk: January–July 2009). The most appropriate equilibration time obtained in the previous experiment (shorter time without negative effect on spermatozoa) was employed in all tests. Semen samples were diluted with DMA as above and loaded into 0.25-mL French straws, and then frozen at 3 freezing rates before being plunged into liquid nitrogen: 1) slow freezing rate, from 5°C to −85°C at 10°C/min; 2) medium freezing rate, from 5°C to −35°C at 7°C/min and then from −35°C to −140°C at 60°C/min; and 3) rapid freezing rate, from 5°C to −180°C at 60°C/min.

The slow freezing rate was achieved by placing the straws directly into nitrogen vapor 5 cm above the surface of liquid nitrogen (1.1 L) in an expanded polythene bath for 10 min as in experiment 1. The medium and rapid freezing rates were achieved using a biological freezer unit (Computer Freezer-Icetube 1810, Minitüb, Tiefenbach, Germany). After 15 d the straws were thawed for 30 s at 37°C in a water bath, as in experiment 1. The fertilization capacity of the sperm cells was estimated from the results of 4 consecutive intravaginal artificial insemination procedures involving 300 million sperm cells/female performed twice weekly. A total of 45 Blue Andaluza hens (15/treatment) were inseminated. All inseminations were performed between 1200 and 1400 h according to the method of Burrows and Quinn (1939). Eggs were collected from d 3 after the first insemination until d 3 after the last insemination. Fertility (% fertile/incubated eggs) was determined by candling eggs on d 7 of incubation.

**Statistical Analyses**

Sperm variables with a skewed distribution (determined by the Shapiro-Wilk test) before and after thawing were arcsine-transformed before statistical analysis. The influence of equilibration time (experiment 1) and freezing rate (experiment 2) on frozen–thawed sperm variables was analyzed by 2 way-ANOVA following the
statistical model $x_{ijk} = m + A_i + B_j + AB_{ij} + e_{ijk}$, where $x_{ijk}$ = the measured sperm variable, $m$ = the overall mean of variable $x$, $A_i$ = the effect of equilibration time (experiment 1) or freezing rate (experiment 2; $i = 1–6$ for experiment 1, with 1 for fresh semen; $i = 1–4$ for experiment 2, with 1 for fresh semen), $B_j$ = the effect of collection month ($j = 1–9$ for experiment 1; $j = 1–7$ for experiment 2), $AB_{ij}$ = the interaction between $A$ and $B$, and $e_{ijk}$ = the residual ($k = 1–39$ for experiment 1; $k = 1–31$ for experiment 2). Because no significant differences were found among months and the interaction month × treatment (or equilibration time) exerted no significant influence, 1-way ANOVA was performed following the statistical model $x_{ij} = m + A_i + e_{ij}$. A post hoc Newman-Keuls test was performed to compare the differences in mean sperm variable values between treatments (freezing rates and equilibration times). In experiment 2, differences between percentage fertility were analyzed using 1-way ANOVA, employing a model similar to that outlined above. All statistical calculations were undertaken using Statistica software for Windows (v. 9.1, series 2010, StatSoft Inc. Tulsa, OK).

### RESULTS

#### Influence of Equilibration Time on Frozen–Thawed Sperm Variables

No significant differences were seen in sperm variable values among months, nor did the interaction month × equilibration time have any significant effect. The freezing–thawing process significantly reduced ($P < 0.001$) the percentage of motile sperm, the quality of motility (according to the hypoosmotic swelling test), the percentage of viable spermatozoa (according to the PI test), and the percentage of normal acrosomes, at all equilibration times (Table 1). The equilibration time affected no sperm variables except for sperm viability (as examined with PI; Table 1). The percentage of spermatozoa showing membrane integrity after the 1-min equilibration time treatment was lower than that after the 30-min treatment ($P < 0.05$).

#### Effect of Freezing Rate on Frozen–Thawed Spermatozoa

No significant differences were seen in sperm variable values among months, nor did the interaction month × freezing rate have any significant effect. The freezing–thawing process significantly reduced ($P < 0.001$) the values of all sperm variables recorded (Table 2). The percentage of motile spermatozoa and acrosome integrity was higher ($P < 0.05$) in spermatozoa that underwent the medium freezing rate 2-step method than in those that underwent the slow nitrogen vapor and rapid 1-step methods. The quality of sperm movement and plasma membrane integrity (assessed by the hypoosmotic swelling test) were higher ($P < 0.05$) in spermatozoa that underwent the medium-rate 2-step method than in those subjected to the slow nitrogen vapor method (Table 2).

The freezing method had no effect on fertility rates. However, the figures achieved with the medium-rate 2-step method tended to be higher ($P = 0.07$) than those achieved with the rapid 1-step method (Table 3).

### DISCUSSION

These results of this work allow for the optimization of a sperm freezing protocol for use in the establishment of an avian cryobank that complements the current program for conserving Spanish poultry breeds (Campo and Orozco, 1982; Campo, 1998). Sperm permeability to a cryoprotectant depends on the cholesterol content of the plasma membrane, which may vary among breeds (Ansah and Buckland, 1982) or even among lines within a breed (Tajima et al., 1990). In addition, the presence of certain proteins in the seminal plasma

<table>
<thead>
<tr>
<th>Sample</th>
<th>Motile sperm (%)</th>
<th>Quality of motility</th>
<th>Positive endosmosis (%)</th>
<th>Viable spermatozoa (%)</th>
<th>Normal acrosome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh sample</td>
<td>76.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FTHS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-min ET</td>
<td>10.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10-min ET</td>
<td>11.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>30-min ET</td>
<td>12.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>21.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60-min ET</td>
<td>12.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>120-min ET</td>
<td>12.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>0.6</td>
<td>0.1</td>
<td>0.8</td>
<td>1.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a–c</sup>Different superscripts within columns indicate significant differences ($P < 0.05$).

<sup>1</sup>The quality of motility was scored on a scale of 0 (lowest) to 5 (highest); 0 = no movement, 1 = tail movements but no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement, and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement.

<sup>2</sup>Viable spermatozoa were propidium iodide negative.
Table 2. Sperm variable values for fresh heterospermic samples and for frozen–thawed heterospermic samples (FTHS) processed at different freezing rates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Motile sperm (%)</th>
<th>Quality of motility</th>
<th>Positive endosmosis (%)</th>
<th>Viable spermatozoa (%)</th>
<th>Normal acrosome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh samples</td>
<td>77.0a</td>
<td>4.2a</td>
<td>86.5a</td>
<td>43.9a</td>
<td>97.4a</td>
</tr>
<tr>
<td>FTHS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow nitrogen vapor method</td>
<td>9.9c</td>
<td>2.0c</td>
<td>13.0c</td>
<td>13.0b</td>
<td>5.9c</td>
</tr>
<tr>
<td>Medium-rate 2-step method</td>
<td>15.2b</td>
<td>2.6b</td>
<td>20.4b</td>
<td>24.1b</td>
<td>10.0b</td>
</tr>
<tr>
<td>Rapid 1-step method</td>
<td>9.7</td>
<td>2.1bc</td>
<td>15.0bc</td>
<td>14.4bc</td>
<td>5.4c</td>
</tr>
<tr>
<td>SEM</td>
<td>0.8</td>
<td>0.1</td>
<td>1.1</td>
<td>2.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Different superscripts within columns indicate significant differences (P < 0.05).

The quality of motility was scored on a scale of 0 (lowest) to 5 (highest); 0 = no movement, 1 = tail movements but no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement, and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement.

2Viable spermatozoa were propidium iodide negative.

Table 3. Fertility rates obtained with frozen–thawed heterospermic samples processed at different freezing rates

<table>
<thead>
<tr>
<th>Freezing rate</th>
<th>Mean fertility (no. of incubated eggs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow nitrogen vapor method</td>
<td>33.6 ± 13.4 (56)</td>
</tr>
<tr>
<td>Medium-rate 2-step method</td>
<td>40.7 ± 9.4 (81)</td>
</tr>
<tr>
<td>Rapid 1-step method</td>
<td>24.5 ± 9.5 (72)</td>
</tr>
</tbody>
</table>

of males of some lines might contribute to increased cryoprotectant permeability (Bentley et al., 1984). The model followed in the present experiments, based on the use of pooled, heterospermic semen samples from each breed, tries to provide a method adequate for use with all breeds. However, the lack of significant differences in semen parameters (e.g., no significant differences among months) may have been related to the extreme heterogeneity of the semen pool.

The type of cryoprotective agent and the equilibration time allowed are known to influence the quality and the fertilizing capacity of frozen–thawed fowl spermatozoa (Sexton and Fewlass, 1976; Bakst and Sexton, 1979). Data on sperm viability after freezing–thawing involving the use of different equilibration times are variable and depend on factors such as the freezing technique followed, the composition of the extender, and prefreezing handling. Some authors report 10 min to be the optimum equilibration time before freezing turkey semen when using dimethyl sulfoxide as a cryoprotectant (Sexton, 1979), whereas others report that no differences can be appreciated within the range of 10 to 90 min when using dimethyl sulfoxide plus ethylene glycol (Graham et al., 1982). High DNA concentrations appear to be very toxic to rooster spermatozoa (Woelders et al., 2006); a minimum equilibration time should therefore be allowed when using this cryoprotectant. Indeed, equilibration times as short as 2 min have been recommended for DMA (Tsutlin et al., 1995) whereas times up to 60 min have been successful with glycerol (Alexander et al., 1993). The present findings show that equilibration time had no overall effect on frozen–thawed sperm variables, although the percentage of spermatozoa showing membrane integ-

rity after the 1-min equilibration treatment was smaller than that achieved with the 30-min treatment. This suggests that 1 min may be insufficient for adequate cryoprotection to be achieved under the present experimental conditions, unlike that reported in previous studies (Tsutlin et al., 1999). Blesbois et al. (2007) recommend freezing for 1 min after the addition of DMA. However, whereas these authors used the same concentration of cryoprotectant as in the present work, they used a different freezing rate (50°C/min). It is well known that the interaction between cooling rate and cryoprotectant concentration has a significant effect on postthawing sperm variables (Woelders et al., 2006). Tsutlin et al. (1999) also recommend an equilibration period of 1 min when using DMA, but in these authors’ work cryopreservation was achieved by dropping fowl semen samples (about 50 μL/drop) directly into nitrogen liquid. This direct plunging method would bring about instant vitrification, or at least a state close to vitrification (Isachenko et al., 2005), in which the effect of cryoprotective agents is controversial. Moreover, the rate of cooling and the concentration of cryoprotectant required to achieve vitrification are inversely related (Isachenko et al., 2003) and vitrification can be achieved even in the absence of cryoprotectants if the rate of cooling is extremely rapid (Nawroth et al., 2002; Isachenko et al., 2003).

Surprisingly, the long equilibration times seemed not to affect any of the studied sperm variables despite the known cytotoxic effect of DMA. However, ultrastructural damage (e.g., in the DNA) that was not assessed in this study might have occurred (Woelders et al., 2006). Thus, in the second experiment, an equilibration time of 10 min was deemed appropriate. A similar equilibration time was successfully used for preserving gander semen when using DMA and packaging straws (Tai et al., 2001). Moreover, the choice of the shorter equilibration time without deleterious effects on sperm allows us to simplify the freezing process.

A slow freezing protocol was chosen in experiment 1 because the sperm freezing in nitrogen vapor was usually used in our laboratory and other laboratories working with avian spermatozoa in Spain. Because data of
rates similar to those achieved when using glycerol or with packaging straws must be improved if fertility cryopreservation method of using DMA in combination plasm bank for Spanish chicken breeds. However, the idea that the alteration of this structure is a primary cause of cryopreservation-associated fertility loss. The best preservation of the acrosome and membrane integrity was also seen with the 2-step freezing method. Rapid freezing is usually employed with DMA (Tselutin et al., 1995), whereas slow freezing rates are usually used with dimethyl sulfoxide (Sexton, 1980) and glycerol (Seigneurin and Blesbois, 1995). In the present study, the values of frozen–thawed sperm variables were poorest when the slow freezing rate was used, supporting the initial hypothesis that slow cooling rates are not recommendable when DMA is employed as a cryoprotectant. Although ultrarapid freezing (i.e., direct plunging into liquid nitrogen) was not contemplated in the present work, previous reports have shown that very good results are achieved with this method when DMA is used as the cryoprotectant (Tselutin et al., 1999). Although previous studies have recommended a rapid freezing rate of 50°C/min up to −140°C (Blesbois et al., 2007) or 59°C/min (Purdy et al., 2009), in the present work the rapid freezing rate of 60°C/min up to −180°C was associated with poorer sperm variable values than those obtained with the medium-rate 2-step freezing protocol.

Finally, thawing rate may affect the sperm variables and the outcome of insemination. It is generally accepted that thawing rates should mimic freezing rates, but all sperm were thawed for 30 s at 37°C. Hence, thawing of frozen sperm for 4 min at 5°C (Seigneurin and Blesbois, 1995) and for 25 s at 60°C (Chalah et al., 1999) should be tested for slow (10°C/min) and rapid (60°C/min) freezing rate protocols, respectively. In conclusion, the present data suggest that slow cooling rates are not recommendable when using DMA as a cryoprotectant. The medium-rate 2-step freezing method may be useful in helping establish the germplasm bank for Spanish chicken breeds. However, the cryopreservation method of using DMA in combination with packaging straws must be improved if fertility rates similar to those achieved when using glycerol or DMA with sperm pellets (around 80–90%; Chalah et al., 1999; Tselutin et al., 1999) are to be obtained.

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