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Is the protective effect of egg yolk against osmotic and cryogenic damage on dog spermatozoa dosedependent?

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Abstract

Egg yolk (EY) is conventionally used to reduce sperm cryodamage, however, there has not be evaluation of whether there is a dose-dependent effect with inclusion of EY in semen extender. To enhance the knowledge about the protective effect of EY during cryopreservation of dog semen, a specific study was designed to evaluate the dose-dependent protection of the EY against osmotic and cryogenic damage of dog sperm. In the first experiment, sperm stored in an extender that contained graded EY concentrations (0%, 5%, 10%, and 20%) were diluted with hypo- or hyper-osmotic solutions (final osmolality of 75, 150, 300, 500, 1000 mOsm/kg). Results from sperm kinetic, membrane integrity (MI), mitochondrial activity, and normal morphology evaluations indicated osmotic stress has especially marked effects on the kinetic capacity of spermatozoa, however, there were no direct effects on mitochondrial activity. In both hypo- and hyper-osmotic conditions, EY had a protective effect regardless of concentration. In the second experiment, semen samples were diluted in extenders at increasing EY concentrations (0%, 5%, 10%, and 20%) and cryopreserved. Effects on sperm kinetics, membrane and acrosome integrity and mitochondrial membrane potential indicated there was improved sperm viability after thawing when the EY concentration was 5% and 10%, and lesser viability when it was 20%. These results indicate, for the first time, that EY reduces osmotic and cryogenic damage when used at 5% or 10% concentrations, and that these concentrations can be used to protect dog spermatozoa more effectively than the conventionally used concentration (20%).

Keywords	Canine; Egg yolk; Cryopreservation; Sperm kinetics
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20 ABSTRACT

21 Egg volk (EY) is conventionally used to reduce sperm cryodamage, however, there has not be evaluation of whether there is a dose-dependent effect with inclusion of EY in semen extender. To 22 enhance the knowledge about the protective effect of EY during cryopreservation of dog semen, a 23 specific study was designed to evaluate the dose-dependent protection of the EY against osmotic and 24 cryogenic damage of dog sperm. In the first experiment, sperm stored in an extender that contained 25 graded EY concentrations (0%, 5%, 10%, and 20%) were diluted with hypo- or hyper-osmotic 26 solutions (final osmolality of 75, 150, 300, 500, 1000 mOsm/kg). Results from sperm kinetic, 27 membrane integrity (MI), mitochondrial activity, and normal morphology evaluations indicated 28 29 osmotic stress has especially marked effects on the kinetic capacity of spermatozoa, however, there were no direct effects on mitochondrial activity. In both hypo- and hyper-osmotic conditions, EY had 30 a protective effect regardless of concentration. In the second experiment, semen samples were diluted 31 32 in extenders at increasing EY concentrations (0%, 5%, 10%, and 20%) and cryopreserved. Effects on sperm kinetics, membrane and acrosome integrity and mitochondrial membrane potential indicated 33 there was improved sperm viability after thawing when the EY concentration was 5% and 10%, and 34 lesser viability when it was 20%. These results indicate, for the first time, that EY reduces osmotic 35 and cryogenic damage when used at 5% or 10% concentrations, and that these concentrations can be 36 37 used to protect dog spermatozoa more effectively than the conventionally used concentration (20%).

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- 39

40 Keywords: Canine; Egg yolk; Cryopreservation; Sperm kinetics

42 **1. Introduction**

Semen cryopreservation allows for long-term storage of viable and functional spermatozoa
(Leroy et al., 2011). This technology has several advantages because semen can be stored for a long
period (i.e., years) without losing fertilizing capacity, transported for great distances, or can also be
used when the female is in oestrus without having a male in close proximity to mate with the female
(Thomassen and Farstad, 2009).

Cryopreservation, however, has detrimental effects on mammalian sperm viability and 48 fertilizing capacity. A reduction in progressive motility (Jones and Stewart, 1979), alterations of 49 membrane permeability and stability (Holt and North, 1986; Watson, 2000), and an increase in the 50 51 radical species of oxygen (ROS) generation (Alvarez and Storey, 1992; Chatterjee and Gagnon, 2001) have been reported in cryopreserved mammalian spermatozoa as compared with the values for these 52 variables in raw semen. To reduce the effects of temperatures that are used for cryopreservation of 53 54 spermatozoa, extenders with specific composition were developed. Among the different components of the freezing extender, egg yolk (EY) appears to be a necessary component of extenders if these are 55 to be effective for semen cryopreservation and maintenance of sperm viability after thawing. Results 56 of previous studies indicate the use of EY in the freezing medium reduces cellular damage (Phillips 57 and Lardy, 1940; Pace and Graham, 1974; De Leeuw et al., 1993). Although the protective function 58 59 of the EY with cryopreservation of sperm is widely recognized, the underlying mechanism of action has not been ascertained. Results of most studies indicate that the protective capacity of EY is related 60 to the content of low density lipoproteins (LDL) (Pace and Graham, 1974; Moussa et al., 2002; 61 62 Bencharif et al., 2010). It has been proposed that these components adhere to and interact with the sperm membrane (Foulkes, 1977; Graham and Foote, 1987; Manjunath et al., 2002; Bergeron et al., 63 2004). In some studies the metabolism of spermatozoa, however, is inhibited by some EY 64 components, and this could affect sperm motility (Pace and Graham, 1974; Wall and Foote, 1999). 65

Traditionally, while EY is added to dog semen extender at a 20% concentration when there is
cryopreservation of sperm (Anderson, 1972; Peña et al., 1998; Silva et al., 2002), there have been few

studies in which there has been assessment of whether the cryoprotective effect of EY is dosedependent. In other males, such as the stallion, EY is effectively used for semen cryopreservation at
a 2% concentration, without a reduction of sperm quality and fertilisation capacity (Pillet et al., 2008).

The damage induced by cryopreservation on spermatozoa is multimodal because in several studies there was a combination of cold shock (Amann and Pickett, 1987), peroxidation (Slaweta et al., 1988), and osmotic stress (Watson, 2000). When the temperature reduction is beyond the freezing point for semen, water forms ice crystals first in the extracellular compartment. This increases the solute concentration in the free uncrystallised water outside the cell, inducing hyperosmotic stress (Sieme et al., 2016). During thawing, however, the ice crystals melt in the free water that enters the plasma membrane, thus sperm undergo hypo-osmotic stress.

Although Foulkes (Foulkes, 1977) suggested that the protective effect of the EY during 78 cryopreservation could contribute to the colloid pressure maintenance of the external medium, few 79 80 studies have been focused on the functions of EY as a protective compound against the osmotic stress. Even though there is broad understanding of the importance of EY as a semen extender component 81 82 and consequent wide use of EY for sperm cryopreservation in dogs, there have been surprisingly few 83 studies conducted to clarify the dose-dependent protective effect of this component. Furthermore, a 84 relevant part of the damage during cryopreservation could be attributed to the osmotic stress, but few 85 studies focused on the protective functions of the EY against osmotic stress in dog spermatozoa. Thus, to increase the knowledge of the biology and manipulation of reproduction in dogs, the present study 86 was designed to ascertain, for the first time, the protective effect of EY at different concentrations 87 (0%, 5%, 10%, and 20%) on dog spermatozoa in different anisosmotic conditions. Furthermore, the 88 aim of the present study was to evaluate the cryoprotective effect of EY, added at the same 89 concentrations (0%, 5%, 10%, and 20%) to the freezing medium, for dog sperm cryopreservation. 90

91

92 2. Materials and methods

93 2.1. Animals and semen collection

The study involved 21 healthy dogs of known fertility aged between 2 and 6 years. The breeds represented were: Newfoundland (n = 6), Pitbull (n = 5), American Staffordshire (n = 5), and Labrador retriever (n = 5). All dogs were admitted for a routine reproductive examination at the Hospital of the University of Veterinary Medicine of Teramo, Italy. For all the dogs included in the study, the consent was obtained from the owner for the use of part of the semen sample of the dogs in the present study. Animals were managed in ways consistent with the Italian legislation concerning animal care (DL n.116, 27/01/1992).

Semen collection was conducted for all dogs using the digital manipulation method by the same individual to reduce the effects of semen collection process on sperm quality. Only the spermrich fraction was evaluated and used for experiments. From each animal, two ejaculates were collected, for a total of 42 samples. Only ejaculates in which sperm motility was > 70% and concentration > 200×10^6 sperm/mL were included in the studies.

106

107 2.2. Experimental designs

108 2.2.1. Experiment 1. Protective effect of EY on dog sperm during osmotic stress

A hyperosmotic solution based on TRIS formula (hyper-TRIS) was prepared using 119.8 g/L 109 TRIS, 67.32 g/L citric acid and 12.375 g/L glucose (pH 6.9; mOsm/kg 1218). This solution was 110 111 diluted using bi-distilled sterile water to achieve isosmotic TRIS (iTRIS, pH, 6.8, Osm 302 mOsm/kg). Semen samples were aliquoted to four groups and diluted at 240 x 10⁶ sperm/mL in iTRIS, 112 then each sample was diluted 1:1 with 40% (final concentration 20% EY - EY20), 20% (final 113 concentration 10% - EY10), 10% (final concentration 5% EY - EY5) EY, or there was no EY added 114 to the extender (0%; EY0 – as control). The 40%, 20%, and 10% EY extenders had an osmolality of 115 116 1864, 1839, and 1821 mOsm/kg, respectively. Each treatment sample (EY20, EY10, EY5, and EY0) was in turn divided into five aliquots, in duplicate. To evaluate the protective effect of different EY 117 concentrations, each treatment sample was then diluted with a combination of hyper-TRIS and bi-118 distilled water to a final osmolality of 75, 150, 300, 500, or 1000 mOsm/kg. The final sperm 119

concentration was 40 x 10⁶ sperm/mL. Part of these samples, immediately after dilution, was used for
objective motility evaluation using the CASA system as subsequently described in this manuscript.
The evaluation was performed at T0, 20 min after the time of dilution (T20) and 45 min after the time
of dilution (T45), following the procedure subsequently described in this manuscript for the kinetic
evaluations. The remaining sample was morphologically assessed using a phase contrast microscopy,
as subsequently described in this manuscript.

There was another portion of the samples with graded EY concentrations placed in two aliquots and used for flow cytometry evaluation of membrane integrity (MI; first aliquot) and mitochondrial potential (second aliquot), as subsequently described in this manuscript. The evaluation was performed after 20 (T20) and 45 (T45) min subsequent to addition of the stain, as subsequently described in this manuscript.

131

132 2.2.2. Experiment 2. Protective effect of EY during cryopreservation

Semen samples were diluted 1:1 (v:v) with iTRIS (pH, 6.7, Osm 304 mOsm/kg), centrifuged 133 for 10 min at 700 g and re-suspended in iTRIS at the concentration of 300 x 10⁶ sperm/mL. The semen 134 was then diluted 1:1 (v:v) with iTRIS supplemented with 8% glycerol (final concentration 4%) (Peña 135 et al., 1998) and 40% (final concentration 20% EY - EY20), 20% (final concentration 10% - EY10), 136 137 10% (final concentration 5% EY - EY5) EY, or there was no EY added to the extender (0%; EY0 as control). The samples were then cooled and there was equilibration at 4 °C for 2 h in a passive 138 refrigerator, packaged in 0.25 straws (IMV Technologies, L'Aigle, France) and sealed mechanically. 139 Straws were suspended 4 cm above the liquid nitrogen surface for 10 min, and were then plunged 140 into liquid nitrogen (Anderson, 1972), and subsequently stored for at least 5 days. Samples were 141 evaluated for sperm objective motility, MI, acrosome integrity, and mitochondrial potential at the end 142 of equilibration (EQ) period and after thawing (FT). For thawing of samples, straws were placed in a 143 water bath at 37 °C for 30 seconds (Bencharif et al., 2008b), the sample was then transferred to a 2-144

ml plastic tube and there was an additional incubation at 37 °C to achieve 5 minutes of total
incubation.

147

148 2.3. Semen evaluation

149 2.3.1. Semen concentration

Raw semen was evaluated within 10 min after collection. Sperm concentration was determined using a Bürker counting chamber (Merck, Leuven, Belgium) after dilution 1:1000 with a formol-saline solution.

153

154 2.3.2. Sperm kinetics

The kinetic evaluations were performed using the computer-assisted sperm analyser (CASA) 155 system IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA, USA) for objective evaluation of 156 157 motility using the guidelines for CASA utilisation (Iguer-ouada and Verstegen, 2001; Rijsselaere et al., 2003). There was correct identification of spermatozoa by using the playback function and 158 adjusting the detection gates accordingly. Samples from Experiment 1 were analysed without further 159 dilution, while frozen/thawed samples were diluted with relevant extender at 40 x 10⁶ sperm/mL. An 160 aliquot of each sample was re-warmed at 37 °C for 5 min and a 5-µL drop was loaded onto a Makler 161 162 chamber (Sefi Medical Instruments, Haifa, Israel). Data for motility variables were collected and recorded by examining sperm cells in 12 non-consecutive fields. The anti-collision algorithm was 163 activated. Motility variables evaluated were total motility (TM; %), progressive motility (PM; %), 164 average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; 165 μm/s), amplitude of lateral head displacement (ALH; μm), beat cross frequency (BCF; Hz), 166 straightness (STR, as VSL/VAP; %), and linearity (LIN, as VSL/VCL; %). Spermatozoa with VAP 167 \geq 80 µm/s and STR \geq 75% were considered to be progressive cells. 168

In Experiment 1, MI in the different osmotic conditions was evaluated using the propidium 171 172 iodide (PI) exclusion test that has been previously described and validated (Ball and Vo, 2001) with some modifications. Semen was diluted to 10×10^6 sperm/mL with the relevant extender, and aliquots 173 (500 µL) being incubated with PI at the final concentration of 12 µM for 5 min at 22 °C and then 174 were analysed using the flow cytometer EPICS XL (Beckman Coulter, San Jose, CA, USA). Data 175 acquisitions occurred with the use of the System II software (Beckman Coulter, USA). The sperm 176 177 population was selected on the basis of the forward- and side-scatter, and a gate was selected based on the values determined from these evaluations. Samples were excited using a 20-mW argon ion 178 488-nm laser, and PI fluorescence was obtained using the FL3 sensor through a 660/20 nm long pass 179 180 filter. Forward and side-scatter values were recorded on a linear scale and fluorescence values on a logarithmic scale. Flow cytometric analysis was performed at a flow rate of 6 to 24 µL/min, and 181 acquisitions were stopped at 30,000 events. Events with red fluorescence were considered to represent 182 183 sperm with membrane damage, while those cells without fluorescence were considered to be spermatozoa with MI. 184

In Experiment 2, sperm MI and acrosome integrity were evaluated simultaneously using flow 185 cytometry, as previously described (Gloria et al., 2018). Briefly, sperm in samples diluted to 10 x 10⁶ 186 187 sperm/mL (1 mL) were stained with 2.4 µM of PI and 5 µg/mL of FITC-conjugated agglutinin derived from Pisum sativum (FITC-PSA). After 10 min of dark incubation at 22 °C, each sample was analysed 188 using a flow cytometer (EPICS XL). The FITC-PSA fluorescence data were collected using an FL1 189 sensor with a 530/28 nm band-pass, while data for PI fluorescence were obtained using the FL3 sensor 190 with a 660/20 nm long pass filter. Adjustment of compensation values for the two emission detectors 191 used was done. The sperm population was selected on the basis of the forward- and side-scatter, and 192 193 a gate was selected based on the values for these variables. Forward- and side-scatter values were recorded on a linear scale and fluorescence values on a logarithmic scale. Flow cytometric analysis 194 was performed at a flow rate of 6 to 24 µL/min, and acquisitions were stopped at 30,000 events. Use 195 of the combination of these two fluorochromes allowed for characterisation of four different 196

subpopulations: sperm with MI and acrosome integrity that had no fluorescence (PI-/PSA-); sperm
with MI and an acrosome reaction (PI-/PSA+); sperm with a damaged membrane and acrosome
integrity (PI+/PSA-); and sperm with a damaged membrane and a reacted acrosome (PI+/PSA+).

200

201 2.3.4. Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) of spermatozoa was evaluated using the 202 fluorescent stain 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimida zolylcarbocyanine chloride (JC-1) 203 as reported by Gloria et al., (Gloria et al., 2018). The sperm suspension was adjusted to a concentration 204 of 5×10^6 sperm/mL and incubated for 45 min at 37 °C in the dark with the JC-1 stain (final stain 205 206 concentration 8 µM). At the end of the incubation period, cells were washed in the same medium that contained no stain and there were evaluations using the flow cytometer EPICS XL (Beckman Coulter) 207 equipped with the System II software (Beckman Coulter) as previously described (Garner and 208 209 Thomas, 1999). The sperm population was selected on the basis of the forward- and side-scatter, and a gate was selected based on the values for these variables. The green fluorescent emissions of the 210 monomeric form of JC-1 (mitochondria with relatively lesser potential - LMMP) were collected using 211 the 530 ± 15 - nm filter (FL 1), and the orange emission of the polymeric form of JC-1 (mitochondria 212 with a relatively greater membrane potential - HMMP) was detected using the $585 \pm 21 - nm$ filter 213 214 (FL 2). The flow cytometric analysis was performed at a flow rate of 8 to 30 µL/min, and the acquisitions were stopped at 30,000 events. No adjustment of compensation values for the two 215 emission detectors was done. 216

217

218 *2.3.5. Sperm morphology*

Sperm morphology was evaluated using a phase contrast microscope (BX-51 - Olympus Italia, Milan, Italy) at 1000 X magnification. Spermatozoa at different osmolalities were immobilized with the addition of 3% glutaraldehyde (Hancock, 1957), and a drop (6 μ l) was placed on a slide and covered with a 22 x 22 mm coverslip. Spermatozoa were then classified as normal sperm, sperm with an abnormal head, sperm with an abnormal midpiece, and sperm with an abnormal tail, that were in
turn subdivided into classifications of sperm with complete coiling (more than the 50% of the tail
length was involved in the twisting/coiling), or partial coiling (the twisting/coiling involved the distal
part of the tail) of the tail, and sperm with other tail abnormalities. Tail abnormalities were evaluated
for at least 400 spermatozoa.

228

229 2.4. Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). The data were evaluated using the Shapiro-Wilk (normal distribution) and Levene (homogeneity of variances) tests. When data were not normally distributed, a log transformation was performed before data analyses were conducted.

In Experiment 1, the effect of the concentration of EY on the different sperm variables (kinetic variables, MI, mitochondrial membrane potential, morphological subclasses) was evaluated using a general linear model (GLM) based on an Univariate ANOVA. Dog was included as a random factor. *Post-hoc* evaluation was performed using the Scheffé's test.

In Experiment 2, the cryoprotective effects of the different concentrations of EY, in terms of kinetic variables, membrane and acrosome integrity, and mitochondrial membrane potential were evaluated using a GLM based on a Univariate ANOVA, with the Scheffé's test being used for the *post-hoc* evaluation. Dog was included as a random factor.

In Experiment 1, correlations between total and progressive motility, MI, and mitochondrial membrane potential were determined using the Pearson's correlation coefficient. For both the experiments, differences were considered significant when P < 0.05. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

246

247 **3. Results**

248 *3.1. Experiment 1*

249

The protective effect of EY against anisosmotic stress was evaluated for dog spermatozoa.

250 Data indicated EY had a protective effect, irrespective of the concentration of EY used.

251 In samples diluted in iTRIS without EY at T0, the values for kinetic variables were similar to those with inclusion of EY in the semen extender, with the exception of ALH, which was less in 252 samples with EY, irrespective of the concentration, compared with those without EY (P < 0.05; Table 253 1). In these samples, values for kinetic variables were less in both hypo- and hyper-osmotic 254 conditions. At 75 and at 1000 mOsm/kg, there was no spermatozoa motility, while at 150 and at 500 255 mOsm/kg, the proportion of sperm with total motility was less than 10% in all samples. In samples 256 diluted with 5% EY, the values for kinetic variables with use of the 150 mOsm/kg were less than 257 258 those with 300 mOsm/kg concentration (P < 0.01). Values at the 500 mOsm/kg concentration were similar to those recorded in the control sample for total motility (Table 1). Moderate hyperosmotic 259 conditions apparently resulted in lesser values for kinetic variables because spermatozoa that were 260 261 motile when these conditions prevailed had lesser velocities (VAP, VSL, and VCL; P < 0.05) and progressiveness (STR, LIN; P < 0.05), with an increase in the amplitude of the movement (ALH; P 262 < 0.05), resulting in a reduced proportion of sperm with progressive motility (P < 0.05). Values for 263 kinetic variables were related to the presence of EY, but were not affected by the concentration of 264 265 this component, because the values when there was use of 5%, 10%, and 20% concentrations of EY 266 were similar (Table 1). Duration of incubation seemed to have a negligible effect on values for sperm kinetic variables. Values recorded after 20 (data not shown) and 45 (Table 2) min were similar to 267 those soon after dilution, with an effect of anisosmotic conditions on samples without EY and on the 268 269 sample in hypo-tonic media.

Membrane integrity and mitochondrial membrane potential were evaluated at 20 and 45 min after dilution, due to the time required for the incubation with the staining for the procedure to be valid. In samples that had no EY added that were incubated for 20 minutes (T20), the MI was greater at 300 mOsm/kg than in those incubated at 150 and 75 mOsm/kg (Table 3). Similarly, as osmolality increased, there was a decrease in MI (P < 0.05; Table 3). In the presence of EY, however, MI was similar at all osmolarities, regardless of EY concentration (Table 3). There was a similar trend for HMMP in samples without EY compared with those with 5%, 10% and 20% EY (Table 3). Values for MI, HMMP, and LMMP at T45 were similar compared with those measured at T20 in the extender with corresponding EY concentration and osmolality. There were similar values for acrosome integrity throughout the experiment and there was an apparent lesser effect of both EY concentration and osmolality on acrosome integrity as compared with MI (data not shown).

281 In samples in which there was no inclusion of EY in the extender, there were correlations between sperm TM, and MI (r = 0.896, P < 0.01), TM and HMMP (r = 0.824, P < 0.01), and MI and 282 HMMP (r = 0.792, P < 0.01) at 300 mOsm/kg, while in anisosmotic conditions, MI was correlated 283 284 with HMMP (r = 0.786, P < 0.01), however, not with TM (r = 0.318, P > 0.05). In samples containing 5%, 10%, and 20% EY, there were correlations at 300 mOsm/kg between TM and MI (r = 0.916, P 285 < 0.01; r = 0.934, P < 0.01; r = 0.928, P < 0.01, respectively), TM and HMMP (r = 0.874, P < 0.01; 286 287 r = 0.836, P < 0.01; r = 0.792, P < 0.01, respectively), and MI and HMMP (r = 0.816, P < 0.01; r = 0.010.842, P < 0.01; r = 0.758, P < 0.01). Similarly, in all the samples with EY there were correlations at 288 500 mOsm/kg for TM and MI (r = 0.826, P < 0.01 for 5% EY; r = 0.842, P < 0.01 for 10% EY; r =289 0.816, P < 0.01 for 20%EY), TM and HMMP (r = 0.682, P < 0.01 for 5% EY; r = 0.648, P < 0.01290 for 10% EY; *r* = 0.586, *P* < 0.05 for 20% EY), and MI and HMMP (*r* = 0.798, *P* < 0.01 for 5% EY; 291 r = 0.816, P < 0.01 for 10% EY; r = 0.786, P < 0.01 for 20% EY). At 75, 150, and 1000 mOsm/kg, 292 MI was correlated, however, with HMMP (r = 0.638, P < 0.05; r = 0.682, P < 0.05; r = 0.568, P < 0.05; r = 0293 0.05, respectively in samples containing 5%; r = 0.672, P < 0.05; r = 0.684, P < 0.05; r = 0.548, P < 0.05; 294 0.05, respectively in samples containing 10% EY; and r = 0.626, P < 0.05; r = 0.584, P < 0.05; r =295 0.526, P < 0.05, respectively in samples containing 20% EY) but not with TM. 296

The percentage of sperm with an abnormal head and midpiece were not affected by the osmolality of the medium, or by EY concentration (Table 4, P > 0.05). There was an effect of dog with use of the model (P < 0.05).

As expected, hyperosmotic conditions marginally affected sperm tail morphology. Soon after 300 dilution (T0), morphological subclasses recorded at 500 and 1000 mOsm/kg in samples were similar 301 to those recorded at 300 mOsm/kg (P > 0.05). Furthermore, spermatozoa in the hypo-osmotic 302 condition had a typical twisting/coiling of the tail. The percentage of spermatozoa with a tail response 303 was similar at both 75 and 150 mOsm/kg, however, the percentage of sperm with complete tail coiling 304 was greater at 75 compared with 150 mOsm/kg (P < 0.05) that was, in turn, greater than in the iso-305 osmotic samples (P < 0.01). The percentage of spermatozoa with partial coiling, however, was greater 306 at 150 compared with 75 mOsm/kg (P < 0.05; Figure 1). The inclusion of EY in the extender seemed 307 to have a partial protective effect against the hypo-osmotic stress because the percentage of complete 308 309 coiling was less in samples when there was inclusion of 5%, 10%, and 20% EY in the extender compared with samples without EY at both 75 and 150 mOsm/kg (P < 0.05). There were no 310 differences (P > 0.05) in the proportion of the morphological tail subclasses, however, in samples 311 312 diluted with EY5, EY10, and EY20 (data not shown). There was no effect of the time on the sperm morphological subclasses because the subclasses were similar soon after the dilution (T0), at T20, 313 and T45 (data not shown). 314

315

316 *3.2. Experiment 2*

317 In Experiment 2, the dose-dependent cryoprotective nature of EY was evaluated in this study. In the samples evaluated soon after dilution, EY apparently had an effect on the velocity of dog sperm 318 movement, because progressive motility, VAP, VSL, VCL, STR, and LIN were all greater in samples 319 diluted with 5% and 10% EY compared with samples without EY (P < 0.05). Samples diluted with 320 20% EY had similar values for these variables as those not diluted with EY, and lesser (P < 0.05) 321 compared with inclusion of 5% and 10% EY in the extender (Table 5). There was negligible effect 322 on total motility, ALH, and BCF of EY concentration because values were similar for all treatments 323 (P > 0.05). Membrane and acrosome integrity, such as sperm with HMMP, were similar in samples 324

without EY and samples where there was inclusion of 5%, 10%, and 20% EY in the extender (Table5).

During equilibration, the values for semen samples diluted at different EY concentrations 327 were similar compared to those of corresponding samples soon after dilution (P > 0.05). The 328 cryopreservation of samples resulted in there being lesser values for sperm variables but the effect on 329 spermatozoa seemed to be related to EY concentration. Samples in extender without EY had lesser 330 values for kinetic variables, MI, and HMMP compared with samples in extender containing 5% and 331 10% EY (P < 0.05; Table 6). Unexpectedly, in samples diluted with 20% EY, total and progressive 332 motilities were less compared with the values of samples diluted in 5% and 10% EY (P < 0.05), even 333 though the values were greater compared with sperm in samples containing no EY (P < 0.05). The 334 values for other kinetic variables for samples diluted in 20% EY were similar to those when the 335 extender contained 5% and 10% EY. Although PI+/PSA- and PI+/PSA+ subpopulations were not 336 337 different between samples diluted in 20% EY compared with 5% and 10% EY (P > 0.05; Table 6) the total amount of sperm with membrane damage (PI+/PSA- plus PI+/PSA+) was greater in samples 338 diluted in 20% EY compared with 5% and 10% EY (P < 0.05). 339

340

341 4. Discussion

342 The data reported in the present study indicate that EY has actions in reducing osmotic stress of spermatozoa. Spermatozoa are able to adapt to the solute concentration in the medium surrounding 343 the cells by transfer of water across the plasma membrane and modification of the cytoskeleton 344 345 (Correa et al., 2007). In a simple salt solution, spermatozoa respond to anisosmotic conditions as linear osmometers, because there is a correlation between bull sperm volume and medium osmolality 346 (Guthrie et al., 2002). In several studies, results indicated there was an osmotic tolerance limit for 347 spermatozoa of different domestic animals, including those of bulls (Liu and Foote, 1998; Guthrie et 348 al., 2002), stallions (Ball and Vo, 2001; Glazar et al., 2009), boars (Gilmore et al., 1998), and rams 349 (Curry and Watson, 1994). Specific studies regarding the osmotic tolerance limit were not conducted 350

on dog spermatozoa. The determination of the osmotic tolerance limit of dog spermatozoa was not the primary aim of the present study; however, data for the response of dog sperm to osmotic stress in the medium without EY indicate dog spermatozoa are similar to those of other species in responding to osmotic stress.

In all the previous studies on the osmotic stress response of spermatozoa, there was not 355 evaluation of sperm in a complex medium supplemented with colloidal components, that could 356 modulate the cellular adaptation. In dogs, the addition of EY seemed to protect cellular structures 357 involved in the regulation of sperm motility at 500 mOsm/kg, even though there was a general 358 reduction in the progressive motility. Similarly, the protective action of EY was more evident on 359 360 sperm MI. In samples diluted with 5%, 10%, and 20% EY, percentages of sperm with MI at 150, and 361 at 500 and 1,000 mOsm/kg were similar compared to the isosmotic condition. Although the protective actions of EY in anisosmotic conditions seemed to be clear, data indicate that this action was neither 362 363 dose-dependent, nor time-dependent, because there were similar values for all the seminal variables when there was inclusion of 5%, 10%, and 20% EY at all incubation times. 364

The marked inconsistency between sperm motility and MI, as indicated by the lack of 365 correlations between the values for these variables, in anisosmotic conditions indicate that sperm 366 functions may cease before there are disruptions in the integrity of the plasmalemma. Results from 367 368 the present study confirm results from previous studies with bulls, in which the proportion of total 369 motile spermatozoa at 100 and 150 mOsm/kg was less compared with the MI when there was similar management of bull semen in the same conditions as that of the present study, however, values for 370 371 both variables were less than those near-isosmotic conditions. In hyperosmotic conditions, sperm motility was markedly less, whereas sperm MI was similar to the values when there were 300 to 936 372 373 mOsm/kg conditions (Liu and Foote, 1998). There was a similar response trend in other studies with human and ram spermatozoa (Curry and Watson, 1994; Gao et al., 1995). 374

Different from what was previously hypothesized (Liu and Foote, 1998), the mechanism resulting in kinetic loss was not related to the mitochondrial dysfunction because the changes in kinetic values were similar to those for MI. This indicates that the lesser kinetic capacity of spermatozoa during osmotic stress could be marginally due to the dysfunction of the metabolic function of mitochondria. Thus, the suppressive effect of the hyperosmotic conditions on kinetics of sperm is through a different mechanism, likely at the cytoskeleton. Reorganization of the cytoskeletal actin when there are hyperosmotic conditions may be responsible for the decreased motility when these conditions prevail (Correa et al., 2007). Specific studies should be designed, however, to verify this second hypothesis.

In a previous study, it was suggested that the response of sperm to the anisosmotic condition 384 could not only be related to plasma MI but also to the membrane permeability to ions and to the 385 386 cytoskeletal integrity (Petrunkina et al., 2004). Results from the present study seem to corroborate the results from this previous study because there were different extents of curving/twisting in sperm 387 incubated in hypo-osmotic conditions when EY was not included in the diluent which may indicate 388 389 there was a different response of spermatozoa at the structural level. The capacity of dog spermatozoa for modification of the tail morphology in hypo-osmotic conditions was reported by Kumi-Diaka 390 (1993). Unfortunately, in this previous study the extent of the sperm response to the hypo-osmotic 391 stress was not reported; thus, it was not possible to compare the data from this previous and the present 392 study directly. In contrast with findings of Kumi-Diaka (1993), the number of sperm that had curled 393 394 tails in the present study was similar soon after dilution, at 20 min and after 45 min of incubation. Thus, in the present study there was osmotic adaptation onset near the time of induction of osmotic 395 stress and there were no subsequent changes during the incubation period. The timing of sperm 396 397 response to the osmotic stress in the present study was similar to that previously reported (Pinto and Kozink, 2008), in which there was no difference in the percentage of sperm with a curled tail at 1 or 398 399 60 min of the incubation period.

Results indicating there was a protective effect of EY for osmotic stress could indicate that EY has actions during cryopreservation because sperm survival after freezing-thawing procedures seemed to be related to the sperm capacity to undergo cell volume regulation (Petrunkina et al., 2004).

As expected, the addition of EY to the medium resulted in a protective effect during 403 404 cryopreservation, as reported in most studies where there was cryopreservation of dog semen using EY (Silva et al., 2002). Although the usual concentration of EY used in dog semen extender is 20% 405 (Anderson, 1972; Peña et al., 1998; Silva et al., 2002), the results of the present study indicate that 406 the concentration of EY is not as important as previously thought for preservation of dog 407 spermatozoa. In samples without EY, there were lesser percentages of motile and membrane intact 408 409 spermatozoa, similar to the data reported in a previous study (Silva et al., 2002). There was an improvement in sperm characteristics when EY was used at the 5% concentration compared with 410 other EY concentrations that were assessed. 411

Unexpectedly, the total amount of motile and progressive sperm was slightly less in samples diluted in extender with 20% EY compared with 5% and 10% EY. This finding indicates that a greater EY concentration could be detrimental for cryopreserved dog spermatozoa, as previously proposed in other species (Moussa et al., 2002; Amirat et al., 2004). To the best of the authors' knowledge, no study has been previous conducted where there was comparison of different EY concentrations in cryopreservation of the same dog semen sample, thus the supposed greater protective effects of EY at the 20% concentration was never previously evaluated.

419 The results in the present study indicate dog spermatozoa could be successfully frozen with 420 there being viable sperm after thawing with use of concentrations of EY that are less than 20%. This finding is consistent with results from studies with other species, in which EY concentration in the 421 extender was 2% (Pillet et al., 2008). The optimal concentration of low-density lipoproteins (LDL), 422 423 which are the active fraction of EY in semen preservation, could be species-specific. In bulls, (Moussa et al., 2002) there were similar post-thaw sperm kinetics with use of EY 20% and 2.5% LDL, while 424 425 the values were greater when there was inclusion of 5% to 10% LDL in the extender (Moussa et al., 2002). With a relatively greater concentration of LDL (15% and 20%), post-thaw sperm motility is 426 less. In dogs, Bencharif et al. (2008) reported there were greater values in the cryopreserved 427 spermatozoa with the use of LDL compared with the conventional EY concentration, but among the 428

different LDL concentrations, there were greater values using 6% LDL. It is possible that the LDL purification conditions used in this previous study could have affected the results, explaining the differences in these previous values from those in the present study. Results of both studies indicate the use of EY at a relatively greater concentration (20%) could reduce dog sperm quality after thawing, and the use of lesser concentrations (5%, 10%) or a purified LDL preparation could result in greater post-thaw dog sperm viability.

The results from the present study indicate that at least, in part, there is a cryoprotective effect 435 of EY of cryopreserved spermatozoa of dogs that is related to the protection against osmotic stress. 436 437 The lack of correlation between total and progressive sperm motility is markedly reduced, and MI, 438 less affected by the anisosmotic environment, in both hypo- and hyper-osmotic conditions, indicating 439 that the damage occurred at the cytoskeleton or to a non-mitochondrial metabolic pathway. The osmoprotective effect of the EY was not dose-dependent because there were similar values at 5%, 10%, 440 441 and 20% concentrations of EY. During cryopreservation of dog sperm, inclusion of EY at 5% and 10% was apparently more effective compared with inclusion of EY at 20% in maintaining sperm 442 viability after thawing. 443

444

445 **5.** Conclusions

446 The results of the present study indicate EY has a protective action during osmotic stress in dog spermatozoa. The protective effect seems not to be dose-dependent because there were no 447 differences in sperm characteristics after dilution with extender at the 5%, 10%, or 20% 448 449 concentrations of EY. Furthermore, spermatozoa in hyperosmotic conditions had a reduction of kinetic capacity to a greater extent than there was loss of membrane integrity, indicating there was 450 451 likely primary cytoskeletal damage that led to a loss of kinetic capacity. Furthermore, inclusion of EY in the semen extender did not have a dose-dependent protection effect during cryopreservation of 452 dog spermatozoa. There was greater viability of frozen-thawed sperm using 5% and 10% EY 453 compared with samples where there was no EY inclusion, however, the viability was only slightly 454

greater than with inclusion of 20% EY, indicating dog spermatozoa could be effectivelycryopreserved at a lesser EY concentration and viability would be retained after thawing.

457

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461

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593 **Figure legend**

- Fig. 1. Bar charts of the tail defect proportions at the different osmolarities (75, 150, 300, 500, and
- 595 1000 mOsm/kg) in dog spermatozoa diluted with 0% (EY 0%) and with 10% (EY 10%) egg yolk;
- 596 Bars with different letters differ (P < 0.05)

599 Values for sperm kinetic characteristics (\pm SEM) of dog semen samples (n = 21) not diluted with

egg yolk (EY 0%), or diluted with 5%, 10%, or 20% EY and incubated in different osmotic

601	conditions (75,	, 150, 300,	500, and	1000 mOsm)	soon after dilution	$(0 \min)$	
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EY	Osmolarity	TM	PM	VAP	VSL	VCL	ALH	
(%)	(mOsm)	(%)	(%)	(µm/s)	(µm/s)	(µm/s)	(µm)	
	75	0	0	0	0	0	0	
	150	4±0.6ª	0	22.3±2.7ª	20.6±2.5ª	95.2±2.9ª	4.4±0.17 ^a	19
0	300	83.9 ± 2.3^{b}	77.8±2 ^a	174.6±4.7 ^b	162.5±3.3 ^b	234.1 ± 4.9^{b}	7.5 ± 0.15^{b}	21
	500	6±0.8ª	0	61.7±2.3°	43.4±2.2 ^{ac}	147.0±5.4 ^{ac}	5.6±0.19 ^a	13
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	13.6±0.8ª	6.1 ± 0.5^{b}	47.1±1.6 ^{ac}	38.2 ± 1.5^{ac}	120.2±4.2 ^{ac}	6±0.17 ^{ab}	37
5	300	91.1±1.5 ^b	83.7±1.8ª	173.9±3.7 ^b	158.2 ± 2.8^{b}	199.7 ± 4.2^{b}	6.2±0.19 ^{ab}	20
	500	82.5 ± 2.5^{b}	36.9±2.1°	96.4±2.2°	78.2±2.5°	167.2±5.3°	8.1 ± 0.19^{b}	24
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	12±0.6ª	5.9 ± 0.7^{b}	41.8±2.3 ^{ac}	35.3±2.1 ^{ac}	113.0±4.6 ^a	5.7±0.19 ^a	38
10	300	90.3±1.1 ^b	83.5±1.7ª	178.6±3.1 ^b	167.2±3.1 ^b	202.3 ± 5.7^{b}	6.3±0.17 ^{ab}	17
	500	81.5±2.3 ^b	38.3±2°	98.1±2.7°	75.9±2.2°	164.5±4.9°	8.6±0.13 ^b	23
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	$10.7{\pm}0.8^{a}$	4.8 ± 0.5^{b}	49.1±2.1 ^{ac}	37.2±1.6 ^{ac}	121.6±4 ^{ac}	5.9±0.24 ^a	33
20	300	88.6 ± 1.8^{b}	83.8±1.8ª	179.6 ± 2.9^{b}	169.1±4.6 ^b	203.1 ± 5^{b}	6.5±0.13 ^{ab}	16
	500	77.3±1.7 ^b	31.2±1.5°	97.6±2.4°	75.5±3.9°	162.7±4.3°	$8.4{\pm}0.24^{b}$	21
	1000	0	0	0	0	0	0	

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL,

603 curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR,

604 Linearity - LIN, sperm viability

605 In the same column, values with different superscript (a/b/c) differ ($P \le 0.05$)

- Values for sperm kinetic characteristics (\pm SEM) of dog semen samples (n = 21) not diluted with
- egg yolk (EY 0%), or diluted with 5%, 10%, or 20% EY and incubated in different osmotic

610 conditions (75, 150, 300, 500, and 1000 mOsm) for 45 min

EY	Osmolarity	ТМ	PM	VAP	VSL	VCL	ALH	
(%)	(mOsm)	(%)	(%)	(µm/s)	(µm/s)	(µm/s)	(µm)	
	75	0	0	0	0	0	0	
	150	0	0	0	0	0	0	
0	300	82.4±2.3ª	71.6±2.3ª	164.2 ± 2.6^{a}	137.8±3ª	201.3±4.1ª	7.8 ± 0.24^{a}	18
	500	1.3±0.3 ^b	0	37.2 ± 2.5^{b}	26.8 ± 2.2^{b}	113.9 ± 5.8^{b}	9.5 ± 0.59^{b}	9
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	18.4±1.4°	9.6 ± 0.5^{b}	50.7 ± 1.6^{bc}	46.4 ± 2.1^{bc}	131.4±2.5 ^b	5.6±0.22°	39
5	300	88.7±2ª	79.8±1.8ª	176.2±2.9 ^a	151.7±2.7ª	198.4±4.6ª	7±0.2ª	28
	500	78.5±2.1ª	36.2±1.7°	94.1±1.9°	76.8±2.3°	168.2 ± 4.5^{ab}	$8.9{\pm}0.22^{b}$	24
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	15.7±0.9°	8.2±0.4b	62.4 ± 1.6^{bc}	43.1±1.7 ^{bc}	128.1 ± 2.8^{b}	5.4±0.17°	42
10	300	90.5 ± 1.8^{d}	$82.8{\pm}1.8^{a}$	179.4±3.6 ^a	158.3±2.5ª	205.7±4.3ª	7.1±0.24 ^a	2
	500	83.8±1.9 ^a	36.1±2°	98.5±2.2°	80.3±2.3°	166.5±4.7 ^{ab}	9.1±0.41 ^b	26
	1000	0	0	0	0	0	0	
	75	0	0	0	0	0	0	
	150	14.2±1.3°	6.3 ± 0.2^{b}	47.8 ± 1.9^{b}	39.8 ± 2^{bc}	126.2±2.4 ^b	5.5±0.26°	40
20	300	89.6±1.9 ^d	81.3±2 ^a	177.4±2.1ª	161.2±5.1ª	204.1±5 ^a	$7.2{\pm}0.24^{a}$	23
	500	75.8±2.2ª	32.4±1.4°	93.7±2.4°	74.8±2.1°	164.1±4.1 ^{ab}	9±0.26 ^b	20
	1000	0	0	0	0	0	0	

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL,

612 curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR,

613 Linearity - LIN, sperm viability

614 In the same column, values with different superscript (a/b/c) differ ($P \le 0.05$)

617 Means for (± SEM) of total sperm motility (TM), sperm membrane integrity (MI), sperm with

relatively greater mitochondrial membrane potential (HMMP), sperm with relatively lesser

619 mitochondrial membrane potential (LMMP) in semen samples of dogs (n = 21) not diluted with egg

yolk (EY 0%), or diluted with 5%, 10%, or 20% EY and incubated in different osmotic conditions

621 (75, 150, 300, 500, and 1000) for 20 min

EY (%)	Osmolarity (mOsm)	TM (%)	MI (%)	HMMP (%)
	75	0	37.3±4.2ª	27.2±2.3ª
	150	1.7±0.4ª	64.8±3.5 ^{bc}	37.9±2.7ª
0	300	81.6±2.6 ^b	88.6±2.8°	74.8±1.9 ^b
	500	2.4±0.5ª	55.9±2.9 ^b	61.2±2.4 ^b
	1000	0	38.7±2.9ª	10.4±1.8ª
	75	0	78.5±2.6°	54.7±2 ^b
	150	13.7±1.1°	83.8 ±2.1°	66.1±2.1 ^b
5	300	90.4±1.5 ^b	86.9±2°	70.6±1.6 ^b
	500	79.7±1.7 ^b	86.4±1.9°	70.1±2 ^b
	1000	0	76.1±2.4°	68.6±2.2 ^b
	75	0	83.7±2.1°	57.6±1.9 ^b
	150	14.8±0.7°	82.5±1.8°	65.8±1.3 ^b
10	300	91.8±1.6 ^b	87.8±1.5°	73.8±2.1 ^b
	500	85.2±2.1 ^b	84.1±1.9°	68.5±2.2 ^b
	1000	0	75.7±3.2°	69.3±2.3 ^b
	75	0	80.5±2.3°	58.1±2.2 ^b
	150	12.3±1.2°	84.1±1.6°	66.4±1.9 ^b
20	300	90.7 ± 2^{b}	89.9±1.7°	71.9±1.4 ^b
	500	74.7 ± 2^{b}	85.1±2.2°	70.8±1.2 ^b
	1000	0	80.6±2.4°	67.5±1.2 ^b

622 In the same column, values with different superscript (a/b/c) differ ($P \le 0.05$)

Percentages of sperm head and midpiece abnormalities (\pm SEM) of dog semen samples (n = 21) not

diluted with egg yolk (EY 0%), or diluted with 5%, 10%, or 20% EY and incubated in different

osmotic conditions (75, 150, 300, 500, and 1000 mOsm) soon after dilution (0 min)

EY (%)	Osmolarity (mOsm)	Abnormal head	Abnormal midpiece
	75	2.6±0.3	3.1±0.41
	150	2.3±0.26	4.3±0.59
0	300	3.2±0.33	2.8±0.46
	500	2.6 ± 0.46	2.2±0.44
	1000	1.9±0.33	2.7±0.41
	75	2.4 ± 0.24	3.4±0.28
	150	2.6±0.35	2.8±0.24
5	300	2.9±0.26	2.9±0.33
	500	2.7±0.3	3±0.2°
	1000	2.2 ± 0.26	2.8±0.46
	75	2.1±0.33	3.2±0.55
	150	2.7±0.24	3.1±0.46
10	300	2.6±0.39	3.4±0.5
	500	3.3±0.46	2.8±0.37
	1000	2.5±0.3	3.1±0.41
	75	3.1±0.28	2.8±0.46
	150	2.7 ± 0.46	4.2±0.33
20	300	3.1±0.44	3.5±0.48
	500	2.3±0.37	3.8±0.59
	1000	2.6±0.35	3.1±0.39

631 Mean (\pm SEM) sperm characteristics of fresh dog semen (n = 21) not extended in egg yolk (0% EY)

	EY 0%	EY 5%	EY 10%	EY 20%
TM (%)	88±0.4ª	91±0.9ª	93±1.1ª	89±0.9ª
PM (%)	76±1.7ª	87±1.1 ^b	87±1.3 ^b	72±1.7ª
VAP (µm/s)	116.7±7.1ª	129.2±3.5 ^b	131.4±5 ^b	118.2±4.7 ^a
VSL (µm/s)	94.6±4.5ª	113.8 ± 3.3^{b}	112.7±5.7 ^b	98.4±5.7ª
VCL (µm/s)	193±13.3ª	238.2 ± 9.5^{b}	226.1±11.2 ^b	208.8±10.1ª
ALH (µm)	8.2±0.3ª	8.4±0.3ª	8.4±1.1ª	8.3±0.2ª
BCF (Hz)	40.8±2.1ª	43.5±1.6 ^a	45.2±2ª	42.7±2ª
STR (%)	74.7±2.8ª	83.9±2.6 ^{ab}	87.2 ± 2.9^{b}	$80.8{\pm}2.8^{ab}$
LIN (%)	41±3.3ª	56.7 ± 1.5^{b}	58.3 ± 1.5^{b}	46.1±1.4 ^a
PI-/PSA- (%)	83.3±0.6 ^a	89.2±0.8ª	88.4±0.6ª	85.6±0.7ª
PI-/PSA+ (%)	1.1±0.1ª	1.9±0.1ª	1.3±0.1ª	1.2±0.1ª
PI+/PSA- (%)	14.6±0.3ª	8.7±0.3ª	9.1±0.2 ^a	11.8±0.2ª
PI+/PSA+ (%)	0.9±0.1ª	1.1±0.1ª	1.8±0.1ª	1±0.1ª
HMMP (%)	67.8±0.1ª	70.9±2ª	71.6±2.2ª	68.2±2.3ª

or extended at different concentrations in egg yolk (, 5%, 10%, or 20% EY)

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL,

curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR,
 Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm

Linearity - Linv, sperin viaonity, sperin with memorane integrity and acrossine integrity - P1-/PSA-, sperin

636 with membrane integrity and acrosome reaction - PI-/PSA+; sperm with membrane damage and acrosome

637 integrity -PI+/PSA+; sperm with membrane damage and acrosome reaction -PI+/PSA+; sperm with high

638 mitochondrial membrane potential – HMMP

639 In the same row, values with different superscript (a/b) differ ($P \le 0.05$)

642 Mean (\pm SEM) sperm characteristics in dog semen (n = 21) not extended in egg yolk (EY 0%) or

extended in EY at different concentrations 5%, 10%, or 20% EY) after equilibration for 2 h at 4 °C

	EY	0%	EY	5%	EY	EY 10%	
	EQ	FT	EQ	FT	EQ	FT	
TM (%)	83±1.3ª	22±3.1 ^b	92±1.1ª	51±2.2°	91±1.5ª	52±1.7°	
PM (%)	68±1.5ª	13±3.9 ^b	89±0.9°	39±2 ^d	89±1.1°	40±1.5 ^d	
VAP (µm/s)	107.3±6.5ª	64.8 ± 6.8^{b}	134.6±4.1°	109.3±5.8ª	132.7±5.2°	112.4±6.4ª	1
VSL (µm/s)	87.3 ± 5.2^{a}	47.7±6.1 ^b	112.2±4.1°	91.8±5.7 ^{ac}	110.6±5.5°	95.4 ± 5.2^{ac}	1
VCL (µm/s)	187±11.1ª	98.8 ± 7^{b}	235.7±9.2°	145.1 ± 8.6^{d}	229.4±8.7°	139.8±9.3 ^d	2
ALH (µm)	8.6±0.3ª	5.1 ± 0.5^{b}	8.1±0.2ª	7.3±0.3ª	8±0.3ª	7.1±0.2ª	
BCF (Hz)	38.7±1.7ª	29.6 ± 1.8^{b}	45.2 ± 1.8^{a}	41.8±2 ^a	44.7±1.9ª	40.1±2.1ª	4
STR (%)	77.2±3.3ª	75.8±2.8ª	85.8 ± 2.6^{b}	82.9 ± 2.6^{ab}	86.1 ± 3.3^{b}	$80.4{\pm}2.9^{ab}$	8
LIN (%)	43.2 ± 2.6^{a}	39.7±2.8ª	57.9 ± 1.5^{b}	49.3±1.3 ^{ab}	57.4±1.6 ^b	49.7 ± 1.8^{ab}	5
PI-/PSA- (%)	82.7±1.3ª	29.6 ± 1.8^{b}	$88.2{\pm}1.6^{a}$	57.7±2.1°	89.1±1.3ª	54.3±1.8°	8
PI-/PSA+ (%)	1±0.1ª	2.3 ± 0.2^{b}	1.2±0.1ª	1.1±0.2ª	1.2±0.1ª	1±0.1ª	
PI+/PSA- (%)	15.3±0.2ª	62.8±1.1 ^b	9.5±0.2ª	36.8±0.7°	8.2±0.1ª	40.8±0.9°	
PI+/PSA+ (%)	1.2±0.1ª	5.6 ± 0.3^{b}	1.2±0.1ª	4.1±0.3 ^b	1.6±0.1ª	$3.9{\pm}0.2^{b}$	
HMMP (%)	61.7±2 ^a	31.4±2.2 ^b	73.2±1.9°	49.3±1.8 ^{ab}	72.5±2.1°	50.6±2.4 ^{ab}	(

644 (EQ) and after freezing/thawing (FT)

645 Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL,

646 curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR,

Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm
 with membrane integrity and acrosome reaction - PI-/PSA+; sperm with membrane damage and acrosome

649 integrity -PI+/PSA-; sperm with membrane damage and acrosome reaction -PI+/PSA+; sperm with high

650 mitochondrial membrane potential – HMMP

651 In the same row, values with different superscript (a/b/c/d/e) differ ($P \le 0.05$)

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EY	Osmolarity	ТМ	PM	VAP	VSL	VCL	ALH	BCF	STR	LIN
(%)	(mOsm)	(%)	(%)	(µm/s)	(µm/s)	(µm/s)	(µm)	(Hz)	(%)	(%)
	75	0	0	0	0	0	0	0	0	0
	150	4±0.6ª	0	22.3±2.7ª	20.6 ± 2.5^{a}	95.2±2.9ª	4.4±0.17 ^a	19.5±1.9 ^a	62 ± 2.4^{a}	13±1.1ª
0	300	83.9 ± 2.3^{b}	77.8 ± 2^{a}	174.6±4.7 ^b	162.5±3.3 ^b	234.1 ± 4.9^{b}	7.5 ± 0.15^{b}	21.4±1.4ª	93 ± 2^{b}	71 ± 2.2^{b}
	500	6±0.8ª	0	61.7±2.3°	43.4±2.2 ^{ac}	147.0±5.4 ^{ac}	5.6±0.19 ^a	13.8 ± 1.3^{b}	71±2.6 ^{ab}	29±2°
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	13.6±0.8ª	6.1 ± 0.5^{b}	47.1±1.6 ^{ac}	38.2±1.5 ^{ac}	120.2±4.2 ^{ac}	6±0.17 ^{ab}	37.6±1.3°	80±2.2ª	$38\pm2^{\circ}$
5	300	91.1 ± 1.5^{b}	$83.7{\pm}1.8^{a}$	173.9±3.7 ^b	158.2 ± 2.8^{b}	199.7 ± 4.2^{b}	6.2±0.19 ^{ab}	20.3±1.2ª	91 ± 2^{b}	84 ± 2.2^{b}
	500	82.5 ± 2.5^{b}	36.9±2.1°	96.4±2.2°	78.2±2.5°	167.2±5.3°	8.1 ± 0.19^{b}	24.6±1.4ª	77 ± 1.7^{ab}	50±1.7°
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	12±0.6ª	5.9 ± 0.7^{b}	41.8±2.3 ^{ac}	35.3±2.1 ^{ac}	113.0±4.6 ^a	5.7±0.19 ^a	38.7±1.5°	85 ± 2^{a}	35±1.7°
10	300	90.3±1.1 ^b	83.5±1.7ª	178.6±3.1 ^b	167.2±3.1 ^b	202.3 ± 5.7^{b}	6.3±0.17 ^{ab}	17.1±1.3 ^{ab}	93±1.7ª	83±2.4 ^b
	500	81.5 ± 2.3^{b}	38.3±2°	98.1±2.7°	75.9±2.2°	164.5±4.9°	8.6 ± 0.13^{b}	23.1±1.6ª	78 ± 2^{ab}	47±2°
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	10.7±0.8ª	4.8 ± 0.5^{b}	49.1 ± 2.1^{ac}	37.2 ± 1.6^{ac}	121.6±4ac	5.9±0.24ª	33.8±1.5°	73±1.5 ^{ab}	29±2.2°
20	300	88.6 ± 1.8^{b}	$83.8{\pm}1.8^{a}$	179.6 ± 2.9^{b}	169.1±4.6 ^b	203.1±5 ^b	6.5±0.13 ^{ab}	16.5 ± 1.6^{ab}	94 ± 2^{b}	83 ± 2^{b}
	500	77.3±1.7 ^b	31.2±1.5°	97.6±2.4°	75.5±3.9°	162.7±4.3°	$8.4{\pm}0.24^{b}$	21.8 ± 1.7^{a}	78 ± 2.2^{ab}	47±1.7°
	1000	0	0	0	0	0	0	0	0	0

Sperm kinetic characteristics (\pm SEM) of dog semen samples (n = 21) diluted with egg yolk (EY) 0%, EY 5%, EY 10%, and EY 20% and incubated in different osmotic conditions (75 mOsm 150 mOsm 300 mOsm 500 mOsm and 1000 mOsm) soon after dilution (0 min)

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL, curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR, Linearity - LIN, sperm viability.

In the same column, values with different superscript (a/b/c) differ significantly ($P \le 0.05$).

Sperm kinetic characteristics (\pm SEM) of dog semen samples (n = 21) diluted with egg yolk (EY) 0%, EY 5%, EY 10%, and EY 20% and incubated in different osmotic conditions (75 mOsm, 150 mOsm, 300 mOsm, 500 mOsm, and 1000 mOsm) for 45 min.

EY	Osmolarity	ТМ	PM	VAP	VSL	VCL	ALH	BCF	STR	LIN
(%)	(mOsm)	(%)	(%)	(µm/s)	(µm/s)	(µm/s)	(µm)	(Hz)	(%)	(%)
	75	0	0	0	0	0	0	0	0	0
	150	0	0	0	0	0	0	0	0	0
0	300	82.4±2.3ª	71.6±2.3ª	164.2 ± 2.6^{a}	137.8±3ª	201.3±4.1ª	7.8 ± 0.24^{a}	18.4±1.4 ^a	87±2ª	61±2.2ª
	500	1.3 ± 0.3^{b}	0	37.2 ± 2.5^{b}	26.8 ± 2.2^{b}	113.9±5.8 ^b	9.5 ± 0.59^{b}	9.8 ± 0.9^{b}	46±2 ^b	19±1.3 ^b
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	18.4±1.4°	9.6±0.5 ^b	50.7 ± 1.6^{bc}	46.4±2.1bc	131.4±2.5 ^b	5.6±0.22°	39.7±1.6°	79±1.7 ^a	36 ± 2^{ab}
5	300	88.7±2ª	79.8±1.8ª	176.2±2.9ª	151.7±2.7ª	198.4±4.6 ^a	7±0.2ª	28.5±1.1ª	90±2ª	81±2.4ª
	500	78.5±2.1ª	36.2±1.7°	94.1±1.9°	76.8±2.3°	168.2±4.5 ^{ab}	8.9 ± 0.22^{b}	24.7±1.5 ^a	75±2ª	46±4.1 ^{ab}
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	15.7±0.9°	8.2±0.4b	62.4±1.6 ^{bc}	43.1±1.7 ^{bc}	128.1 ± 2.8^{b}	5.4±0.17°	42.3±1.4°	87±2ª	39±2.2 ^{ab}
10	300	90.5±1.8 ^d	82.8±1.8ª	179.4±3.6ª	158.3±2.5ª	205.7±4.3ª	7.1±0.24 ^a	27.2±1ª	92±2.2ª	83±2.2ª
	500	83.8±1.9 ^a	36.1±2°	98.5±2.2°	80.3±2.3°	166.5±4.7 ^{ab}	9.1±0.41 ^b	26.7±1.6 ^a	83±2ª	56±1.7ª
	1000	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	150	14.2±1.3°	6.3±0.2 ^b	47.8±1.9 ^b	39.8 ± 2^{bc}	126.2±2.4 ^b	5.5±0.26°	40.6±1.5°	79±2.2ª	34 ± 2^{ab}
20	300	89.6±1.9 ^d	81.3±2 ^a	177.4±2.1ª	161.2±5.1ª	204.1±5 ^a	$7.2{\pm}0.24^{a}$	23.8±1.5 ^a	90±1.7 ^a	78 ± 2^{a}
	500	75.8±2.2ª	32.4±1.4°	93.7±2.4°	74.8±2.1°	164.1±4.1 ^{ab}	9±0.26 ^b	20.6±1.5ª	77±2.2ª	45 ± 2^{ab}
	1000	0	0	0	0	0	0	0	0	0

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL, curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR, Linearity - LIN, sperm viability.

In the same column, values with different superscript (a/b/c) differ significantly ($P \le 0.05$).

Mean (\pm SEM) of total motility (TM), membrane integrity (MI), sperm with high mitochondrial membrane potential (HMMP), sperm with low mitochondrial membrane potential (LMMP) in canine samples (n = 21) diluted with egg yolk (EY) 0%, EY 5%, EY 10%, and EY 20% and incubated in different osmotic conditions (75 mOsm, 150 mOsm, 300 mOsm, 500 mOsm, and 1000 mOsm) for 20 min.

EY (%)	Osmolarity (mOsm)	TM (%)	MI (%)	HMMP (%)	LMMP (%)
(70)	(IIIOSIII) 75	0	37 3+4 2ª	27 2+2 3ª	17 7+1 /a
	150	0 1 7+0 4a	57.5 ± 4.2	27.2 ± 2.3 27.0 $\pm2.7a$	17.7 ± 1.4
0	150	1./±0.4*	04.8±3.3**	37.9±2.7=	23.0±2.4*
0	300	81.6±2.6 ^b	88.6±2.8°	74.8±1.9 ^b	3.6 ± 0.9^{6}
	500	2.4±0.5 ^a	55.9±2.9 ^b	61.2±2.4 ^b	11.8 ± 0.7^{ab}
	1000	0	38.7 ± 2.9^{a}	10.4 ± 1.8^{a}	5.8 ± 0.7^{b}
	75	0	78.5±2.6°	54.7±2 ^b	27.1±1.7ª
	150	13.7±1.1°	83.8 ±2.1°	66.1±2.1 ^b	16.3±2ª
5	300	90.4±1.5 ^b	86.9±2°	70.6±1.6 ^b	10.6±1.2 ^{ab}
	500	79.7±1.7 ^b	86.4±1.9°	70.1 ± 2^{b}	13.4±1.3 ^{ab}
	1000	0	76.1±2.4°	68.6 ± 2.2^{b}	11.7±1.1 ^{ab}
	75	0	83.7±2.1°	57.6±1.9 ^b	21.9±1.4ª
	150	14.8±0.7°	82.5±1.8°	65.8±1.3 ^b	15.1±2.1ª
10	300	91.8±1.6 ^b	87.8±1.5°	73.8±2.1 ^b	8.4±0.5 ^b
	500	85.2±2.1 ^b	84.1±1.9°	68.5±2.2 ^b	12.2±0.7 ^{ab}
	1000	0	75.7±3.2°	69.3±2.3 ^b	12.8 ± 0.6^{ab}
	75	0	80.5±2.3°	58.1 ± 2.2^{b}	24.2±0.8ª
	150	12.3±1.2°	84.1±1.6°	66.4±1.9 ^b	13.7±0.6 ^{ab}
20	300	90.7±2 ^b	89.9±1.7°	71.9±1.4 ^b	9.2±0.6 ^b
	500	74.7±2 ^b	85.1±2.2°	70.8±1.2 ^b	$7.9{\pm}0.4^{b}$
	1000	0	80.6±2.4°	67.5±1.2 ^b	14.5±0.5 ^{ab}

In the same column, values with different superscript (a/b/c) differ significantly ($P \le 0.05$).

Percentage of sperm head and midpiece abnormalities (\pm SEM) of dog semen samples (n = 21) diluted with egg yolk (EY) 0%, EY 5%, EY 10%, and EY 20% and incubated in different osmotic conditions (75 mOsm, 150 mOsm, 300 mOsm, 500 mOsm, and 1000 mOsm) soon after dilution (0 min).

EY	Osmolarity	Abnormal head	Abnormal midpiece
(%)	(mOsm)	(%)	(%)
	75	2.6±0.3	3.1±0.41
	150	2.3±0.26	4.3±0.59
0	300	3.2±0.33	2.8±0.46
	500	2.6 ± 0.46	2.2±0.44
	1000	1.9±0.33	2.7±0.41
	75	$2.4{\pm}0.24$	3.4±0.28
	150	2.6±0.35	2.8±0.24
5	300	2.9±0.26	2.9±0.33
	500	2.7±0.3	3±0.2°
	1000	2.2 ± 0.26	2.8 ± 0.46
	75	2.1±0.33	3.2±0.55
	150	2.7±0.24	3.1±0.46
10	300	2.6±0.39	3.4±0.5
	500	3.3±0.46	2.8±0.37
	1000	2.5±0.3	3.1±0.41
	75	3.1±0.28	2.8 ± 0.46
	150	2.7 ± 0.46	4.2±0.33
20	300	3.1±0.44	3.5±0.48
	500	2.3±0.37	3.8±0.59
	1000	2.6±0.35	3.1±0.39

Mean (\pm SEM) seminal characteristics of fresh canine semen (n = 21) extended at different concentration of egg yolk (0% EY, 5% EY, 10% EY, and 20% EY).

	EY 0%	EY 5%	EY 10%	EY 20%
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TM (%)	88±0.4ª	91±0.9ª	93±1.1ª	89±0.9ª
PM (%)	76±1.7 ^a	87±1.1 ^b	87±1.3 ^b	72±1.7ª
VAP (µm/s)	116.7±7.1ª	129.2±3.5 ^b	131.4±5 ^b	118.2±4.7 ^a
VSL (µm/s)	94.6±4.5ª	113.8±3.3 ^b	112.7±5.7 ^b	98.4±5.7ª
VCL (µm/s)	193±13.3ª	238.2±9.5 ^b	226.1±11.2 ^b	208.8±10.1ª
ALH (µm)	8.2±0.3ª	8.4±0.3ª	8.4±1.1ª	8.3±0.2ª
BCF (Hz)	40.8±2.1ª	43.5±1.6 ^a	45.2±2ª	42.7±2 ^a
STR (%)	74.7±2.8ª	83.9 ± 2.6^{ab}	87.2 ± 2.9^{b}	$80.8{\pm}2.8^{ab}$
LIN (%)	41±3.3ª	56.7 ± 1.5^{b}	58.3 ± 1.5^{b}	46.1±1.4 ^a
PI-/PSA- (%)	83.3±0.6 ^a	$89.2{\pm}0.8^{a}$	88.4±0.6 ^a	85.6±0.7ª
PI-/PSA+ (%)	1.1±0.1ª	1.9±0.1ª	1.3±0.1ª	1.2±0.1ª
PI+/PSA- (%)	14.6±0.3ª	8.7±0.3ª	9.1±0.2ª	11.8±0.2ª
PI+/PSA+ (%)	0.9±0.1ª	1.1±0.1ª	1.8±0.1ª	1±0.1ª
HMMP (%)	67.8±0.1ª	70.9±2ª	71.6±2.2 ^a	68.2±2.3ª

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL, curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR, Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm with membrane integrity and acrosome reaction - PI-/PSA+; sperm with membrane damage and acrosome integrity - PI+/PSA-; sperm with membrane damage and acrosome reaction - PI+/PSA+; sperm with high mitochondrial membrane potential – HMMP.

In the same row, values with different superscript (a/b) differ significantly ($P \le 0.05$).

	EY 0%		EY	5%	EY 10% F		EY	EY 20%	
	EQ	FT	EQ	FT	EQ	FT	EQ	FT	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
TM (%)	83±1.3ª	22±3.1 ^b	92±1.1ª	51±2.2°	91±1.5ª	52±1.7°	88±1.1ª	43±2 ^d	
PM (%)	68±1.5ª	13±3.9 ^b	89±0.9°	39 ± 2^{d}	89±1.1°	40±1.5 ^d	80±1.3°	32 ± 2^{e}	
VAP (µm/s)	107.3±6.5ª	64.8 ± 6.8^{b}	134.6±4.1°	109.3±5.8ª	132.7±5.2°	112.4±6.4ª	120.4±5.5 ^{ac}	97.5±7.1ª	
VSL (µm/s)	87.3±5.2ª	47.7±6.1 ^b	112.2±4.1°	91.8±5.7 ^{ac}	110.6±5.5°	95.4 ± 5.2^{ac}	102.3±5.7 ^{ac}	80.6±5.9ª	
VCL (µm/s)	187±11.1ª	98.8 ± 7^{b}	235.7±9.2°	145.1 ± 8.6^{d}	229.4±8.7°	139.8±9.3 ^d	206.2±9.1°	116.7±8.4 ^b	
ALH (µm)	8.6±0.3ª	5.1±0.5 ^b	8.1±0.2ª	7.3±0.3ª	8±0.3ª	7.1±0.2ª	8.3±0.3ª	7.3±0.2ª	
BCF (Hz)	38.7±1.7ª	29.6±1.8b	45.2±1.8 ^a	41.8±2 ^a	44.7±1.9ª	40.1±2.1ª	41.9±1.9 ^a	37.8±1.8ª	
STR (%)	77.2±3.3ª	75.8±2.8ª	85.8 ± 2.6^{b}	82.9 ± 2.6^{ab}	86.1±3.3 ^b	$80.4{\pm}2.9^{ab}$	81.6±3.5 ^{ab}	77.9±1ª	
LIN (%)	43.2 ± 2.6^{a}	39.7±2.8ª	57.9±1.5 ^b	49.3±1.3 ^{ab}	57.4±1.6 ^b	49.7 ± 1.8^{ab}	51.4 ± 1.6^{ab}	45.8±1.5 ^{ab}	
PI-/PSA- (%)	82.7±1.3ª	29.6±1.8b	88.2±1.6ª	57.7±2.1°	89.1±1.3ª	54.3±1.8°	84.7±1.5 ^a	47.7±1.5°	
PI-/PSA+ (%)	1±0.1ª	2.3±0.2 ^b	1.2±0.1ª	1.1±0.2ª	1.2±0.1ª	1±0.1ª	1.1±0.1ª	1.2±0.1ª	
PI+/PSA- (%)	15.3±0.2ª	62.8±1.1 ^b	9.5±0.2ª	36.8±0.7°	8.2±0.1ª	40.8±0.9°	12.7±0.2ª	46.8±0.8°	
PI+/PSA+ (%)	1.2±0.1ª	5.6±0.3 ^b	1.2±0.1ª	4.1±0.3 ^b	1.6±0.1ª	3.9 ± 0.2^{b}	1.3±0.1ª	4.2 ± 0.3^{b}	
HMMP (%)	61.7 ± 2^{a}	31.4±2.2 ^b	73.2±1.9°	49.3±1.8 ^{ab}	72.5±2.1°	50.6±2.4 ^{ab}	68.6±2.1°	48.9±1.9 ^{ab}	

Mean (\pm SEM) seminal characteristics in canine semen (n = 21) extended at different concentration of egg yolk (EY 0%, EY 5%, EY 10%, and EY 20%) after an emilibration for 2 h at 4%C (EQ) and after fracting (ET)

Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL, curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR, Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm with membrane damage and acrosome reaction - PI-/PSA+; sperm with membrane damage and acrosome reaction - PI+/PSA+; sperm with high mitochondrial membrane potential – HMMP. In the same row, values with different superscript (a/b/c/d/e) differ significantly ($P \le 0.05$).

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Paper: Is the protective effect of EY against osmotic and cryogenic damage on canine spermatozoa dose-dependent?

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