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

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Abstract

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Keywords	Canine; Egg yolk; Cryopreservation; Sperm kinetics
Taxonomy	Cryopreservation, Canine Reproduction

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

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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

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 fertilizing capacity. A reduction in progressive motility (Jones and Stewart, 1979), alterations of membrane permeability and stability (Holt and North, 1986; Watson, 2000), and an increase in the 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 variables in raw semen. To reduce the effects of temperatures that are used for cryopreservation of 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 to be effective for semen cryopreservation and maintenance of sperm viability after thawing. Results of previous studies indicate the use of EY in the freezing medium reduces cellular damage (Phillips and Lardy, 1940; Pace and Graham, 1974; De Leeuw et al., 1993). Although the protective function 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 to the content of low density lipoproteins (LDL) (Pace and Graham, 1974; Moussa et al., 2002; 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., 2004). In some studies the metabolism of spermatozoa, however, is inhibited by some EY components, and this could affect sperm motility (Pace and Graham, 1974; Wall and Foote, 1999).

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

68 studies in which there has been assessment of whether the cryoprotective effect of EY is dose-
69 dependent. In other males, such as the stallion, EY is effectively used for semen cryopreservation at
70 a 2% concentration, without a reduction of sperm quality and fertilisation capacity (Pillet et al., 2008).
71 The damage induced by cryopreservation on spermatozoa is multimodal because in several
72 studies there was a combination of cold shock (Amann and Pickett, 1987), peroxidation (Slaweta et
73 al., 1988), and osmotic stress (Watson, 2000). When the temperature reduction is beyond the freezing
74 point for semen, water forms ice crystals first in the extracellular compartment. This increases the
75 solute concentration in the free uncrystallised water outside the cell, inducing hyperosmotic stress
76 (Sieme et al., 2016). During thawing, however, the ice crystals melt in the free water that enters the
77 plasma membrane, thus sperm undergo hypo-osmotic stress.
78 Although Foulkes (Foulkes, 1977) suggested that the protective effect of the EY during
79 cryopreservation could contribute to the colloid pressure maintenance of the external medium, few
80 studies have been focused on the functions of EY as a protective compound against the osmotic stress.
81 Even though there is broad understanding of the importance of EY as a semen extender component
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,
86 to increase the knowledge of the biology and manipulation of reproduction in dogs, the present study
87 was designed to ascertain, for the first time, the protective effect of EY at different concentrations
88 (0%, 5%, 10%, and 20%) on dog spermatozoa in different anisosmotic conditions. Furthermore, the
89 aim of the present study was to evaluate the cryoprotective effect of EY, added at the same
90 concentrations (0%, 5%, 10%, and 20%) to the freezing medium, for dog sperm cryopreservation.
91
92 **2. Materials and methods**
93 *2.1. Animals and semen collection*

94 The study involved 21 healthy dogs of known fertility aged between 2 and 6 years. The breeds
95 represented were: Newfoundland ($n = 6$), Pitbull ($n = 5$), American Staffordshire ($n = 5$), and Labrador
96 retriever ($n = 5$). All dogs were admitted for a routine reproductive examination at the Hospital of the
97 University of Veterinary Medicine of Teramo, Italy. For all the dogs included in the study, the consent
98 was obtained from the owner for the use of part of the semen sample of the dogs in the present study.
99 Animals were managed in ways consistent with the Italian legislation concerning animal care (DL
100 *n*.116, 27/01/1992).
101 Semen collection was conducted for all dogs using the digital manipulation method by the
102 same individual to reduce the effects of semen collection process on sperm quality. Only the sperm-
103 rich fraction was evaluated and used for experiments. From each animal, two ejaculates were
104 collected, for a total of 42 samples. Only ejaculates in which sperm motility was > 70% and
105 concentration > 200 x 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*
109 A hyperosmotic solution based on TRIS formula (hyper-TRIS) was prepared using 119.8 g/L
110 TRIS, 67.32 g/L citric acid and 12.375 g/L glucose (pH 6.9; mOsm/kg 1218). This solution was
111 diluted using bi-distilled sterile water to achieve isosmotic TRIS (iTRIS, pH, 6.8, Osm 302
112 mOsm/kg). Semen samples were aliquoted to four groups and diluted at 240 x 10^6 sperm/mL in iTRIS,
113 then each sample was diluted 1:1 with 40% (final concentration 20% EY - EY20), 20% (final
114 concentration 10% - EY10), 10% (final concentration 5% EY - EY5) EY, or there was no EY added
115 to the extender (0%; EY0 – as control). The 40%, 20%, and 10% EY extenders had an osmolality of
116 1864, 1839, and 1821 mOsm/kg, respectively. Each treatment sample (EY20, EY10, EY5, and EY0)
117 was in turn divided into five aliquots, in duplicate. To evaluate the protective effect of different EY
118 concentrations, each treatment sample was then diluted with a combination of hyper-TRIS and bi-
119 distilled water to a final osmolality of 75, 150, 300, 500, or 1000 mOsm/kg. The final sperm

concentration was 40 x 10^6 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.

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 for 10 min at 700 *g* and re-suspended in iTRIS at the concentration of 300 x 10^6 sperm/mL. The semen was then diluted 1:1 (v:v) with iTRIS supplemented with 8% glycerol (final concentration 4%) (Peña et al., 1998) and 40% (final concentration 20% EY - EY20), 20% (final concentration 10% - EY10), 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 refrigerator, packaged in 0.25 straws (IMV Technologies, L'Aigle, France) and sealed mechanically. Straws were suspended 4 cm above the liquid nitrogen surface for 10 min, and were then plunged into liquid nitrogen (Anderson, 1972), and subsequently stored for at least 5 days. Samples were evaluated for sperm objective motility, MI, acrosome integrity, and mitochondrial potential at the end of equilibration (EQ) period and after thawing (FT). For thawing of samples, straws were placed in a water bath at 37 °C for 30 seconds (Bencharif et al., 2008b), the sample was then transferred to a 2-

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

2.3. Semen evaluation

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.

2.3.2. Sperm kinetics

The kinetic evaluations were performed using the computer-assisted sperm analyser (CASA) system IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA, USA) for objective evaluation of 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 adjusting the detection gates accordingly. Samples from Experiment 1 were analysed without further dilution, while frozen/thawed samples were diluted with relevant extender at 40 x 10^6 sperm/mL. An aliquot of each sample was re-warmed at 37 °C for 5 min and a 5-µL drop was loaded onto a Makler 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 activated. Motility variables evaluated were total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH; µm), beat cross frequency (BCF; Hz), straightness (STR, as VSL/VAP; %), and linearity (LIN, as VSL/VCL; %). Spermatozoa with VAP ≥80 µm/s and STR ≥75% were considered to be progressive cells.

2.3.3. Sperm membrane and acrosome integrity

In Experiment 1, MI in the different osmotic conditions was evaluated using the propidium iodide (PI) exclusion test that has been previously described and validated (Ball and Vo, 2001) with some modifications. Semen was diluted to 10 x 10^6 sperm/mL with the relevant extender, and aliquots (500 µL) being incubated with PI at the final concentration of 12 µM for 5 min at 22 °C and then were analysed using the flow cytometer EPICS XL (Beckman Coulter, San Jose, CA, USA). Data acquisitions occurred with the use of the System II software (Beckman Coulter, USA). The sperm 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 488-nm laser, and PI fluorescence was obtained using the FL3 sensor through a 660/20 nm long pass 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 acquisitions were stopped at 30,000 events. Events with red fluorescence were considered to represent sperm with membrane damage, while those cells without fluorescence were considered to be spermatozoa with MI.

In Experiment 2, sperm MI and acrosome integrity were evaluated simultaneously using flow cytometry, as previously described (Gloria et al., 2018). Briefly, sperm in samples diluted to 10 x 10^6 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 using a flow cytometer (EPICS XL). The FITC-PSA fluorescence data were collected using an FL1 sensor with a 530/28 nm band-pass, while data for PI fluorescence were obtained using the FL3 sensor with a 660/20 nm long pass filter. Adjustment of compensation values for the two emission detectors used was done. 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. 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 acquisitions were stopped at 30,000 events. Use of the combination of these two fluorochromes allowed for characterisation of four different

197 subpopulations: sperm with MI and acrosome integrity that had no fluorescence (PI-/PSA-); sperm
198 with MI and an acrosome reaction (PI-/PSA+); sperm with a damaged membrane and acrosome
199 integrity (PI+/PSA-); and sperm with a damaged membrane and a reacted acrosome (PI+/PSA+).
200
201 *2.3.4. Mitochondrial membrane potential assay*
202 The mitochondrial membrane potential (MMP) of spermatozoa was evaluated using the
203 fluorescent stain 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimida zolylcarbocyanine chloride (JC-1)
204 as reported by Gloria et al. (Gloria et al., 2018). The sperm suspension was adjusted to a concentration
205 of 5×10^6 sperm/mL and incubated for 45 min at 37 °C in the dark with the JC-1 stain (final stain
206 concentration 8 μM). At the end of the incubation period, cells were washed in the same medium that
207 contained no stain and there were evaluations using the flow cytometer EPICS XL (Beckman Coulter)
208 equipped with the System II software (Beckman Coulter) as previously described (Garner and
209 Thomas, 1999). The sperm population was selected on the basis of the forward- and side-scatter, and
210 a gate was selected based on the values for these variables. The green fluorescent emissions of the
211 monomeric form of JC-1 (mitochondria with relatively lesser potential - LMMP) were collected using
212 the 530 ± 15 - nm filter (FL 1), and the orange emission of the polymeric form of JC-1 (mitochondria
213 with a relatively greater membrane potential - HMMP) was detected using the 585 ± 21 – nm filter
214 (FL 2). The flow cytometric analysis was performed at a flow rate of 8 to 30 μL/min, and the
215 acquisitions were stopped at 30,000 events. No adjustment of compensation values for the two
216 emission detectors was done.
217
218 *2.3.5. Sperm morphology*
219 Sperm morphology was evaluated using a phase contrast microscope (BX-51 - Olympus Italia,
220 Milan, Italy) at 1000 X magnification. Spermatozoa at different osmolalities were immobilized with
221 the addition of 3% glutaraldehyde (Hancock, 1957), and a drop (6 μl) was placed on a slide and
222 covered with a 22 x 22 mm coverslip. Spermatozoa were then classified as normal sperm, sperm with

223 an abnormal head, sperm with an abnormal midpiece, and sperm with an abnormal tail, that were in
224 turn subdivided into classifications of sperm with complete coiling (more than the 50% of the tail
225 length was involved in the twisting/coiling), or partial coiling (the twisting/coiling involved the distal
226 part of the tail) of the tail, and sperm with other tail abnormalities. Tail abnormalities were evaluated
227 for at least 400 spermatozoa.
228
229 *2.4. Statistical analysis*
230 Data are presented as mean ± standard error of the mean (SEM). The data were evaluated
231 using the Shapiro-Wilk (normal distribution) and Levene (homogeneity of variances) tests. When
232 data were not normally distributed, a log transformation was performed before data analyses were
233 conducted.
234 In Experiment 1, the effect of the concentration of EY on the different sperm variables (kinetic
235 variables, MI, mitochondrial membrane potential, morphological subclasses) was evaluated using a
236 general linear model (GLM) based on an Univariate ANOVA. Dog was included as a random factor.
237 *Post-hoc* evaluation was performed using the Scheffé's test.
238 In Experiment 2, the cryoprotective effects of the different concentrations of EY, in terms of
239 kinetic variables, membrane and acrosome integrity, and mitochondrial membrane potential were
240 evaluated using a GLM based on a Univariate ANOVA, with the Scheffé's test being used for the
241 *post-hoc* evaluation. Dog was included as a random factor.
242 In Experiment 1, correlations between total and progressive motility, MI, and mitochondrial
243 membrane potential were determined using the Pearson's correlation coefficient. For both the
244 experiments, differences were considered significant when $P < 0.05$. Statistical analyses were
245 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
252 those with inclusion of EY in the semen extender, with the exception of ALH, which was less in
253 samples with EY, irrespective of the concentration, compared with those without EY ($P < 0.05$; Table
254 1). In these samples, values for kinetic variables were less in both hypo- and hyper-osmotic
255 conditions. At 75 and at 1000 mOsm/kg, there was no spermatozoa motility, while at 150 and at 500
256 mOsm/kg, the proportion of sperm with total motility was less than 10% in all samples. In samples
257 diluted with 5% EY, the values for kinetic variables with use of the 150 mOsm/kg were less than
258 those with 300 mOsm/kg concentration ($P < 0.01$). Values at the 500 mOsm/kg concentration were
259 similar to those recorded in the control sample for total motility (Table 1). Moderate hyperosmotic
260 conditions apparently resulted in lesser values for kinetic variables because spermatozoa that were
261 motile when these conditions prevailed had lesser velocities (VAP, VSL, and VCL; $P < 0.05$) and
262 progressiveness (STR, LIN; $P < 0.05$), with an increase in the amplitude of the movement (ALH; P
263 < 0.05), resulting in a reduced proportion of sperm with progressive motility ($P < 0.05$). Values for
264 kinetic variables were related to the presence of EY, but were not affected by the concentration of
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
267 kinetic variables. Values recorded after 20 (data not shown) and 45 (Table 2) min were similar to
268 those soon after dilution, with an effect of anisosmotic conditions on samples without EY and on the
269 sample in hypo-tonic media.

270 Membrane integrity and mitochondrial membrane potential were evaluated at 20 and 45 min
271 after dilution, due to the time required for the incubation with the staining for the procedure to be
272 valid. In samples that had no EY added that were incubated for 20 minutes (T20), the MI was greater
273 at 300 mOsm/kg than in those incubated at 150 and 75 mOsm/kg (Table 3). Similarly, as osmolality
274 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).

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 HMMP ($r = 0.792$, $P < 0.01$) at 300 mOsm/kg, while in anisosmotic conditions, MI was correlated 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 ($r = 0.916$, $P < 0.01$; $r = 0.934$, $P < 0.01$; $r = 0.928$, $P < 0.01$, respectively), TM and HMMP ($r = 0.874$, $P < 0.01$; $r = 0.836$, $P < 0.01$; $r = 0.792$, $P < 0.01$, respectively), and MI and HMMP ($r = 0.816$, $P < 0.01$; $r = 0.842$, $P < 0.01$; $r = 0.758$, $P < 0.01$). Similarly, in all the samples with EY there were correlations at 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; $r = 0.816$, $P < 0.01$ for 20%EY), TM and HMMP ($r = 0.682$, $P < 0.01$ for 5% EY; $r = 0.648$, $P < 0.01$ 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; $r = 0.816$, $P < 0.01$ for 10% EY; $r = 0.786$, $P < 0.01$ for 20% EY). At 75, 150, and 1000 mOsm/kg, MI was correlated, however, with HMMP ($r = 0.638$, $P < 0.05$; $r = 0.682$, $P < 0.05$; $r = 0.568$, $P < 0.05$, respectively in samples containing 5%; $r = 0.672$, $P < 0.05$; $r = 0.684$, $P < 0.05$; $r = 0.548$, $P < 0.05$, respectively in samples containing 10% EY; and $r = 0.626$, $P < 0.05$; $r = 0.584$, $P < 0.05$; $r = 0.526$, $P < 0.05$, respectively in samples containing 20% EY) but not with TM.

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 dilution (T0), morphological subclasses recorded at 500 and 1000 mOsm/kg in samples were similar to those recorded at 300 mOsm/kg ($P > 0.05$). Furthermore, spermatozoa in the hypo-osmotic condition had a typical twisting/coiling of the tail. The percentage of spermatozoa with a tail response was similar at both 75 and 150 mOsm/kg, however, the percentage of sperm with complete tail coiling was greater at 75 compared with 150 mOsm/kg ($P < 0.05$) that was, in turn, greater than in the iso-osmotic samples ($P < 0.01$). The percentage of spermatozoa with partial coiling, however, was greater at 150 compared with 75 mOsm/kg ($P < 0.05$; Figure 1). The inclusion of EY in the extender seemed to have a partial protective effect against the hypo-osmotic stress because the percentage of complete 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 differences ($P > 0.05$) in the proportion of the morphological tail subclasses, however, in samples 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, and T45 (data not shown).

3.2. Experiment 2

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 movement, because progressive motility, VAP, VSL, VCL, STR, and LIN were all greater in samples diluted with 5% and 10% EY compared with samples without EY ($P < 0.05$). Samples diluted with 20% EY had similar values for these variables as those not diluted with EY, and lesser ($P < 0.05$) compared with inclusion of 5% and 10% EY in the extender (Table 5). There was negligible effect on total motility, ALH, and BCF of EY concentration because values were similar for all treatments ($P > 0.05$). Membrane and acrosome integrity, such as sperm with HMMP, were similar in samples

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

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

4. Discussion

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 the cells by transfer of water across the plasma membrane and modification of the cytoskeleton (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 (Guthrie et al., 2002). In several studies, results indicated there was an osmotic tolerance limit for spermatozoa of different domestic animals, including those of bulls (Liu and Foote, 1998; Guthrie et al., 2002), stallions (Ball and Vo, 2001; Glazar et al., 2009), boars (Gilmore et al., 1998), and rams (Curry and Watson, 1994). Specific studies regarding the osmotic tolerance limit were not conducted

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 evaluation of sperm in a complex medium supplemented with colloidal components, that could modulate the cellular adaptation. In dogs, the addition of EY seemed to protect cellular structures involved in the regulation of sperm motility at 500 mOsm/kg, even though there was a general reduction in the progressive motility. Similarly, the protective action of EY was more evident on sperm MI. In samples diluted with 5%, 10%, and 20% EY, percentages of sperm with MI at 150, and 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 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.

The marked inconsistency between sperm motility and MI, as indicated by the lack of correlations between the values for these variables, in anisosmotic conditions indicate that sperm functions may cease before there are disruptions in the integrity of the plasmalemma. Results from the present study confirm results from previous studies with bulls, in which the proportion of total 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 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 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).

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 could not only be related to plasma MI but also to the membrane permeability to ions and to the 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 incubated in hypo-osmotic conditions when EY was not included in the diluent which may indicate 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 (1993). Unfortunately, in this previous study the extent of the sperm response to the hypo-osmotic stress was not reported; thus, it was not possible to compare the data from this previous and the present study directly. In contrast with findings of Kumi-Diaka (1993), the number of sperm that had curled 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 stress and there were no subsequent changes during the incubation period. The timing of sperm 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 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 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% (Anderson, 1972; Peña et al., 1998; Silva et al., 2002), the results of the present study indicate that the concentration of EY is not as important as previously thought for preservation of dog spermatozoa. In samples without EY, there were lesser percentages of motile and membrane intact 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 other EY concentrations that were assessed.

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.

The results in the present study indicate dog spermatozoa could be successfully frozen with 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 extender was 2% (Pillet et al., 2008). The optimal concentration of low-density lipoproteins (LDL), 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 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 less. In dogs, Bencharif et al. (2008) reported there were greater values in the cryopreserved spermatozoa with the use of LDL compared with the conventional EY concentration, but among the

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 of EY of cryopreserved spermatozoa of dogs that is related to the protection against osmotic stress. The lack of correlation between total and progressive sperm motility is markedly reduced, and MI, less affected by the anisosmotic environment, in both hypo- and hyper-osmotic conditions, indicating that the damage occurred at the cytoskeleton or to a non-mitochondrial metabolic pathway. The osmo-protective effect of the EY was not dose-dependent because there were similar values at 5%, 10%, 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 viability after thawing.

5. Conclusions

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 differences in sperm characteristics after dilution with extender at the 5%, 10%, or 20% 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 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 dog spermatozoa. There was greater viability of frozen-thawed sperm using 5% and 10% EY compared with samples where there was no EY inclusion, however, the viability was only slightly

455 greater than with inclusion of 20% EY, indicating dog spermatozoa could be effectively
456 cryopreserved at a lesser EY concentration and viability would be retained after thawing.
457
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593 **Figure legend**

594 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 ($P < 0.05$)

597

598 **Table 1**

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

600 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)

EY (%)	Osmolarity (mOsm)	TM (%)	PM (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	
0	75	0	0	0	0	0	0	
	150	4±0.6^{a}	0	22.3±2.7^{a}	20.6±2.5^{a}	95.2±2.9^{a}	4.4±0.17^{a}	19
	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±0.8^{a}	0	61.7±2.3^{c}	43.4±2.2^{ac}	147.0±5.4^{ac}	5.6±0.19^{a}	13
	1000	0	0	0	0	0	0	
5	75	0	0	0	0	0	0	
	150	13.6±0.8^{a}	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
	300	91.1±1.5^{b}	83.7±1.8^{a}	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^{c}	96.4±2.2^{c}	78.2±2.5^{c}	167.2±5.3^{c}	8.1±0.19^{b}	24
	1000	0	0	0	0	0	0	
10	75	0	0	0	0	0	0	
	150	12±0.6^{a}	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
	300	90.3±1.1^{b}	83.5±1.7^{a}	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^{c}	98.1±2.7^{c}	75.9±2.2^{c}	164.5±4.9^{c}	8.6±0.13^{b}	23
	1000	0	0	0	0	0	0	
20	75	0	0	0	0	0	0	
	150	10.7±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
	300	88.6±1.8^{b}	83.8±1.8^{a}	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^{c}	97.6±2.4^{c}	75.5±3.9^{c}	162.7±4.3^{c}	8.4±0.24^{b}	21
	1000	0	0	0	0	0	0	

602 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 ($P \leq 0.05$)
606

Table 2

Values for sperm kinetic characteristics (± 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) for 45 min

EY (%)	Osmolarity (mOsm)	TM (%)	PM (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	
0	75	0	0	0	0	0	0	
	150	0	0	0	0	0	0	
	300	82.4±2.3^{a}	71.6±2.3^{a}	164.2±2.6^{a}	137.8±3^{a}	201.3±4.1^{a}	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	
5	75	0	0	0	0	0	0	
	150	18.4±1.4^{c}	9.6±0.5^{b}	50.7±1.6^{bc}	46.4±2.1^{bc}	131.4±2.5^{b}	5.6±0.22^{c}	39
	300	88.7±2^{a}	79.8±1.8^{a}	176.2±2.9^{a}	151.7±2.7^{a}	198.4±4.6^{a}	7±0.2^{a}	28
	500	78.5±2.1^{a}	36.2±1.7^{c}	94.1±1.9^{c}	76.8±2.3^{c}	168.2±4.5^{ab}	8.9±0.22^{b}	24
	1000	0	0	0	0	0	0	
10	75	0	0	0	0	0	0	
	150	15.7±0.9^{c}	8.2±0.4b	62.4±1.6^{bc}	43.1±1.7^{bc}	128.1±2.8^{b}	5.4±0.17^{c}	42
	300	90.5±1.8^{d}	82.8±1.8^{a}	179.4±3.6^{a}	158.3±2.5^{a}	205.7±4.3^{a}	7.1±0.24^{a}	2
	500	83.8±1.9^{a}	36.1±2^{c}	98.5±2.2^{c}	80.3±2.3^{c}	166.5±4.7^{ab}	9.1±0.41^{b}	26
	1000	0	0	0	0	0	0	
20	75	0	0	0	0	0	0	
	150	14.2±1.3^{c}	6.3±0.2^{b}	47.8±1.9^{b}	39.8±2^{bc}	126.2±2.4^{b}	5.5±0.26^{c}	40
	300	89.6±1.9^{d}	81.3±2^{a}	177.4±2.1^{a}	161.2±5.1^{a}	204.1±5^{a}	7.2±0.24^{a}	23
	500	75.8±2.2^{a}	32.4±1.4^{c}	93.7±2.4^{c}	74.8±2.1^{c}	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, 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 ($P \leq 0.05$)

Table 3

Means for (± SEM) of total sperm motility (TM), sperm membrane integrity (MI), sperm with relatively greater mitochondrial membrane potential (HMMP), sperm with relatively lesser 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 (75, 150, 300, 500, and 1000) for 20 min

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

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

624 **Table 4**

625 Percentages of sperm head and midpiece abnormalities (± SEM) of dog semen samples (n = 21) not
626 diluted with egg yolk (EY 0%), or diluted with 5%, 10%, or 20% EY and incubated in different
627 osmotic conditions (75, 150, 300, 500, and 1000 mOsm) soon after dilution (0 min)

EY (%)	Osmolarity (mOsm)	Abnormal head (%)	Abnormal midpiece (%)
0	75	2.6±0.3	3.1±0.41
	150	2.3±0.26	4.3±0.59
	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
5	75	2.4±0.24	3.4±0.28
	150	2.6±0.35	2.8±0.24
	300	2.9±0.26	2.9±0.33
	500	2.7±0.3	3±0.2[c]
	1000	2.2±0.26	2.8±0.46
10	75	2.1±0.33	3.2±0.55
	150	2.7±0.24	3.1±0.46
	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
20	75	3.1±0.28	2.8±0.46
	150	2.7±0.46	4.2±0.33
	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

628
629

630 **Table 5**

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

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

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

633 Total motility - TM, Progressive motility - PM, Average path velocity - VAP, Straight line velocity - VSL,
634 curvilinear velocity - VCL, lateral head displacement - ALH, beat cross frequency - BCF, straightness - STR,
635 Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm
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 ($P \leq 0.05$)
640

641 **Table 6**

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

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

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

	EY 0%		EY 5%		EY 10%		
	EQ	FT	EQ	FT	EQ	FT	
TM (%)	83±1.3^{a}	22±3.1^{b}	92±1.1^{a}	51±2.2^{c}	91±1.5^{a}	52±1.7^{c}	
PM (%)	68±1.5^{a}	13±3.9^{b}	89±0.9^{c}	39±2^{d}	89±1.1^{c}	40±1.5^{d}	
VAP (µm/s)	107.3±6.5^{a}	64.8±6.8^{b}	134.6±4.1^{c}	109.3±5.8^{a}	132.7±5.2^{c}	112.4±6.4^{a}	1
VSL (µm/s)	87.3±5.2^{a}	47.7±6.1^{b}	112.2±4.1^{c}	91.8±5.7^{ac}	110.6±5.5^{c}	95.4±5.2^{ac}	1
VCL (µm/s)	187±11.1^{a}	98.8±7^{b}	235.7±9.2^{c}	145.1±8.6^{d}	229.4±8.7^{c}	139.8±9.3^{d}	2
ALH (µm)	8.6±0.3^{a}	5.1±0.5^{b}	8.1±0.2^{a}	7.3±0.3^{a}	8±0.3^{a}	7.1±0.2^{a}	
BCF (Hz)	38.7±1.7^{a}	29.6±1.8^{b}	45.2±1.8^{a}	41.8±2^{a}	44.7±1.9^{a}	40.1±2.1^{a}	4
STR (%)	77.2±3.3^{a}	75.8±2.8^{a}	85.8±2.6^{b}	82.9±2.6^{ab}	86.1±3.3^{b}	80.4±2.9^{ab}	8
LIN (%)	43.2±2.6^{a}	39.7±2.8^{a}	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±1.3^{a}	29.6±1.8^{b}	88.2±1.6^{a}	57.7±2.1^{c}	89.1±1.3^{a}	54.3±1.8^{c}	8
PI-/PSA+ (%)	1±0.1^{a}	2.3±0.2^{b}	1.2±0.1^{a}	1.1±0.2^{a}	1.2±0.1^{a}	1±0.1^{a}	
PI+/PSA- (%)	15.3±0.2^{a}	62.8±1.1^{b}	9.5±0.2^{a}	36.8±0.7^{c}	8.2±0.1^{a}	40.8±0.9^{c}	1
PI+/PSA+ (%)	1.2±0.1^{a}	5.6±0.3^{b}	1.2±0.1^{a}	4.1±0.3^{b}	1.6±0.1^{a}	3.9±0.2^{b}	
HMMP (%)	61.7±2^{a}	31.4±2.2^{b}	73.2±1.9^{c}	49.3±1.8^{ab}	72.5±2.1^{c}	50.6±2.4^{ab}	6

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,
647 Linearity - LIN, sperm viability; sperm with membrane integrity and acrosome integrity - PI-/PSA-; sperm
648 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 ($P \leq 0.05$)
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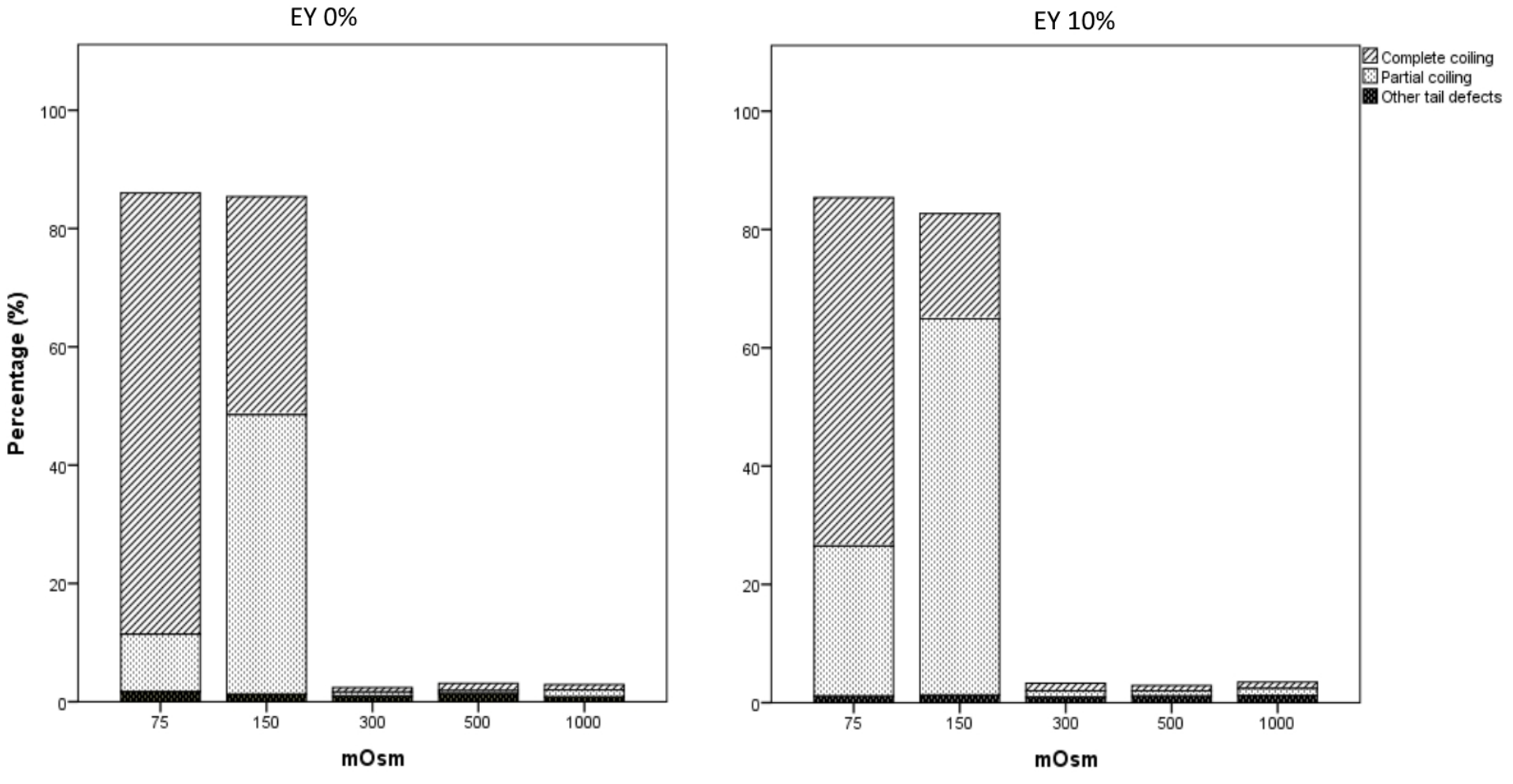
EY 0%
EY 10%
Complete coiling
Partial coiling
Other tail defects
Percentage (%)
100
80
60
40
20
0
75
150
300
500
1000
mOsm

Table 1

Sperm kinetic characteristics (± 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 (mOsm)	TM (%)	PM (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
0	75	0	0	0	0	0	0	0	0	0
	150	4±0.6^{a}	0	22.3±2.7^{a}	20.6±2.5^{a}	95.2±2.9^{a}	4.4±0.17^{a}	19.5±1.9^{a}	62±2.4^{a}	13±1.1^{a}
	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±1.4^{a}	93±2^{b}	71±2.2^{b}
	500	6±0.8^{a}	0	61.7±2.3^{c}	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^{c}
	1000	0	0	0	0	0	0	0	0	0
5	75	0	0	0	0	0	0	0	0	0
	150	13.6±0.8^{a}	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^{c}	80±2.2^{a}	38±2^{c}
	300	91.1±1.5^{b}	83.7±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±1.2^{a}	91±2^{b}	84±2.2^{b}
	500	82.5±2.5^{b}	36.9±2.1^{c}	96.4±2.2^{c}	78.2±2.5^{c}	167.2±5.3^{c}	8.1±0.19^{b}	24.6±1.4^{a}	77±1.7^{ab}	50±1.7^{c}
	1000	0	0	0	0	0	0	0	0	0
10	75	0	0	0	0	0	0	0	0	0
	150	12±0.6^{a}	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^{c}	85±2^{a}	35±1.7^{c}
	300	90.3±1.1^{b}	83.5±1.7^{a}	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±1.7^{a}	83±2.4^{b}
	500	81.5±2.3^{b}	38.3±2^{c}	98.1±2.7^{c}	75.9±2.2^{c}	164.5±4.9^{c}	8.6±0.13^{b}	23.1±1.6^{a}	78±2^{ab}	47±2^{c}
	1000	0	0	0	0	0	0	0	0	0
20	75	0	0	0	0	0	0	0	0	0
	150	10.7±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}	33.8±1.5^{c}	73±1.5^{ab}	29±2.2^{c}
	300	88.6±1.8^{b}	83.8±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^{c}	97.6±2.4^{c}	75.5±3.9^{c}	162.7±4.3^{c}	8.4±0.24^{b}	21.8±1.7^{a}	78±2.2^{ab}	47±1.7^{c}
	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 ($P \leq 0.05$).

Table 2

Sperm kinetic characteristics (± 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 (mOsm)	TM (%)	PM (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
0	75	0	0	0	0	0	0	0	0	0
	150	0	0	0	0	0	0	0	0	0
	300	82.4±2.3^{a}	71.6±2.3^{a}	164.2±2.6^{a}	137.8±3^{a}	201.3±4.1^{a}	7.8±0.24^{a}	18.4±1.4^{a}	87±2^{a}	61±2.2^{a}
	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
5	75	0	0	0	0	0	0	0	0	0
	150	18.4±1.4^{c}	9.6±0.5^{b}	50.7±1.6^{bc}	46.4±2.1^{bc}	131.4±2.5^{b}	5.6±0.22^{c}	39.7±1.6^{c}	79±1.7^{a}	36±2^{ab}
	300	88.7±2^{a}	79.8±1.8^{a}	176.2±2.9^{a}	151.7±2.7^{a}	198.4±4.6^{a}	7±0.2^{a}	28.5±1.1^{a}	90±2^{a}	81±2.4^{a}
	500	78.5±2.1^{a}	36.2±1.7^{c}	94.1±1.9^{c}	76.8±2.3^{c}	168.2±4.5^{ab}	8.9±0.22^{b}	24.7±1.5^{a}	75±2^{a}	46±4.1^{ab}
	1000	0	0	0	0	0	0	0	0	0
10	75	0	0	0	0	0	0	0	0	0
	150	15.7±0.9^{c}	8.2±0.4b	62.4±1.6^{bc}	43.1±1.7^{bc}	128.1±2.8^{b}	5.4±0.17^{c}	42.3±1.4^{c}	87±2^{a}	39±2.2^{ab}
	300	90.5±1.8^{d}	82.8±1.8^{a}	179.4±3.6^{a}	158.3±2.5^{a}	205.7±4.3^{a}	7.1±0.24^{a}	27.2±1^{a}	92±2.2^{a}	83±2.2^{a}
	500	83.8±1.9^{a}	36.1±2^{c}	98.5±2.2^{c}	80.3±2.3^{c}	166.5±4.7^{ab}	9.1±0.41^{b}	26.7±1.6^{a}	83±2^{a}	56±1.7^{a}
	1000	0	0	0	0	0	0	0	0	0
20	75	0	0	0	0	0	0	0	0	0
	150	14.2±1.3^{c}	6.3±0.2^{b}	47.8±1.9^{b}	39.8±2^{bc}	126.2±2.4^{b}	5.5±0.26^{c}	40.6±1.5^{c}	79±2.2^{a}	34±2^{ab}
	300	89.6±1.9^{d}	81.3±2^{a}	177.4±2.1^{a}	161.2±5.1^{a}	204.1±5^{a}	7.2±0.24^{a}	23.8±1.5^{a}	90±1.7^{a}	78±2^{a}
	500	75.8±2.2^{a}	32.4±1.4^{c}	93.7±2.4^{c}	74.8±2.1^{c}	164.1±4.1^{ab}	9±0.26^{b}	20.6±1.5^{a}	77±2.2^{a}	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 ($P \leq 0.05$).

Table 3

Mean (± 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 (%)
0	75	0	37.3±4.2^{a}	27.2±2.3^{a}	17.7±1.4^{a}
	150	1.7±0.4^{a}	64.8±3.5^{bc}	37.9±2.7^{a}	23.6±2.4^{a}
	300	81.6±2.6^{b}	88.6±2.8^{c}	74.8±1.9^{b}	3.6±0.9^{b}
	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}
5	75	0	78.5±2.6^{c}	54.7±2^{b}	27.1±1.7^{a}
	150	13.7±1.1^{c}	83.8 ±2.1^{c}	66.1±2.1^{b}	16.3±2^{a}
	300	90.4±1.5^{b}	86.9±2^{c}	70.6±1.6^{b}	10.6±1.2^{ab}
	500	79.7±1.7^{b}	86.4±1.9^{c}	70.1±2^{b}	13.4±1.3^{ab}
	1000	0	76.1±2.4^{c}	68.6±2.2^{b}	11.7±1.1^{ab}
10	75	0	83.7±2.1^{c}	57.6±1.9^{b}	21.9±1.4^{a}
	150	14.8±0.7^{c}	82.5±1.8^{c}	65.8±1.3^{b}	15.1±2.1^{a}
	300	91.8±1.6^{b}	87.8±1.5^{c}	73.8±2.1^{b}	8.4±0.5^{b}
	500	85.2±2.1^{b}	84.1±1.9^{c}	68.5±2.2^{b}	12.2±0.7^{ab}
	1000	0	75.7±3.2^{c}	69.3±2.3^{b}	12.8 ±0.6^{ab}
20	75	0	80.5±2.3^{c}	58.1±2.2^{b}	24.2±0.8^{a}
	150	12.3±1.2^{c}	84.1±1.6^{c}	66.4±1.9^{b}	13.7±0.6^{ab}
	300	90.7±2^{b}	89.9±1.7^{c}	71.9±1.4^{b}	9.2±0.6^{b}
	500	74.7±2^{b}	85.1±2.2^{c}	70.8±1.2^{b}	7.9±0.4^{b}
	1000	0	80.6±2.4^{c}	67.5±1.2^{b}	14.5±0.5^{ab}

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

Table 4

Percentage of sperm head and midpiece abnormalities (± 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 (mOsm)	Abnormal head (%)	Abnormal midpiece (%)
0	75	2.6±0.3	3.1±0.41
	150	2.3±0.26	4.3±0.59
	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
5	75	2.4±0.24	3.4±0.28
	150	2.6±0.35	2.8±0.24
	300	2.9±0.26	2.9±0.33
	500	2.7±0.3	3±0.2[c]
	1000	2.2±0.26	2.8±0.46
10	75	2.1±0.33	3.2±0.55
	150	2.7±0.24	3.1±0.46
	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
20	75	3.1±0.28	2.8±0.46
	150	2.7±0.46	4.2±0.33
	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

Table 5

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

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 ($P \leq 0.05$).

Table 6

Mean (± 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 equilibration for 2 h at 4°C (EQ) and after freezing/thawing (FT).

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

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/c/d/e) differ significantly ($P \leq 0.05$).

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

Authors: Alessia Gloria, Daniele Zambelli, Augusto Carluccio, Marco Cunto, Patrizia Ponzio, Alberto Contri

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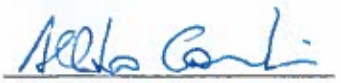
Alberto Contri	

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