Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm

Fabio Mosca[a,*], Manuela Madeddu[a], Ahmad Abdel Sayed[a], Luisa Zaniboni[a], Nicolaia Iaffaldano[b], Silvia Cerolini[a]

[a]*Department of Veterinary Medicine, University of Milan, via Trentacoste 2, 20134 Milan, Italy.*

[b]*Department of Agricultural, Environmental and Food Science, University of Molise, via De Sanctis, 86100 Campobasso, Italy.*

*Corresponding author. E-mail address: fabio.mosca1@unimi.it (F. Mosca)

ABSTRACT

The aim of this study was to assess the combined effect of dimethylacetamide (DMA) and two non-permeating cryoprotective agents, trehalose and sucrose, on the quality of post-thaw chicken semen. Adult Lohmann roosters (n=27) were used. Semen was processed according to the following treatments: Lake pre-freezing extender + 6% DMA (LPF, control treatment), LPF + 0.1 M trehalose (LPF-T treatment), LPF + 0.1 M sucrose (LPF-S treatment) and LPF + 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). Semen was loaded into straws and frozen in nitrogen vapor. Sperm quality (viability, mobility and kinetic parameters) was assessed immediately after thawing (T0) and at 5 (T5), 10 (T10) and 15 minutes (T15) thereafter. The different cryodiluent combinations significantly affected the kinetic parameters. The presence of trehalose, alone or with sucrose, combined with DMA improved the quality of motion in cryopreserved sperm in comparison to DMA alone (LPF) and DMA with sucrose (LPF-S). In particular, the highest values in linearity (LIN) and wobble (WOB) were measured in the treatment LPF-T. The treatments significantly affected the recovery rate of progressive motile sperm that presented the best value soon after thawing in the LPF-T treatment; moreover, the presence of trehalose, alone (LPF-T) or with sucrose (LPF-TS), significantly improved the recovery rate of progressive motile sperm also at T5 and T10 compared to LPF and LPF-S. The present results show a positive synergic action of DMA and trehalose on motile function of thawed chicken sperm.

Keywords: Chicken sperm; Semen cryopreservation; Cryoprotectants; DMA; Trehalose; Sucrose

1. INTRODUCTION

The most feasible method for *ex situ* management of genetic resources in birds is semen cryopreservation [7; 20; 22], which has firstly been studied in the chicken and then in other domesticated birds, such as turkey, duck and goose [6; 25; 40]. Despite years of research, the cryopreservation of poultry sperm still cannot be carried out efficiently [6; 28]. The low quality of frozen-thawed poultry semen as well as the poor fertilization rates, obtained in avian as opposed to mammalian species, are attributable to the unique morphological characteristics of avian sperm, which make them more susceptible to freezing damage [12; 28]. A variety of semen cryopreservation protocols involving different cryoprotective agents (CPAs), packaging methods and freezing and thawing rates have been developed in different poultry species [6; 8; 24]. The choice of the CPA is certainly among the most important factors for an effective poultry semen freezing protocol. Despite decades of research on the use of permeant CPAs (P-CPAs) [4; 39; 44], quality of avian sperm after freezing/thawing procedures and relative fertilization rates remain highly variable. In chickens, the average fertility after artificial insemination of frozen/thawed semen is equal to 60%, ranging from 0 to 90% [8]. In various mammalian species, sperm cryosurvival has been improved by combining P-CPAs with non-permeant CPAs (N-CPAs) [1; 21; 47]. The combination of P-CPAs and N-CPAs provides different mechanisms to protect spermatozoa during freezing/thawing procedures. P-CPAs increase membrane fluidity through rearrangement of membrane lipid and protein and partially dehydrate the cell, lowering the freezing point and thus reducing the formation of intracellular ice crystals, one of the main biophysical mechanisms of sperm death [23; 41]. However, P-CPA themselves could paradoxically have a toxic effect, related to its concentration and the time of cell exposure, causing sperm membrane destabilization and protein denaturation [41]. In contrast, N-CPAs are generally large, nontoxic, hydrophilic molecules (sugars, proteins or aminoacids) playing a different

protective effect: because of the inability to diffuse across the plasma membrane, these substances create an osmotic pressure that lowers the freezing temperature of the medium and decrease extra-cellular ice formation [2]. The use of N-CPAs, that act mainly as osmoprotectants, could reduce the amount of P-CPAs needed in sperm cryopreservation. Among the disaccharides, sucrose and trehalose are N-CPAs widely studied in different mammalian species: bulls [47], goats [1] boars [21] and rabbits [32]. In contrast, the effect of trehalose and sucrose on the post-thaw quality of poultry sperm was poorly studied and few reports are available. Recently, Blanco et al [4] tested trehalose and/or sucrose in combination with the P-CPA dimethylacetamide (DMA) and reported an improved post-thawing motility of turkey semen, which was dependent upon DMA concentration. Although sucrose and trehalose have received some attention as osmoprotectants for chicken sperm in the past [39; 43], there is a lack of current original studies on the effect of N-CPAs in this species.

The aim of this study was to assess the combined effect of DMA and the N-CPA trehalose and sucrose on the quality of post-thaw chicken semen.

2. MATERIAL AND METHODS

2.1 Bird management and semen collection

Twenty-seven adult Lohmann male fowl (*Gallus gallus domesticus*) were housed at 28 weeks of age in individual cages and kept at 20° C and 14L:10D photoperiod at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were fed *ad libitum* a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% CP) and drinking water. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching [14]. After 2-week semen collection

training period, all males were routinely collected twice a week from May to June. Semen was collected according to the technique initially described by Burrows and Quinn [11]. Each day of collection, males were divided in three different groups (nine birds/group) and all ejaculates collected within one group were pooled into one semen sample. Pools obtained in different days were always formed with different ejaculates to reduce the effect of the bird.

2.2 Semen processing for cryopreservation

The ejaculates were pooled into graduated tubes, semen volume was recorded and sperm concentration was measured after 1:200 dilution in 0.9% NaCl using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm [10]. Then, each pooled semen sample was split into four aliquots, each one assigned to one treatment. Semen aliquots were diluted to a concentration of 1.5 x 10^9 sperm/ml using 4 different cryodiluents: Lake pre-freezing modified extender (LPF, control treatment; 8 g D-fructose, 5 g potassiumacetate,19.2 g sodium glutamate, 3 g polyvinylpyrrolidone, 0.7 g magnesium acetate, 3.75 g glycine, adjusted to 1L with distilled water; pH 7.0, osmolality 340 mOsmol/kg), LPF added with 0.1 M trehalose (LPF-T treatment), LPF added with 0.1 M sucrose (LPF-S treatment) and LPF added with 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). The diluted semen was immediately cooled and kept at 4° C for 30 minutes. During this incubation, semen samples were transferred to the laboratory for further quality assessment and freezing processing. Sperm quality assessment included viability and motility. Sperm viability was measured using the dual fluorescent staining SYBR14/propidium iodide (PI) procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano [33] with minor modifications. In brief, the incubations were done at room temperature and the 7.1 diluent [26] was used. Assessment of 200 spermatozoa was made in duplicate aliquots for

every sample and evaluated microscopically at 1000X total magnification using a Zeiss (Axioskop 40- AxioCamICc 1) microscope and FITC filter fluorescence. Sperm motility was assayed using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh pooled semen samples were further diluted in refrigerated 0.9% NaCl to a sperm concentration of 100 x 10^6/ml and incubated for 20 minutes 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 motion parameters recorded were: motile spermatozoa (%), progressive motile spermatozoa (%), curvilinear velocity [VCL, (µm/s)], straight-line velocity [VSL, (µm/s)], average path velocity [VAP, (µm/s)], amplitude of lateral head displacement [ALH, (µm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)]. A minimum of 3 fields and 500 sperm tracks were analyzed at 100X total magnification for each sample. After the assessment of sperm quality, semen aliquots were further diluted to 1 x 10^9 sperm/ml with the corresponding extender (LPF, LPF-T, LPF-S and LPF-TS) containing 18% dimethylacetamide (DMA) to 6% final DMA concentration [48], equilibrated at 5°C for 1 min and loaded into 0.25-ml French straws (IMV Technologies, France). Four different straw colors were used according to the 4 different treatments. Straws were transferred on racks (made of wire netting supported by a Styrofoam frame) floating over a nitrogen bath at 3 cm of height [29], frozen for 10 min and then plunged into liquid nitrogen. Straws were stored in cryotank for at least 7 days. Semen collection was repeated on four days to process 12 pooled semen samples (12 replicates per treatment) and a total of 24 straws were stored per treatment. The straws were thawed in water bath at 38°C for 30 s and sperm quality was assessed in thawed semen. Sperm viability was recorded immediately after thawing (T0), and after 10 min; sperm

142 motility was recorded immediately after thawing (T0), and after 5 (T5), 10 (T10) and 15
143 minutes (T15). Sperm viability and motility were measured as previously described, with the
144 exception of using 0.9% NaCl at room temperature for sample dilution before sperm motility
145 analysis.
146
147 ***2.3 Statistical analysis***
148
149 Analysis of variance on sperm quality parameters recorded in fresh and frozen/thawed semen
150 samples was performed using the MIXED procedure of SAS [37]. Treatment (DMA;
151 DMA+trehalose; DMA+sucrose; DMA+trehalose+sucrose), time (fresh semen; 0, 5, 10 and
152 15 min after thawing), and the relative interaction (treatment * time) were considered as fixed
153 effects and the pooled semen sample was considered as random effect. The *t* test was used to
154 compare LSMeans.

155 The recovery rates (%) of sperm viability at different time (T0, T10) after cryopreservation
156 were calculated as follows: [(mean on thawed semen*100)/mean on fresh semen]. The same
157 formula was used to calculate the recovery rates (%) of sperm motility and progressive
158 motility at different time (T0, T5, T10, T15) after cryopreservation. Analysis of variance on
159 the recovery variables was performed using the GLM procedure of SAS [37], and the
160 treatment was the only source of variation included in the model. The *t* test was used to
161 compare LSMeans.
162
163 **3. RESULTS**
164
165 The mean volume and sperm concentration recorded in fresh ejaculates were 0.2 ± 0.06 ml
166 and 3.70 ± 0.78 x 10^9 sperm/ml respectively.

167 The results of the analysis of variance showed that the effect of time ($P < 0.001$) and of the
168 treatment ($P < 0.05$) significantly affected the majority of the semen quality parameters; in
169 contrast, the interaction time*treatment did not affected semen quality measured before and
170 after cryopreservation.
171 The majority of the sperm quality parameters were decreased by the freezing-thawing process,
172 and the further *in vitro* incubation after thawing, as reported in Table 1. A major decrease in
173 the mean values was recorded between fresh and frozen-thawed semen samples (T0) and a
174 further progressive significant decrease was recorded during 15 min interval after thawing.
175 The mean proportion of viable sperm recorded in fresh semen was 72% and a significant
176 progressive decrease to 36% and 28% was recorded after freezing/thawing at T0 and T10
177 respectively. The mean proportion of motile sperm showed a major significant decrease
178 between fresh and cryopreserved semen (T0) and a further progressive significant decrease at
179 5, 10 and 15 min after thawing (Table 1). A similar trend was also observed for all kinetic
180 parameters, with the exception of VCL and BCF. VCL mean values recorded during the
181 whole *in vitro* processing did not show significant changes, and BCF mean values recorded
182 before cryopreservation, at T0 and T5 were similar and a significant decrease occurred at T10
183 and T15 (Table 1).
184 The cryoprotectants did not significantly affected sperm viability, motility and progressive
185 motility, and similar mean values were recorded in all treatments (Table 2). In contrast, the
186 different DMA and N-CPAs, trehalose and sucrose, combinations significantly affected the
187 kinetic parameters, with the only exception of VCL. The presence of trehalose, alone or with
188 sucrose, combined with DMA improved the quality of motion in cryopreserved sperm in
189 comparison to DMA alone (LPF) and DMA with sucrose (LPF-S). In particular, the highest
190 values in LIN and WOB were measured in the treatment LPF-T (Table 2).

191 According to the analysis of variance, the treatment significantly affected the recovery rate of
192 progressive motile sperm only. High proportions of viable sperm were recovered immediately
193 after thawing (range 47-53%) and after ten minutes incubation (35-44%) and no differences
194 were found between treatments. The same result was found for the recovery rate of motile
195 sperm and the range value recorded at T0, T5, T10 and T15 was 33-42%, 27-34%, 20-27%
196 and 16-21% respectively. Low recovery rates of progressive motile sperm were found in all
197 treatments (Table 3). The best recovery rate of progressive motile sperm, 24%, was recorded
198 soon after thawing in the LPF-T treatment; moreover, the presence of trehalose, in LPF-T and
199 LPF-TS, significantly improved the recovery rate of progressive motile sperm also at T5 and
200 T10 compared to LPF and LPF-S. The recovery of progressive motile sperm progressively
201 decreased after thawing and very low similar values were found at T15 in all treatments,
202 included those with trehalose (Table 3).
203
204 **4. DISCUSSION**
205
206 Currently, the need for *ex situ* conservation of avian genetic resources is widely recognized
207 [19], but the storage of semen collected from rare breeds and/or pure lines in sperm cryobanks
208 has been so far considered in few national programs for conservation of animal genetic
209 resources, i.e. in France and The Netherland in Europe and in North America [3; 5; 8; 46]. In
210 2012, FAO guidelines for cryopreservation of animal genetic resources describe two
211 procedures for chicken semen characterized by different diluents, cryoprotectants and
212 temperature gradient [13]. A further cryoprotectant, methylacetamide, was successfully used
213 to develop a procedure for freezing chicken sperm in Japanese [38] and Korean breeds [27]
214 designated as 'Natural Monument'. Recent reviews confirmed the difficulty of obtaining
215 cryopreserved avian semen without significant loss of fertilizing potential [9] and the need

that future studies should be conducted with the aim of improving relative sperm fertility, dependent by sperm quality, and fertility duration after freezing [42]. Therefore, the standardization of cryopreservation procedures for chicken semen is still a matter of research.

In the present study, non-permeant cryoprotectants were tested in combination with DMA to improve chicken sperm survival and function, corresponding to the motion ability, after cryopreservation in semen packaged in straws, according to the FAO cryopreservation guidelines [13]. The effect of the concomitant presence of permeant- and non permeant cryoprotectants was assessed soon after thawing and after a short *in vitro* storage period in order to study the survival of sperm to the cryopreservation process and the potential cell lifespan after thawing. The final goal was to identify a reference procedure to be implemented in a sperm cryobank of Italian chicken breeds.

The control cryopreservation procedure used in the present study was adapted from a pellet procedure previously set up in our laboratory [48]. In order to improve the post-thaw quality of sperm packaged into straws, freezing was performed in nitrogen vapour 3 cm above the liquid nitrogen bath and thawing was performed at 38°C for 30 s [29]. The sperm viability, motility and progressive motility recorded in chicken semen samples frozen/thawed according to the control procedure were 35, 33 and 3% respectively. Using a similar cryopreservation method, lower viability (24%) and motility (15%) values were reported in frozen/thawed semen from Spanish chicken breeds [36]. Purdy et al. [31] also reported lower motility (15%) and progressive motility (1.8%) in chicken semen added with DMA and frozen over nitrogen vapour.

The proportions of viable, motile and progressive motile sperm recovered soon after thawing undergo a fast and progressive decrease within a very short interval. The loss is much more severe for sperm motility compared to viability; in fact, the proportion of motile and progressive motile sperm decrease by 52% and 74% respectively within 15 min after thawing,

241 whereas the decrease of viable sperm was almost 20% within 10 min. The kinetic parameters
242 indicative of the sperm quality motion follow a similar trend after thawing, even if the rate of
243 decrease is less consistent.
244 In various mammalian species, sperm cryosurvival was improved by combining P-CPAs with
245 N-CPAs [1; 21; 47] and, among N-CPAs, trehalose and sucrose were widely studied [1; 21;
246 32; 47]. A significant positive synergic action between glycerol and trehalose was reported in
247 1989 on motility of thawed chicken semen also [43]. However, since then no exhaustive
248 reports were published on the effect of sucrose and trehalose on the success of
249 cryopreservation in chicken sperm. The present study aimed to investigate the potential
250 positive synergic action of DMA and N-CPAs, trehalose and sucrose, on the quality of
251 cryopreserved chicken sperm. The results show that trehalose, but not sucrose, play a positive
252 protective action during the cryopreservation of chicken sperm. In particular, even if sperm
253 viability, motility and progressive motility were not affected, many kinetic parameters
254 indicative of the quality of sperm movement were improved in frozen/thawed semen samples
255 processed in presence of trehalose or trehalose and sucrose.
256 The lack of effect of trehalose combined with P-CPAs, DMA and glycerol, on the proportion
257 of viable sperm after cryopreservation was previously reported in the *Gallus gallus* and
258 *Alectoris Barbara* species [30]. Trehalose and/or sucrose combined to DMA did not improve
259 post-thaw sperm motility also in the *Gru canadiensis* and, in contrast, a positive effect was
260 found in the *Meleagris gallopavo* species [4].
261 The presence of trehalose, alone or combined to sucrose, into the freezing diluent had a
262 positive effect on VSL, LIN, WOB and BCF of post-thaw chicken semen. VSL was reported
263 as the most accurate estimate of sperm cell velocity [17] and it was also identified as an
264 important quantitative trait related to fertility [18]. In fresh semen, VSL must be > 30 μm/s
265 for a sperm to be able to penetrate a dense Accudenz solution [16]. Lower VSL values were

found in frozen/thawed chicken sperm, corresponding to 11-12 μm/s, in agreement with the results reported in a previous scientific study where only P-CPAs were used [35]. The improvement of VSL in semen samples protected with trehalose and DMA during freezing and thawing might suggest, as a consequence, the potential improvement of the fertilizing ability of cryopreserved sperm. The kinetic parameters LIN and WOB, indicative of progressiveness [34], were also improved in semen samples processed in presence of trehalose. LIN has been largely investigated in mammalian species [45], where a sperm subpopulation with high values of VAP and LIN could make the difference between high or low fertility. BCF has been associated to the ability of mammalian sperm of penetration into the *zona pellucida* of the oocyte. In humans in particular, VCL and BCF were significantly higher in sperm able to perform penetration in *in vitro* assay compared to those failing [15]. Finally, the recovery of progressive motile sperm soon after thawing and 5 and 10 min thereafter increased in semen samples provided with trehalose before freezing, thus the sensitivity of the progressive motile sperm present in the fresh sample to the freezing/thawing process was decreased.

5. CONCLUSIONS

Cryopreservation of living germplasm for the purpose of storing genetic resources is one of the most complex challenges in *ex situ* animal conservation programs. In avian species, despite many years of research on the use of permeating agents to cryoprotect semen, survival of sperm after freezing/thawing procedures remains highly variable. According to the experience in mammals, the investigation on the interaction between permeating cryoprotectants, like dimethylacetamide, and natural osmoprotectants, such as sucrose or

trehalose, can be a suitable strategy to improve the success of sperm cryopreservation in birds also.

Our study found a positive synergic action of trehalose and DMA on motile function of frozen/thawed chicken sperm; in contrast, sucrose combined with DMA did not show a similar positive effect. The positive cryoprotective action of trehalose was on the quality of sperm motion, not on the proportion of viable and motile sperm, and on the recovery of progressive motile sperm after cryopreservation. In particular, trehalose improves some key parameters of sperm motion positively related to the fertilizing ability of male gametes. Further studies are required to build on fundamental knowledge about the mechanism of the cryoprotective action of trehalose and to study its full potential as cryoprotectant alone or combined to DMA in chicken semen. It will be of interest to deeper investigate the quantitative DMA/trehalose ratio with the goal to study if a consistent decrease in DMA is possible in presence of trehalose to fully prevent the toxic effect directly related to its concentration.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENTS

The authors thank Regione Lombardia (CoVAL project, n.1723) for the financial support to the research activities, and Mrs. Maria Grazia Mangiagalli for bird management and semen collections.

REFERENCES

315 [1] E.M. Aboagla, T. Terada, Trehalose-enhanced fluidity of the goat sperm membrane and its
316 protection during freezing, Biol. Reprod. 69 (2003) 1245-1250.
317 [2] E. Aisen, V. Medina, A. Venturino, Cryopreservation and post thawed fertility of ram
318 semen frozen in different trehalose concentration, Theriogenology 57 (2002) 1801-1808.
319 [3] H.D. Blackburn, The national animal germplasm program: challenges and opportunities
320 for poultry genetic resources, Poult. Sci. 85 (2006) 210-215.
321 [4] J.M. Blanco, J.A. Long, G. Gee, D.E. Wildt, A.M. Donoghue,, Comparative
322 cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP
323 on turkey and crane sperm cryosurvival, Anim. Reprod. Sci. 123 (2011) 242-248.
324 [5] E. Blesbois, F. Seigneurin, I. Grasseau, C. Limouzin, J. Besnard, D. Gourichon, G.
325 Coquerelle, P. Rault, M. Tixier-Boichard, Semen cryopreservation for ex situ management of
326 genetic diversity in chicken: creation of the French avian cryobank, Poult. Sci. 86 (2007) 555-
327 564.
328 [6] E. Blesbois, Current status in avian semen cryopreservation, World. Poult. Sci. J. 63
329 (2007) 213-222.
330 [7] E. Blesbois, I. Grasseau, F. Signeurin, S. Mignon-Grasteau, M. Saint Jalme, M.M.
331 Mialon-Richard, Predictors of success of semen cryopreservation in chickens,
332 Theriogenology 69, 2 (2008) 252-261.
333 [8] E. Blesbois, Freezing avian semen, Avian Biol. Res. 4 (2011) 52-58.
334 [9] E. Blesbois, Biological features of the avian male gamete and their application to
335 biotechnology of conservation, J. Poult. Sci. 49 (2012) 141-149.
336 [10] J.P. Brillard, G.R. McDaniel, The reliability and efficiency of various methods for
337 estimating spermatozoa concentration, Br. Poultry Sci. 64 (1985) 155–158.

[11] W.H. Burrows, J.P. Quinn, A method of obtaining spermatozoa from the domestic fowl, Poult. Sci. 14 (1935) 253-254.

[12] A.M. Donoghue, G.J. Wishart, Storage of poultry semen, Anim. Reprod. Sci. 62 (2000) 213-232.

[13] FAO, Animal production and health guidelines n. 12- Cryopreservation of animal genetic resources, 2012, available at http://www.fao.org/docrep/016/i3017e/i3017e00.pdf.

[14] FASS, Guide for the Care and Use of agricultural Animals in Research and Teaching. Federation of Animal Science Societies, Champaign, USA, Third Edition, 2010, available at http://www.fass.org.

[15] P.M. Fetterolf, B.J. Rogers, Prediction of human sperm penetrating ability using computerized motion parameters, Mol. Repr. Devel. 27, 4 (1990) 326-331.

[16] D.P. Froman, Sperm motility in birds: insights from fowl sperm, Soc. Reprod. Fertil. Suppl. 65 (2007) 293-308.

[17] D.P. Froman, A.J. Feltmann, Sperm mobility: phenotype in roosters (Gallus domesticus) determined by concentration of motile sperm and straight line velocity, Biol. Reprod. 62 (2000) 303-309.

[18] D.P. Froman, A.J. Feltmann, M.L. Rhoads, J.D. Kirby, Sperm mobility: a primary determinant of fertility in the domestic fowl (Gallus domesticus), Biol. Reprod. 61 (1999) 400-405.

[19] J.E. Fulton, Avian genetic stock preservation: an industry perspective, Poult. Sci. 85 (2006) 227-231.

[20] G.F. Gee, Artificial insemination and cryopreservation of semen from nondomestic birds, In: Proceedings First International Symposium on the Artificial Insemination of Poultry. MR Bakst and GJ Wishart, Ed. Poultry Science Association, Savoy, Illinois, 1995, p. 262 – 280.

362 [21] O. Gutiérrez-Pérez, M.L. Juàrez-Mosqueda, S.U. Carvajal, M.E: Ortega, Boar
363 spermatozoa cryopreservation in low glycerol/trehalose enriched freezing media improves
364 cellular integrity, Cryobiology 58 (2009), 287-292.
365 [22] R.H. Hammerstedt, Cryopreservation of poultry semen - Current status and economics
366 In: Proceedings First International Symposium on the Artificial Insemination of Poultry. MR
367 Bakst and GJ Wishart, Ed. Poultry Science Association, Savoy, Illinois, 1995, p. 229 – 250.
368 [23] W.V: Holt, Basic aspects of frozen semen storage, Anim. Reprod. Sci. 62 (2000) 3-22.
369 [24] N. Iaffaldano, Storage of turkey semen with special focus on cryopreservation. In:
370 Proceedings of the 9th Turkey Science and Production Conference. Chester, UK, 2015, p. 34-
371 40.
372 [25] P.E. Lake, The history and future of the cryopreservation of avian germplasm, Poult. Sci.
373 65 (1986) 1-15.
374 [26] P.E. Lake, O. Ravie, An attempt to improve the fertility of stored fowl semen with
375 certain additivies in a basic diluent, Reprod. Nutr. Develop. 21, 6B (1981) 1077–1084.
376 [27] H.J. Lee, S.K. Kim, H.J. Jang, K.S. Kang, J.H. Kim, S.B. Choi, J.Y. Han,
377 Cryopreservation of Korean Oge chicken semen using N-methylacetamide, CryoLetters 33, 6
378 (2012) 427-434.
379 [28] J.A. Long, Avian semen cryopreservation: what are the biological challenges?, Poult.
380 Sci. 85 (2006) 232-236.
381 [29] M. Madeddu, F. Mosca, A. Abdel Sayed, L. Zaniboni, M.G. Mangiagalli, E. Colombo, S.
382 Cerolini, Effect of cooling rate on the survival of cryopreserved rooster sperm: comparison of
383 different distances in the vapor above the surface of the liquid nitrogen, Anim. Reprod. Sci.,
384 (2016) http://dx.doi.org/10.1016/j.anireprosci.2016.05.014.
385 [30] M. Madeddu, F. Berlinguer, V. Pasciu, S. Succu, V. Satta, G.G. Leoni, A. Zinellu, M.
386 Muzzeddu, C. Carru, S. Naitana, S., Differences in semen freezability and intracellular ATP

387 content between the rooster (*Gallus gallus domesticus*) and the Barbary partridge (*Alectoris*
388 *Barbara*), Theriogenology 74 (2010) 1010-1018.

389 [31] P.H. Purdy, Y. Song, F.G. Silversides, H.D. Blackburn, Evaluation of glycerol removal
390 techniques, cryoprotectants, and insemination methods for cryopreserving rooster sperm with
391 implications of regeneration of breed or line or both, Poult. Sci. 88, 10 (2009) 2184-2191.

392 [32] M.P. Rosato, N. Iaffaldano, Cryopreservation of rabbit semen: comparing the effects of
393 differing cryoprotectants, cryoprotectants-free vitrification, and the use of albumin plus
394 osmoprotectants on sperm survival and fertility after standard vapour freezing and
395 vitrification, Theriogenology 79 (2013) 508-516.

396 [33] M.P. Rosato, N. Iaffaldano, Effect of chilling temperature on the long term survival of
397 rabbit spermatozoa held either in a trisbased or a jellifield extender, Reprod. Domest. Anim.
398 46 (2011) 301-308.

399 [34] J. Santiago-Moreno, C. Castano, A. Toledano-Diaz, M.C: Esteso, A. Lpoez-Sebastian, N.
400 Ganan, M.J. Hierro, F. Marchal, J.L. Campo, E. Blesbois, Characterization of red-legged
401 partridge (Alectoris rufa) sperm: seasonal changes and influence of genetic purity, Poult. Sci.
402 94, 1 (2015), 80-87.

403 [35] J. Santiago-Moreno, C. Castano, A. Toledano-Diaz, M.A. Coloma, A. Lopez-Sebastian,
404 M.T Prieto, J.L. Campo, Influence of season on the freezability of free-range poultry semen,
405 Reprod. Domest. Anim. 47, 4 (2012), 578-583.

406 [36] J. Santiago-Moreno, C. Castano, A. Toledano-Diaz, M.A. Coloma, A. Lopez-Sebastian,
407 M.T. Prieto, J.L. Campo, Semen cryopreservation for the creation of a Spanish poultry breeds
408 cryobank: optimization of freezing rate and equilibration time, Poult. Sci. 90, 9 (2011) 2047-
409 2053.

410 [37] SAS, SAS User's Guide Statistics, Version 9.1 ed. SAS Institute Inc., Cary, NC, SA,
411 1999.

412 [38] K. Sasaki, T. Tatsumi, M. Tsutsui, T. Niinomi, T. Imai, M. Naito, A. Tajima, Y. Nishi, A
413 method for cryopreserving semen from Yakido roosters using N-methylacetamide as a
414 cryoprotective agent, J. Poult. Sci. 47 (2010) 297-301.

415 [39] T.J. Sexton, Comparison of various cryoprotective agents on washed chicken
416 spermatozoa. Effect of glucose, sucrose and polyvinylpyrrolidone, Poult. Sci. 54 (1975) 1297-
417 1299.

418 [40] P. Surai, G.J. Wishart, Poultry artificial insemination technology in the country of the
419 former USSR, World Poult. Sci. J. 52 (1996) 27-43.

420 [41] J.E. Swain, G.D. Smith, Cryoprotectants. In: Chian RC, Quinn P (Eds), Fertility
421 Cryopreservation. Cambridge University Press, New York, 2010, p. 24.38.

422 [42] A. Tajima, Conservation of avian genetic resources, J. Poult. Sci., 50 (2013) 1-8.

423 [43] T. Terada, K Ashizawa, T. Maeda, Y Tsutsumi, Efficacy of Trehalose in
424 cryopreservation of chicken spermatozoa, Jpn. J. Anim. Reprod. 35, 1 (1989) 20-24.

425 [44] K. Tselutin, F. Seigneurin, E. Blesbois, Comparison of cryoprotectants and method of
426 cryopreservation of fowl spermatozoa, Poult. Sci. 78 (1999) 586-590.

427 [45] A.J.F. Vazquez, M.J. Cedillo, V.J. Quezada, A.C. Rivas, E.C.L. Morales, E.M.E. Ayala,
428 M.J. Hernandez, R.A. Gonzalez, M.A. Aragon, Effects of repeated electroejaculations on
429 kinematic sperm subpopulations and quality markers of Mexican creole goats, Anim. Reprod.
430 Sci. 154 (2015) 29-38.

431 [46] H. Woelders, C.A. Zuidberg, S.J. Hiemstra, Animal genetic resources conservation in
432 The Netherlands and Europe: poultry perspective, Poult. Sci. 85 (2006) 216-222.

433 [47] H. Woelders, A. Matthijs, B. Engel, Effects of trehalose and sucrose, osmolarity of the
434 frozen medium, and cooling rate on viability and intactness of bull sperm after freezing and
435 thawing, Cryobiology 35 (1997) 93-105.
436 [48] L. Zaniboni, C. Cassinelli, M.G. Mangiagalli, T.M. Gliozzi, S. Cerolini, Pellet
437 cryopreservation of chicken semen: effects of sperm working concentration, cryoprotectants
438 concentration, and equilibration time during in vitro processing, Theriogenology 82 (2014)
439 251-258.

440

441 **Table 1**. Sperm quality parameters (LSMeans ± S.E.) measured in fresh and frozen/thawed
442 semen soon after (T0) thawing and after five(T5), ten (T10) and fifteen (T15) minutes.

Sperm parameters[1]	Fresh	T0	T5	T10	T15	S.E.
Viability (%)	71.8 ^{A}	35.9 ^{B}	-	28.4 ^{C}	-	1.3
Motility (%)	86.8 ^{A}	33.3 ^{B}	25.8 ^{C}	19.9 ^{D}	15.9 ^{E}	1.5
Progressive motility (%)	18.6 ^{A}	3.1 ^{B}	1.9 ^{C}	1.2 ^{CD}	0.8 ^{D}	0.5
VCL (μm/s)	57.2	37.5	33.9	31.5	29.9	1.2
VSL (μm/s)	20.6 ^{A}	11.0 ^{B}	9.6 ^{C}	8.5 ^{D}	7.8 ^{D}	0.4
VAP (μm/s)	35.1 ^{A}	19.7 ^{B}	17.5 ^{C}	15.9 ^{D}	14.7 ^{D}	0.7
LIN (%)	36.9 ^{A}	29.0 ^{B}	27.5 ^{C}	26.8 ^{CD}	25.8 ^{D}	0.8
STR (%)	59.4 ^{A}	55.1 ^{B}	54.1 ^{BC}	52.8 ^{CD}	52.2 ^{D}	0.8
WOB (%)	62.0 ^{A}	52.4 ^{B}	51.1 ^{BC}	50.2 ^{CD}	48.9 ^{D}	0.8
ALH (μm)	3.3 ^{A}	2.9 ^{B}	2.8 ^{BC}	2.6 ^{CD}	2.5 ^{D}	0.1
BCF (Hz)	5.9 ^{A}	6.4 ^{A}	6.2 ^{A}	5.5 ^{B}	4.9 ^{B}	0.3

443 [1]Viability, the percentage of viable spermatozoa; Motility, the percentage of motile spermatozoa;
444 Progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity;
445 VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement;
446 BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and
447 WOB (VAP/VCL x 100), wobble.
448 ^{A,B,C,D,E} Values within a row with different superscripts differ significantly at $P<0.001$

449

Table 2. Influence of cryodiluent composition on post-thaw sperm quality parameters (LSMeans ± S.E.) of chicken semen.

Sperm parameters[1]	LPF[2]	LPF-S[2]	LPF-ST[2]	LPF-T[2]	S.E.
Viability (%)	46.9	44.7	43.4	46.4	1.3
Motility (%)	35.8	35.3	35.9	38.4	1.5
Progressive motility (%)	4.8	4.9	5.2	5.6	0.5
VCL (μm/s)	36.8	37.6	38.1	39.5	1.2
VSL (μm/s)	11.1 ^{A}	10.8 ^{A}	11.7 ^{B}	12.4 ^{B}	0.4
VAP (μm/s)	20.0 ^{A}	19.8 ^{A}	20.7 ^{AB}	21.9 ^{B}	0.7
LIN (%)	29.2 ^{A}	27.5 ^{B}	29.7 ^{A}	30.8 ^{C}	0.8
STR (%)	54.4 ^{A}	52.8 ^{B}	55.3 ^{AC}	56.2 ^{C}	0.8
WOB (%)	53.0 ^{A}	51.3 ^{B}	52.9 ^{A}	54.5 ^{C}	0.8
ALH (μm)	2.8 ^{a}	2.8 ^{a}	2.9 ^{ab}	3.1 ^{B}	0.1
BCF (Hz)	5.4 ^{A}	5.2 ^{A}	6.1 ^{B}	6.4 ^{B}	0.3

[1]Viability, the percentage of viable spermatozoa; Motility, the percentage of motile spermatozoa; Progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble.

[2]LPF, Lake pre-freezing diluent containing DMA alone; LPF-S, LPF containing DMA and sucrose; LPF-ST, LPF containing DMA, sucrose and trehalose; LPF-T, LPF containing DMA and trehalose.

^{A,B,C} Values within a row with different superscripts differ significantly at $P<0.001$

^{a,b} Values within a row with different superscripts differ significantly at $P<0.05$

462 **Table 3**. Influence of cryodiluent composition on recovery rates of progressive motile sperm
463 (LSMeans ± S.E.) from 0 to 15 minutes after thawing in chicken semen.

Time after thawing (min)	LPF[1]	LPF-S[1]	LPF-ST[1]	LPF-T[1]	S.E.
T0	15.1 [a]	13.7 [a]	13.6 [a]	23.6 [b]	2.1
T5	7.1 [a]	6.6 [a]	13.1 [b]	14.5 [b]	1.4
T10	3.9 [a]	4.1 [a]	9.1 [b]	8.7 [b]	1.1
T15	3.1	3.0	5.5	4.8	0.8

464 [1]LPF, Lake pre-freezing diluent containing DMA alone; LPF-S, LPF containing DMA and sucrose;
465 LPF-ST, LPF containing DMA, sucrose and trehalose; LPF-T, LPF containing DMA and trehalose.
466 [a,b] Values within a row with different superscripts differ significantly at $P<0.05$