The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown bear (Ursus arctos) sperm

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1 **The antioxidant effects of soybean lecithin- or low-density lipoprotein-**
2 **based extenders for the cryopreservation of brown bear (*Ursus arctos*)**
3 **sperm**

4

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23

24 **Abstract**

25

26 Egg yolk low-density lipoproteins (LDL) and soybean lecithin were evaluated as
27 replacements for egg yolk in extenders used for the cryopreservation of brown bear
28 sperm. The motility, viability and acrosomal status of post-thawed sperm were
29 analysed, and an egg yolk extender was used as a control. The total antioxidant
30 capacity of these extenders was tested. Soybean lecithin showed an effect that was
31 dependent on the soybean concentration (2%, 3.5%, and 5%) and source (type A:
32 24% L-α-phosphatidylcholine, and type B: 14-23% L-α-phosphatidylcholine). Only
33 semen cryopreserved with 5% type A soybean exhibited a sperm motility similar to
34 that of semen cryopreserved in egg yolk-based extender after thawing, although
35 the sperm viability and acrosome status were not as high. Semen frozen in an
36 extender containing LDL (10-15%) exhibited improved sperm viability in
37 comparison with the control, but sperm motility was lower. The LDL-based extender
38 exhibited a higher anti-oxidant activity than the egg yolk extender and soy lecithin-
39 based extenders. The extenders with higher anti-oxidant activity showed
40 improvements in frozen sperm viability but lower semen motility. These results
41 indicate that soybean lecithin did not have the same protective effect as egg yolk
42 during the freezing of brown bear sperm but suggest that LDL (10-15%) could be a
43 useful substitute for egg yolk in these extenders.

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47 **Keywords:** brown bear, egg yolk LDL, soybean, sperm, cryopreservation

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49 **Introduction**

50

51 The Cantabrian brown bear (*Ursus arctos*) in Spain is seriously endangered (Anel *et*
52 *al.* 2008), and it is considered to be at risk of extinction (Real Decreto 439/1990 of
53 the Spanish law, regulation of the National Catalogue of Endangered Species). The
54 optimisation of freezing protocols for the sperm of wild species would help to
55 improve strategies to establish and manage Genome Resource Banks (GRB) that
56 support efforts to conserve endangered species (Holt and Lloyd, 2009).
57 Egg yolk is commonly used in sperm cryopreservation media because of its
58 protective effects against cold shock, particularly with respect to the preservation of
59 sperm motility and the maintenance of acrosomal integrity (Salamon and Maxwell,
60 2000). Egg yolk has routinely been included, albeit at different concentrations (e.g.,
61 from 5% to 20% for Cuvier's gazelle semen; Garde *et al.* 2008), in the majority of
62 extenders for frozen semen from domestic animals and wild species. The
63 cryopreservation of bear sperm has always been carried out with egg yolk-
64 containing extenders. Ishikawa *et al.* (2002) reported the use of 15% egg yolk for
65 freezing semen from Hokkaido brown bears. Okano *et al.* (2004, 2006a, 2006b)
66 cryopreserved semen from Japanese black bears at a final egg yolk concentration of
67 20%. Giant panda spermatozoa have been cryopreserved using 20% egg yolk
68 (Spindler *et al.* 2004). In a study of brown bear semen, Anel *et al.* (2010) found
69 that sperm motility and the percentages of viable spermatozoa and spermatozoa
70 with intact acrosomes were higher in extenders containing 20% egg yolk than in
71 those containing 10% egg yolk.
72 Several studies have shown that high concentrations of egg yolk have a negative
73 effect on motility and acrosome integrity after thawing (Mohor gazelle: Holt *et al*,
74 1996) and on post-thawing viability (goat: Ritar and Salamon, 1991). Other
75 disadvantages of using egg yolk in freezing extenders are that a) the animal origin
76 of egg yolk introduces a potential risk of bacterial contamination, which may reduce
77 the fertilisation potential of the spermatozoa (Bousseau *et al*, 1998); b) the egg

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78 yolk composition is variable and may differ between batches; and c) certain
79 components of egg yolk have been reported to inhibit spermatozoa respiration or
80 reduce their motility (Pace and Graham, 1974; Watson and Martin, 1975; Almirat *et*
81 *al.* 2004). Therefore egg yolk should be replaced by another material with similar
82 activity.
83 Soybean lecithin, the main component of which is phosphatidylcholine, has been
84 used to replace egg yolk, with good results (ram: de Paz *et al.* 2010; bull: Aires *et*
85 *al*, 2003 and Stradioli *et al.* 2007; horse: Riker *et al.* 2006 and Aurich *et al.* 2007).
86 Riker *et al.* (2006) described the mechanism of membrane stabilisation in
87 spermatozoa that were cryopreserved in soy phosphatidylcholine-based diluent;
88 they observed lipid aggregates on the membrane surface and protective effects on
89 motility, viability and fertility.
90 Low-density lipoproteins (LDL) are another alternative to egg yolk. LDL are now
91 considered to be the main factor responsible for the cryoprotective properties of
92 egg yolk. Pace and Graham (1974) purified egg yolk using ultracentrifugation and
93 observed that LDL had a cryoprotective effect on bull spermatozoa. The use of LDL
94 may avoid the issues caused by the heterogeneity of the extenders prepared with
95 whole egg yolk, facilitating extender standardisation. The suitability of LDL as a
96 cryoprotectant has been confirmed in the bull (Hu JH *et al.* 2008, 2011; Vera-
97 Munoz *et al.* 2009), stallion (Pillet *et al.* 2011), ram (Moustacas *et al.* 2011) and
98 dog (Varela Junior *et al.* 2009).
99 Cooling and freezing-thawing cycles cause physical and chemical stresses on the
100 sperm membrane that reduce sperm viability and fertilisation capability. Both cold
101 shock and freezing damage are associated with reactive oxygen species (ROS) and
102 oxidative stress generation (Stradioli *et al.* 2007). Hu *et al.* (2011) stated that an
103 extender containing LDL reduced the negative impact of ROS on bull sperm. Thus,
104 we carried out a total antioxidant capacity (TAC) assay on the extenders used in the
105 present study to evaluate the relationship between sperm viability and exogenous
106 antioxidant capacity.

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107 The aim of the present study was to analyse the use of LDL or soybean lecithin in
108 place of egg yolk in extenders for the cryopreservation of brown bear semen. We
109 evaluated the effects of these extenders on sperm quality (motility, membrane and
110 acrosome integrity), and we determined the antioxidant capacity of the tested
111 extenders.
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113 **Materials and methods**

114

115 *Reagents and animal regulation*

116 All products were obtained from Sigma (Madrid, Spain) unless otherwise specified.
117 The Equex STM Paste was obtained from Minitüb (Tiefenbach, Germany).

118

119 *Animals and sample collection*

120 A total of 31 ejaculate samples from 23 sexually mature male brown bears were
121 obtained by electroejaculation during the breeding season (late April to early July).
122 The animals were housed in a half-freedom regime in Cabárceno Park (Cantabria,
123 Spain; 43º 21´ N, 3º 50´ W, altitude: 142 meters) and fed a diet based on chicken
124 meat, bread and fruits. Animal manipulations were performed in accordance with
125 Spanish Animal Protection Regulation RD1201/2005, which conforms to European
126 Union Regulation 2003/65.
127 The male bears were immobilised by teleanaesthesia using 750 mg of zolazepam
128 HCl+tiletamine HCl (Zoletil 100®; Virbac, Carros, France) and 6 mg of
129 medetomidine (Zalopine®, Orion Pharma Animal Health, Finland, 10 mg/mL). After
130 immobilisation, the bears were weighed and monitored (pulse, oxygen saturation
131 and respiration). Prior to electroejaculation, the prepucial area was shaved and
132 washed with physiological saline serum, and the rectum was emptied of faeces. The
133 bladder was catheterised during semen collection to prevent urine contamination.
134 Electroejaculation was carried out with a PT Electronics® electroejaculator (PT
135 Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a
136 diameter of 26 mm. Electric stimuli were provided until ejaculation occurred (10 V
137 and 250 mA, on average).
138 To prevent urine contamination or a low cell concentration, the ejaculates were
139 collected as isolated fractions in 15 mL graduated glass tubes. Immediately after
140 collection, the volume of each fraction was recorded, its osmolality was measured
141 using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany), and

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142 the pH value was determined using a CG 837 pH meter (Schott Instruments, Main,
143 Germany). For each fraction, subjective motility was assessed with a phase contrast
144 microscope (100x), and urospermia was evaluated using a Merckgnost Urea Rapid
145 Screening test (Merck, Barcelona, Spain). Fractions with low cell concentrations
146 (<200x10^6 cell/mL), low motility (< 50%) or urine contamination (>80 mg
147 urea/dL) were discarded. All valid fractions from the same electroejaculation were
148 mixed and constituted one ejaculate. Selected samples were centrifuged at 600×*g*
149 for 6 minutes, and the pellet was processed as described below.

150

151 *Experimental design*

152 *Experiment 1. Effect of soybean lecithin on sperm cryopreservation*

153 Four extenders were prepared. The control extender (EY20) consisted of a TTF-Base
154 (TES-Tris-fructose, 300 mOsm/kg, pH 7.1, with 6% glycerol, 2% EDTA and 1%
155 Equex STM paste) with 20% egg yolk (de Paz *et al.* 2012). Soybean lecithin
156 extenders were prepared with TTF-Base supplemented with 20 g/L (2%, S2), 35
157 g/L (3.5%, S3.5) and 50 g/L (5%, S5) soybean (L-α-phosphatidylcholine, Soy PC
158 (20%) from Avanti Polar Lipids®, Alabaster, Alabama, USA) instead of egg yolk.
159 The Soy PC (20%) contained phosphatidylcholine (PC, 24%),
160 phosphatidylethanolamine (PE, 18.6%), phosphatidylinositol (PI, 11.5%),
161 lysophosphatidylcholine (LPC, 4.6%) and other components (41.3%).
162 Fifteen brown bear ejaculates were used. After each ejaculate had been
163 centrifuged, the sperm pellet was divided into four aliquots, diluted with the same
164 volume of each extender at room temperature, and processed for cryopreservation
165 (see section 2.4).

166

167 *Experiment 2. Effect of soybean lecithin source on sperm cryopreservation*

168 Several commercial sources of soybean lecithin are available, and each source has
169 a unique phospholipid composition. The different lipids in the diluents may
170 differentially affect sperm lipid packing at the membrane surface and consequently

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171 modify the sperm's chilling sensitivity (Riker *et al.*, 2006). To evaluate these
172 differences, we tested two different sources of soybean lecithin. Type A soybean
173 was the lecithin used in experiment 1, and type B was L-α-phosphatidylcholine
174 purchased from Sigma (cat. no. P5638), reported to contain from 14% to 23%
175 phosphatidylcholine (other major phospholipid components included
176 phosphatidylethanolamine and inositol phosphatides).
177 Three extenders were prepared using 50 g/L soybean lecithin, according to the
178 results of the previous experiment: 1) EY20 extender, 2) TTF-Base extender with 50
179 g/L type A soybean (S5-A) or 3) TTF-Base extender with 50 g/L type B soybean
180 (S5-B).
181 Eight brown bear ejaculates were used. The pellet was divided into three aliquots,
182 which were diluted with the same volume of each extender at room temperature
183 and processed for cryopreservation (see section 2.4).
184

185 *Experiment 3. Effect of LDL on sperm cryopreservation*

186 Four extenders were prepared using extender EY20 as a control (see 2.3.1). LDL
187 extenders were prepared with TTF-Base supplemented (vol/vol) with 5% (LDL5),
188 10% (LDL10) or 15% (LDL15) LDL instead of egg yolk. For the extraction of LDL
189 (Moussa *et al.* 2002), egg yolk was diluted 1:1 (vol/vol) in a 1.7 M NaCl solution
190 and homogenised. The LDL was then slowly precipitated out with ammonium
191 sulphate. The solution was dialysed for at least 6 hours to remove the ammonium
192 sulphate and then frozen at -20 °C. After this step, LDL was obtained by
193 lyophilisation of the frozen solution.
194 Eight brown bear ejaculates were used. The sperm pellet was divided into four
195 aliquots, which were diluted with the same volume of TTF-Base extender at room
196 temperature and processed for cryopreservation (see section 2.4).
197

198 *Study of the Total Antioxidant Capacity (TAC) of the extenders*

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199 The extenders used in the cryopreservation experiments were tested for their
200 antioxidant capacities. TTF-Base was included in the analysis as a control. The other
201 extenders tested were EY20, S2, S3.5, S5, S5-A, S5-B, LDL5, LDL10 and LDL15.
202 Lecithin extenders were prepared in duplicate at a concentration of 5% (wt/vol)
203 using type A and type B soybean lecithin (see 2.3.1 and 2.3.2). TAC assessment
204 was carried out with the Total Antioxidant Capacity Assay Kit® (BioVision, Mountain
205 View, USA) following the assay procedure recommended by the manufacturer, and
206 the outcome was recorded in mM Trolox equivalents. The extenders were analysed
207 after storage at 5 °C for one week.
208
209 *Cryopreservation protocol*
210 The tubes with the extended samples (1:1 at 3% glycerol) were placed in beakers
211 containing 100 mL of water at room temperature and transferred to a refrigerator,
212 where they were slowly cooled to 5 °C. Then, the samples were diluted with the
213 same volume of each extender containing 9% glycerol to achieve a final glycerol
214 concentration of 6%. A final sperm concentration of 100 $\times 10^6$ spermatozoa/mL was
215 achieved by adding the appropriate volume of each extender to 6% glycerol. After
216 packaging into 0.25-mL plastic straws and equilibrating for 1 h at 5 °C, the samples
217 were frozen in a programmable freezer (Kryo 10 Series III; Planer plc., Sunbury-
218 On-Thames, UK) at -20 °C/min to -100 °C and then transferred to liquid nitrogen
219 containers. The cryopreserved samples remained in liquid nitrogen for a minimum
220 of 1 week. Thawing was performed by plunging the straws into water at 65 °C for 6
221 s.
222
223 *Evaluation of semen motility*
224 Sperm quality was evaluated immediately before freezing and after thawing. The
225 motility parameters were assessed using a computer-assisted semen motility
226 analysis system (CASA) (ISAS, Integrated Semen Analyser System; Proiser,
227 Valencia, Spain). Samples were diluted (10–20 $\times 10^6$ cells/mL) in buffered medium

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228 (HEPES 20 mm/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose, 1% egg
229 yolk; pH 7; 300 mOsm/kg) and warmed on a 37 °C plate for 5 min. Then, a 5-µL
230 sperm sample was placed in a Makler counting cell chamber (10 µm depth; Sefi
231 Medical Instruments, Haifa, Israel). The sample was examined at ×100 (negative
232 phase contrast) using a microscope with a warmed stage (38 °C). To classify a
233 spermatozoon as motile, it had to meet the following standard parameters: 25
234 frames/s, a head area of 5 to 80 μm^2 and a VCL > 10 µm/s. At least five sequences
235 or 200 spermatozoa were saved and analysed. The computer-assisted sperm
236 analysis provided the following parameters: total motility (TM, %; defined as the
237 percentage of spermatozoa with VCL > 10 µm/s), progressive motility (PM, %;
238 spermatozoa were considered progressive if VCL>25 and STR>80%), average path
239 velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL,
240 µm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, µm) and
241 straightness (STR, %).
242
243 *Flow Cytometry*
244 Sperm viability and acrosomal status were assessed using PNA-FITC/PI double
245 staining and flow cytometry (Martínez-Pastor *et al.* 2010). Stock solutions of the
246 fluorochromes were prepared in PBS at 1 µg/mL PNA-FITC and 6 µM PI. Sperm
247 samples were diluted to 1-2×10^6 spermatozoa/mL with fluorochrome solution in
248 polypropylene tubes (300 µL/tube). Flow cytometry analysis yielded the percentage
249 of red events (PI−) and the percentage of green events (PNA-FITC+). These
250 percentages were corrected according to Petrunkina & Harrison (2010) to remove
251 the signals for non-DNA containing particles. The number of non-sperm particles in
252 samples was analysed with SYBR14/PI (LIVE/DEAD Sperm Viability Kit: Invitrogen,
253 Barcelona, Spain) in parallel. Sperm samples were diluted to 5x10^6
254 spermatozoa/mL with PBS, incubated with 24 µM PI and 100 nM SYBR-14, and
255 stored in the dark at 37 °C for 20 min. We detected two populations, corresponding
256 to spermatozoa (staining events in quadrants Q3 and Q4, red or green) and foreign

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257 particles (non-staining events). Finally, the percentages of viable spermatozoa (PI–,
258 data corrected, named VIAB) and spermatozoa with damaged acrosomes (PNA-
259 FITC+, data corrected, named dACR) were calculated.
260 Flow cytometry evaluations were carried out using a FACSCalibur flow cytometer
261 (Becton Dickinson BioSciences, San Jose, CA, USA) equipped with an argon-ion
262 laser tuned at 488 nm and running at 200 mW with standard optics (DM 560SP and
263 DM 640 LP). The levels of red (670 LP filter, FL3 photodetector) and green
264 fluorescence (530/30BP filter, FL1 photodetector) of the acquired particles were
265 recorded. In all cases, 10 000 events per sample were assessed using Cell Quest
266 Pro v. 3.1 software (BD Biosciences) with a flow rate of 200 cells/s. Calibration was
267 carried out periodically using standard beads (Calibrite; Becton Dickinson
268 BioSciences). The flow cytometry data were analysed using Weasel v.3.0.2 (WEHI,
269 Melbourne, Australia).
270
271 *Statistical analysis*
272 The data were analysed using the SAS™ V.9.0. package (SAS Institute Inc., Cary,
273 NC, USA). The results are shown as the means and standard errors of the mean,
274 unless otherwise stated. The pre-freezing and post-thawing data were analysed
275 using linear mixed-effects models (MIXED procedure). Models were built with the
276 extender as the fixed effect and the sample as the random effect. Significant fixed
277 effects were further analysed using multiple comparisons of the means with Tukey
278 contrasts. A significance level of $P<0.05$ was used.

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279 **Results**

280

281 The following quality parameters were measured in fresh brown bear semen
282 samples (mean±SEM): volume (2.1±0.2 mL), cell concentration (327.5±59.4 $\times 10^6$
283 spermatozoa/mL), pH (8.2±0.1) and osmolarity (293.0±4.0 mOsm).

284

285 *Experiment 1. The effect of soybean lecithin on sperm cryopreservation*
286 The before-freezing evaluation showed differences in all parameters analysed
287 (except STR) between the control and S2 extender (Table 1). Soybean-based
288 extenders caused a significant reduction in sperm quality in comparison with the
289 control, as indicated by the PM, VSL and cell viability, whereas LIN, ALH and
290 acrosome status did not differ between the control and S5 extender. After thawing
291 (Table 1), the control presented better results for all the motility parameters
292 ($p<0.05$) except LIN (STR and ALH showed the lowest values in S2) and for sperm
293 viability. Moreover, both the control and the S5 samples exhibited lower acrosome
294 damage values.

295

296 *Experiment 2. The effect of soybean lecithin source on sperm cryopreservation*
297 The before-freezing analysis showed that the samples diluted in the egg yolk and
298 S5-A extenders contained significantly higher percentages of total motile
299 spermatozoa and progressive motile spermatozoa in comparison with the sperm
300 diluted in the S5-B extender (Table 2). The kinematic parameters of the
301 spermatozoa, the sperm viability and the proportion of damaged acrosomes were
302 not significantly different among the three extenders. After thawing, the
303 spermatozoa motilities in the S5-A extender group and control group were higher
304 than that in sperm frozen in S5-B, although no significant differences were
305 observed in any variables except TM (Table 2). The percentage of viable sperm was
306 lower in diluents containing soybean lecithin than in the control, but these diluents
307 did not affect the percentage of damaged acrosomes.

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309 *Experiment 3. The effect of LDL on sperm cryopreservation*

310 In the before-freezing study, the egg yolk extender had (Table 3) the highest values
311 for the motility parameters ($p<0.05$) including TM, PM, VAP and VCL. No differences
312 were observed in TM or PM between the LDL extenders. After thawing (Table 3), the
313 sperm motility was higher in the control than in the LDL extenders. The number of
314 damaged acrosomes was highest in the LDL5 extender ($p<0.05$), and the
315 percentages of viable sperm were highest in the LDL10 and LDL15 extenders.

316

317 *Study of the Total Antioxidant Capacity (TAC) of the extenders*

318 The LDL extenders showed higher Trolox equivalent antioxidant activity values after
319 refrigerated storage than those observed for the EY20 extender (Figure 1). Among
320 the soybean extenders, higher values were observed for S5-A and S5-B than for the
321 EY20, S2 and S3.5 extenders.

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324 **Discussion**

325

326 The replacement of egg yolk in diluents for freezing semen to obtain chemically
327 standardised extenders (e.g., replacement with LDL) or to avoid the use of animal
328 proteins (e.g., replacement with soybean) is a major priority in sperm
329 cryopreservation. To address these objectives, we carried out three different
330 experiments to compare sperm motility, acrosome status and viability parameters
331 in freeze-thawed brown bear semen preserved in TTF-based extenders containing
332 soybean lecithin, low-density lipoproteins or egg yolk.

333

334 *Soybean-based extenders*

335 To our knowledge, this is the first study in which soybean lecithin was used in an
336 extender for the cryopreservation of brown bear semen. The effects of three
337 concentrations of soybean lecithin (2%, 3.5% and 5%) on post-thaw sperm
338 physiology were tested in comparison with conventional egg yolk extender (Anel *et*
339 *al.* 2008). We could not assess concentrations above 50 g/L soybean lecithin (5%)
340 due to the size of the particles and aggregates in the soybean lecithin-TTF extender
341 emulsion (de Paz *et al.* 2010).

342 In the present study, the motility of post-thawed sperm and the percentages of
343 viable spermatozoa and spermatozoa with intact acrosomes increased when the
344 concentration of soybean lecithin in the extender was increased. Papa *at el.* (2011)
345 compared different concentrations of soybean lecithin (between 10 and 20 g/L) in
346 freezing extenders for stallion semen and showed that sperm frozen in extenders
347 containing any concentration of soybean lecithin maintained similar percentages of
348 motile spermatozoa. Our results showed that sperm motility, sperm viability and
349 the percentage of intact acrosomes were significantly lower for brown bear semen
350 that was diluted using a soybean lecithin extender than for semen that was diluted
351 in an egg yolk extender. In bovine semen, van Wagtendonk-de Leeuw *et al.* (2000)
352 observed that semen diluted with a commercial soybean-based extender (Biociphos

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353 Plus®), in comparison with semen diluted in other extenders based on egg yolk,
354 showed significantly lower post-thawing motility. This result can be explained by the
355 higher viscosity and presence of particulate debris in the Biociphos Plus extender.
356 However, stallion semen frozen in Botu-Crio®, which contains soybean lecithin
357 rather than egg, exhibited similar percentages of total motile sperm and sperm with
358 intact plasma membranes (Papa *et al.* 2011). These data suggest that the
359 protective effect of soybean lecithin in semen cryopreservation, as observed for egg
360 yolk, varies across species.
361 A deleterious effect on fertilisation potential has been observed in bull semen frozen
362 with soybean lecithin compared with sperm frozen in egg yolk-containing diluent,
363 although both types of semen showed similar sperm motility and plasma membrane
364 integrity values (Papa *et al.* 2011). However, another fertility study determined that
365 equine sperm cryopreserved in soy phosphatidylcholine-based medium were
366 capable of fertilisation at the same rate as sperm frozen in egg yolk-based medium,
367 and noting that sperm viability and motility were maintained in the soy
368 phosphatidylcholine-based medium (Riker *et al.* 2006). However, according to our
369 results, extenders based on soybean lecithin cannot protect the sperm membrane
370 during freezing as well as extenders based on EY20.
371 The lipid compositions of different commercial sources of soybean lecithin vary
372 significantly, and this variation affects the preparation of media and the media's
373 effects on semen quality (de Paz et al, 2010). In the present study, two sources of
374 soybean lecithin (50 g/L) were tested: S5-A (24% PC) and S5-B (14-23% PC).
375 After thawing, we observed that spermatozoa showed higher total and progressive
376 motility in the S5-A soybean-based extender than in the S5-B-based extender (only
377 the TM was statistically significantly different between these groups), but the
378 percentages of viable spermatozoa and spermatozoa with intact acrosomes and the
379 kinematic parameters were similar. Our results suggest that phosphatidylcholine
380 plays an important role in protecting sperm motility, although this effect could be
381 due to other components present in the commercial preparation of soybean lecithin.

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382 However, the sperm viability in frozen samples was not protected by the soybean
383 extenders compared with the egg yolk extender. Riker *et al.* (2006) found that pure
384 soy phosphatidylcholine-based diluents can prevent membrane damage during the
385 cryopreservation of equine spermatozoa and that these diluents perform as well as
386 a standard egg-yolk-based diluent in preserving sperm viability, motility, and
387 fertility.
388 In our study, the extender containing 5% type A soybean lecithin protected sperm
389 motility against cold shock damage as well as the egg yolk-based extender did, but
390 no protective effect was observed for sperm viability or acrosome integrity.
391 Different protective effects of additives on sperm motility and other spermatozoa
392 quality parameters have been observed (Leite *et al.* 2010). Del Valle *et al.* (2011)
393 showed that soy lecithin is able to effectively protect certain quality characteristics
394 of ram spermatozoa against freezing–induced damage; however, lecithin induced
395 the loss of mitochondrial membrane potential or mitochondrial loss which, in fresh
396 semen, was not reflected by changes in sperm motility. These authors suggest that
397 purified soy lecithin (L-α-phosphatidylcholine) might work only through interactions
398 with the plasma membrane, while egg yolk, which contains not only L-α-
399 phosphatidylcholine but also lipoproteins, might be better able to protect
400 mitochondria.
401
402 *LDL extender*
403 Our results showed that, before freezing, the motility parameters (TM and PM) and
404 some kinematic parameters (VAP, VCL and VSL) were lower in sperm preserved
405 with the LDL-containing extender (5, 10 or 15%) than in sperm preserved with the
406 extender containing 20% egg yolk. However, the percentages of viable spermatozoa
407 and spermatozoa with damaged acrosomes were similar in the LDL-based extender
408 and in the egg yolk extender. These differential protective effects on sperm quality
409 after the addition of LDL instead of egg yolk to the extender have not been
410 observed by other authors. Akhter *et al.* (2011), working with buffalo bull semen

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411 cooled to 4 °C, found that sperm progressive motility, plasma membrane integrity
412 and viability were similar in sperm preserved with an extender containing 10%
413 LDLs and those preserved with an extender containing 20% egg yolk.
414 Yamuchi *et al.* (2009) reported that the sperm cryopreserved in an extender
415 supplemented with LDL instead of egg yolk showed better resistance to cold shock
416 damage during cryopreservation in comparison with semen cryopreserved in an
417 extender supplemented with egg yolk. These authors found that the percentage of
418 total motile sperm, the extent of rapid progressive motility, the integrities of the
419 plasmalemma and DNA, mitochondrial activity and the proteolytic activity of the
420 acrosomal content in the post-thaw sperm were higher in Agu pig sperm treated
421 with LDL (4 or 6%) than in sperm frozen in an extender containing 20% egg yolk.
422 However, our results showed that the motility of post-thaw sperm was decreased in
423 semen treated with LDL during freezing in comparison with semen in an extender
424 containing 20% egg yolk. In contrast, the percentage of spermatozoa with normal
425 acrosomes was not affected by replacing the egg yolk with LDL, and the percentage
426 of viable sperm was higher.
427 The post-thaw reduction in sperm motility, which is directly proportional to the
428 percentage of LDL in the diluent, could indicate that the compounds present in the
429 extender have different effects on the plasma membrane and flagellum (Vera-
430 Munoz *et al.* 2009). The LDL extender was more effective in preserving plasma
431 membrane integrity than the egg yolk extender was, but sperm motility was
432 negatively affected. Amirat *et al.* (2004) found that LDL protected sperm cells
433 against cold shock by preventing the efflux of phospholipids and cholesterol from
434 the sperm cell membrane. According to Riker *et al.* (2006), exogenous lipids from
435 the diluents are not incorporated into the sperm membrane but are strongly
436 associated with the membrane surface; thus, these lipids may provide a physical
437 barrier to freeze-thaw damage and prevent membrane phase separation by
438 influencing lipid packing at the membrane surface. Additionally, the improved
439 cryoprotection of LDL treatments may also be related to the removal of certain

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440 substances present in the egg yolk (high-density lipoproteins and aggregates) that
441 could have deleterious effects on sperm viability (Pace and Graham, 1974).
442

443 *The total antioxidant capacity (TAC) of the extenders*

444 As expected, the TTF diluent used in this experiment to obtain different extenders
445 showed no antioxidant capacity according in the TAC assay; therefore, the
446 antioxidant capacity of the extender was due to the addition of membrane
447 stabilisers. The egg yolk-based extender showed a total antioxidant capacity similar
448 to those of the soy lecithin-based extenders (S2 and S3.5). Consistent with this
449 observation, the greatest sperm viability and the lowest percentage of damaged
450 acrosomes were observed in samples preserved with egg yolk-based extender. In
451 this regard, the LDL-based extenders, which were associated with higher TAC
452 values than egg yolk-based extenders, are less able to protect the sperm motility in
453 post-thawed samples but have a greater protective effect on spermatozoa viability.
454 These results suggest that there is no direct relationship between the antioxidant
455 capacity of the extender and its protective effect on post-thawed sperm quality.
456 The presence of polyunsaturated fatty acids in plasma membranes is accepted as
457 the main cause of spermatozoa membrane oxidation during cryopreservation. Thus,
458 it is possible that the loss of cell function observed in post-thawed spermatozoa
459 involves the aforementioned oxidative stress. Furthermore, although Kasimanickam
460 *et al.* (2007) observed that lipid peroxidation and plasma membrane integrity had a
461 cause and effect relationship, sperm motility was an independent factor. One way to
462 prevent oxidative stress in spermatozoa after thawing is to add antioxidants to the
463 freezing extender (Fernández-Santos *et al.* 2007), but variable results have been
464 obtained with the addition of antioxidants to freezing diluents for different species
465 (bovine: Stradioli *et al.* 2007; red deer: Fernández-Santos *et al.* 2007; ram: Maia
466 *et al.* 2010; pig: Buranaamnuay *et al.* 2011). Hu *et al.* (2011) found that an
467 extender containing LDL reduced the negative impact of ROS on bull sperm and

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468 that 8% LDL was associated with better semen quality parameters and anti-oxidant
469 enzyme activity than was egg yolk.
470 In summary, our results confirm that LDL extenders have greater anti-oxidant
471 activity than egg yolk extenders. High concentrations of LDL (10 or 15 %) could be
472 useful as a substitute for egg yolk in extenders used for cryopreservation because
473 LDL at high concentrations is better able to protect the viability of brown bear
474 sperm and does not affect the acrosomal integrity, although sperm motility is
475 negatively affected. Soybean lecithin did not protect the viability of freeze-thawed
476 brown bear sperm, although the sperm motility was similar in the extender
477 containing 5% type A soybean and the extender containing egg yolk. Further
478 studies are needed to more precisely determine the type and concentration of
479 soybean lecithin suitable for use in freezing diluents for brown bear semen.
480

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488

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Table 1. Brown bear semen quality parameters (mean±SEM) before freezing and after thawing in extenders containing different soybean concentrations. Control (EY20), 2% soybean (S2), 3.5% soybean (S3.5) and 5% soybean (S5).

	Pre-freeze				Post-thawing			
	EY20	**S2**	**S3.5**	**S5**	**EY20**	**S2**	**S3.5**	**S5**
TM	77.6±4.4^{a}	2.1±0.6^{b}	69.3±4.1^{a}	70.4±5.4^{a}	54.2±3.5^{a}	1.0±0.4^{b}	42.9±4.7^{a}	52.4±6.3^{a}
PM	44.0±4.2^{a}	0.4±0.1^{b}	24.0±2.3^{c}	31.6±3.1^{c}	21.5±2.7^{a}	0.2 ±0.1 ^{b}	4.4±0.8^{b}	17.1±7.1^{b}
VAP	60.9±3.2^{a}	11.8±3.9^{b}	55.2±2.8^{a}	50.7±2.9^{a}	56.6±3.5^{a}	6.6±1.3^{b}	42.6±2.4^{c}	42.7±2.8^{c}
VCL	100.3±4.9^{a}	22.2±7.5^{b}	102.8±5.6^{a}	86.4±5.2^{a}	107.0±6.6^{a}	12.0±2.6^{b}	86.4±6.0^{a}	83.5±6.4^{a}
VSL	42.7±2.8^{a}	8.3±3.2^{b}	30.7±1.6^{c}	32.2±1.7^{c}	35.1±2.3^{a}	4.2±1.0^{b}	25.7±1.8^{c}	26.1±2.1^{c}
LIN	40.9±2.3^{a}	30.4±4.3^{b}	30.3±1.3^{b}	36.3±1.6^{a,b}	32.3±0.9	23.6±4.6	28.9±0.5	30.2±0.7
STR	65.5±1.8	55.2±5.8	55.1±1.7	60.9±1.5	58.7±0.8^{a}	40.0±6.8^{b}	56.0±1.1^{a}	56.2±1.5^{a}
ALH	4.4±0.3^{a}	1.3±0.3^{b}	4.6±0.2^{a}	3.8±0.2^{a}	5.0±0.3^{a}	0.8±0.2^{b}	4.4±0.3^{a}	4.2±0.3^{a}
VIAB	77.7±2.3^{a}	2.3±1.1^{b}	23.7±4.4^{c}	56.5±6.0^{d}	50.1±3.3^{a}	3.3±2.0^{b}	16.4±2.8^{c}	21.0±4.1^{c}
dACR	2.8±0.4^{a}	26.1±5.2^{b}	21.1±2.9^{bc}	8.6±2.6^{ac}	11.7±1.8^{a}	43.4±8.3^{b}	29.7±2.6^{bc}	23.7±3.0^{ac}

Total motility (TM; %), progressive motility (PM; %), average velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), linearity (LIN; %), straightness (STR; %), amplitude of lateral head displacement (ALH; μm), viable spermatozoa (VIAB: PI-) and spermatozoa with damaged acrosomes (dACR: PNA-FITC+). Different letters within rows indicate significant differences between the control and the soybean-based extenders ($P<0.05$).

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655 **Table 2.** Brown bear semen quality parameters (mean±SEM) before freezing and after thawing in extenders containing soybean lecithin
656 from two sources. Control (EY20), 5% type A soybean (S5-A) and 5% type B soybean (S5-B).

	Pre-freeze			Post-thawing		
	EY20	**S5-A**	**S5-B**	**EY20**	**S5-A**	**S5-B**
TM	55.2±11.6[ab]	68.6±3.8[b]	33.9±8.8[a]	42.2±7.2[a]	47.2±4.8[a]	28.7±4.4[b]
PM	32.2±7.0[ab]	49.9±3.3[b]	26.2±8.0[a]	24.6±4.9	27.4±3.2	17.5±4.0
VAP	55.6±7.5	51.5±5.4	38.6±8.4	59.8±7.1	50.2±2.9	43.1±7.3
VCL	85.1±12.9	84.1±11.6	56.1±11.8	98.9±12.9	88.9±7.1	69.9±12.8
VSL	37.4±5.4	38.3±4.3	30.4±7.3	41.2±5.3	34.7±3.0	30.7±6.2
LIN	43.1±2.4	47.9±2.4	45.9±7.8	41.8±2.8	39.3±2.3	40.1±8.1
STR	63.5±1.5	72.2±0.8	64.1±9.7	64.6±2.1	65.0±2.2	58.2±9.5
ALH	3.5±0.5	3.6±0.5	2.4±0.4	4.2±0.5	4.0±0.4	3.0±0.6
VIAB	77.5±4.3	77.6±6.0	70.8±11.2	39.9±2.1[a]	26.6±3.9[b]	25.0±3.5[b]
dACR	2.9±0.5	3.9±0.5	7.5±4.8	6.4±0.9	12.6±2.4	10.5±3.5

657

658 Total motility (TM; %), progressive motility (PM; %), average velocity (VAP; µm/s), curvilinear
659 velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), linearity (LIN; %), straightness (STR;
660 %), amplitude of lateral head displacement (ALH; µm), viable spermatozoa (VIAB: PI-) and
661 spermatozoa with damaged acrosomes (dACR: PNA-FITC+). Different letters within rows
662 indicate significant differences between the control and the soybean-based extenders ($P<0.05$).

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665 **Table 3.** Brown bear semen quality parameters (mean±SEM) before freezing and after thawing in extenders containing different LDL
666 concentrations. Control (EY20), low-density lipoproteins (LDL) at three different concentrations (5, 10 and 15%: LDL5, LDL10 and LDL 15,
667 respectively).

	Pre-freeze				Post-thawing			
	EY20	**LDL5**	**LDL10**	**LDL15**	**EY20**	**LDL5**	**LDL10**	**LDL15**
TM	60.3±6.4[a]	31.7±6.1[b]	35.1±6.0[b]	28.7±6.4[b]	41.2±6.4[a]	28.9±4.7[ab]	29.2±5.8[ab]	16.9±3.8[b]
PM	34.8±3.9[a]	16.7±3.8[b]	16.1±2.7[b]	13.4±2.6[b]	22.6±4.2[a]	11.6±1.6[b]	11.9±2.1[b]	7.5±1.5[b]
VAP	56.7±3.6[a]	41.5±3.5[b]	33.5±2.4[bc]	26.5±2[c]	53.1±5.6[a]	38.1±1.9[b]	32.6±1[b,c]	26.5±0.6[c]
VCL	82.2±4.7[a]	68.4±5.4[ab]	56.2±4.9[bc]	41.5±4.3[c]	90.2±7.4[a]	66.6±3[b]	53.6±2.1[c]	40±2.1[d]
VSL	38.4±2.4[a]	26.6±2.8[b]	20.8±1.5[bc]	15.9±1[c]	34.8±3.9[a]	22±1.6[b]	18.5±0.9[b]	15.4±0.4[b]
LIN	46.9±2.0	41.9±2.3	40.4±1.9	45.1±3.3	38.9±2.5[ab]	34.7±0.8[a]	36.5±0.8[ab]	43.6±2.8[b]
STR	65.8±1.5	63.7±2.1	61.2±1.6	62.1±2.2	63.0±1.4[a]	56.6±1[b]	56.0±0.9[b]	57.5±1.8[b]
ALH	3.2±0.2	3.1±0.2	2.9±0.2	2.5±0.2	3.9±0.2[a]	3.3±0.1[b]	2.9±0.1[c]	2.6±0.1[c]
VIAB	72.5±4.6	80.2±2.8	81.9±2.4	77.3±5.2	40.7±6.5[a]	45.5±3.3[a]	66.2±3.0[b]	73.4±3.9[b]
dACR	3.8±0.7	6.3±1.9	1.8±0.3	4.5±2.8	6.6±1.1[ab]	9.6±2.6[b]	4.6±0.6[a]	4.9±1.0[a]

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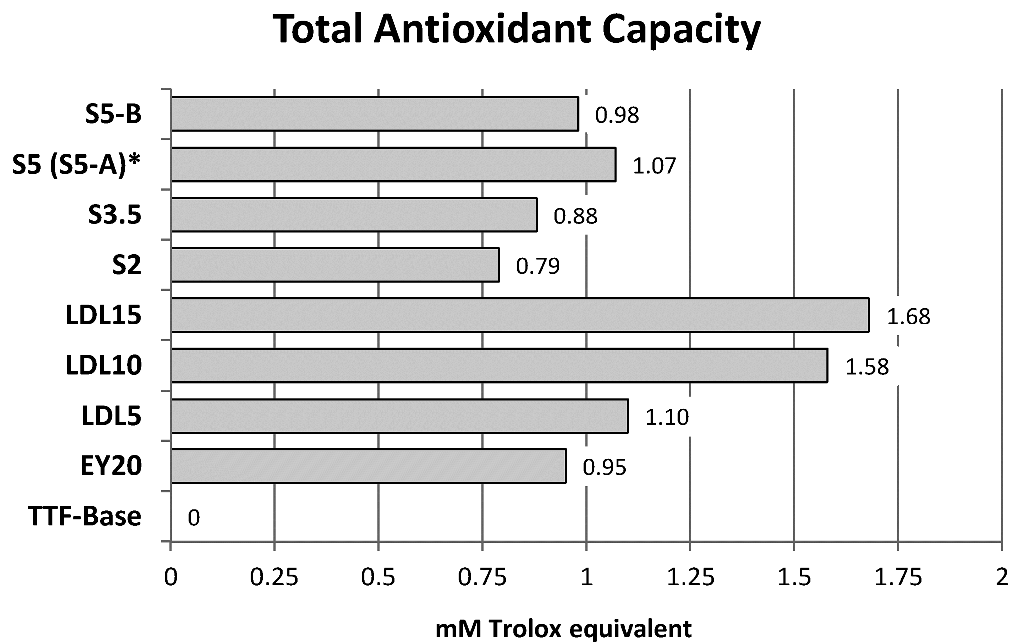
669 Total motility (TM; %), progressive motility (PM; %), average velocity (VAP; µm/s), curvilinear velocity
670 (VCL; µm/s), straight-line velocity (VSL; µm/s), linearity (LIN; %), straightness (STR; %), amplitude of
671 lateral head displacement (ALH; µm), viable spermatozoa (VIAB: PI-) and spermatozoa with damaged
672 acrosomes (dACR: PNA-FITC+). Different letters within rows indicate significant differences between the
673 control and the soybean-based extenders ($P<0.05$).

674

675 **Figure 1.** Total antioxidant capacity (TAC, mM Trolox equivalents) of the extenders used in the
676 present study: S5-B (5% type B soybean); S5 (5% type A soybean); S3.5 (3.5% soybean); S2
677 (2% soybean); LDL15 (15% low-density lipoproteins); LDL10 (10%); LDL5 (5%); EY20 (Control).

678



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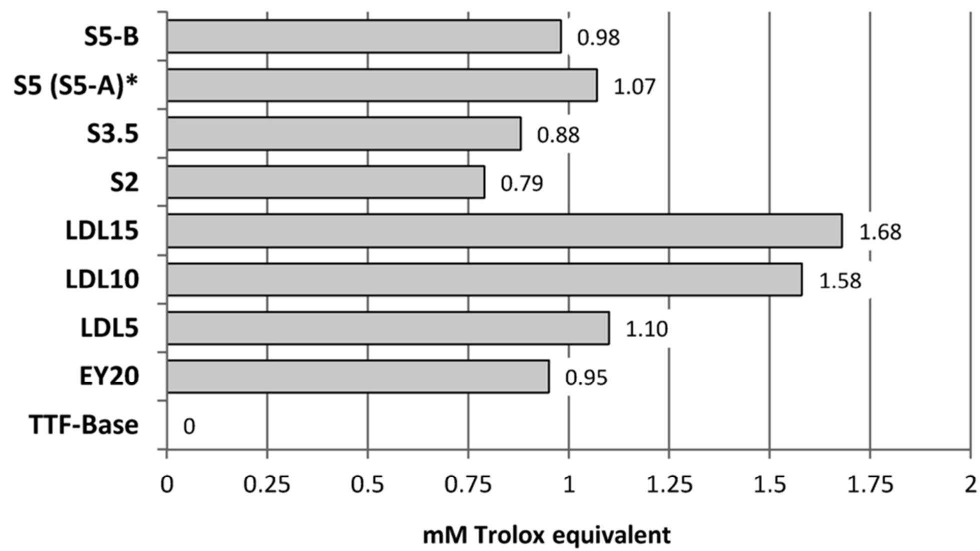

81x54mm (300 x 300 DPI)

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1) RE-CALCULATE THE SUBSETS DATA FROM FLOW CYTOMETRY EXPERIMENTS.

1.1) PNA/IP analysis.

TABLE 1.

	Pre-freezing				Post-thawing			
	EY20	**S2**	**S3.5**	**S5**	**EY20**	**S2**	**S35**	**S5**
VIAB	79.9±2.2[a]	2.4±1.2[b]	24.3±4.6[c]	58.2±6.1[d]	51.7±3.7[a]	3.4±2.1[b]	16.8±2.9[c]	21.6±4.2[c]
dACR	2.8±0.4[a]	26.0±5.2[b]	21.0±2.9[bc]	8.5±2.6[a,c]	11.6±1.6[a]	43.4±8.2[b]	29.7±2.6[bc]	23.7±3.0[ac]

TABLE 2.

	Pre-freezing			Post-thawing		
	EY20	**S5-A**	**S5-B**	**EY20**	**S5-A**	**S5-B**
VIAB	78.5±4.4	79.7±6.3	73.4±11.6	40.8±3.2[a]	27.5±5.9[b]	25.6±5.2[b]
dACR	2.8±0.5	3.9±0.5	7.4±4.8	6.2±1	11.2±2	9.8±2.6

TABLE 3.

	Pre-freezing				Post-thawing			
	EY20	**LDL5**	**LDL10**	**LDL15**	**EY20**	**LDL5**	**LDL10**	**LDL15**
VIAB	73.2±4.9	81.3±2.8	83.4±2.4	80±5.2	41.0±6.8[a]	46.1±3.3[a]	67.8±3[b]	76.3±3.7[b]
dACR	3.8±0.8	6.3±1.9	2.8±0.3	4.5±2.7	6.6±1.1[ab]	9.5±2.6[b]	4.6±0.6[a]	4.9±0.9[a]

1.2) SYBR-14/IP analysis: % of DEBRIS.

Results obtained after the elimination of the portion included inside the FSC/SSC gate. The SYBR-14/IP plot discriminates the % of non-DNA-containing alien particles. We divided the SYBR-14/IP plot into three different regions: SYBR-14+/IP-, SYBR-14+/IP+ and SYBR-14-/IP+. The non-DNA-containing alien particles correspond to the SYBR-14-/IP- region. Mean±S.E.M.

Experiment		**Pre-freezing**	**Post-thawing**
Soybean concentration	EY20 (Control)	2.4±0.5	2.9±0.8
	S2	2.1±0.7	1.9±0.3
	S3.5	1.8±0.5	2.0±0.4
	S5	2.7±0.6	2.6±0.3
Soybean source	EY20 (Control)	1.3±0.2	2.1±0.4
	S5-A	2.5±0.4	2.9±0.3
	S5-B	3.2±0.4	2.1±0.3
LDL concentration	EY20 (Control)	1.4±0.4	1.1±0.2
	LDL5	1.4±0.4	1.4±0.2
	LDL10	1.8±0.3	2.4±0.5
	LDL15	2.9±0.7	3.1±0.9

1.3) Re-calculate.

The misestimation of percentages is corrected applying mathematical formulae and equations from Theriogenology 73 (2010) 839-847.

TABLE 1 Corrected.

	Pre-freezing				Post-thawing			
	EY20	**S2**	**S3.5**	**S5**	**EY20**	**S2**	**S35**	**S5**
VIAB	77.7±2.3[a]	2.3±1.1[b]	23.7±4.4[c]	56.5±6.0[d]	50.1±3.3[a]	3.3±2.0[b]	16.4±2.8[c]	21.0±4.1[c]
dACR	2.8±0.4[a]	26.1±5.2[b]	21.1±2.9[bc]	8.6±2.6[a,c]	11.7±1.8[a]	43.4±8.3[b]	29.7±2.6[bc]	23.7±3.0[ac]

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TABLE 2 Corrected.

	Pre-freezing			Post-thawing		
	EY20	S5-A	S5-B	EY20	S5-A	S5-B
VIAB	77.5±4.3	77.6±6.0	70.8±11.2	39.9±2.1[a]	26.6±3.9[b]	25.0±3.5[b]
dACR	2.9±0.5	3.9±0.5	7.5±4.8	6.4±0.9	12.6±2.4	10.5±3.5

TABLE 3 Corrected.

	Pre-freezing				Post-thawing			
	EY20	LDL5	LDL10	LDL15	EY20	LDL5	LDL10	LDL15
VIAB	72.5±4.6	80.2±2.8	81.9±2.4	77.3±5.2	40.7±6.5[a]	45.5±3.3[a]	66.2±3.0[b]	73.4±3.9[b]
dACR	3.8±0.7	6.3±1.9	1.8±0.3	4.5±2.8	6.6±1.1[ab]	9.6±2.6[b]	4.6±0.6[a]	4.9±1.0[a]

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2) FLOW CYTOMETRIC DOT PLOTS OF SYBR-14/IP ANALYSIS DATA.

Figure 1. CONTROL. R4=2.7%.

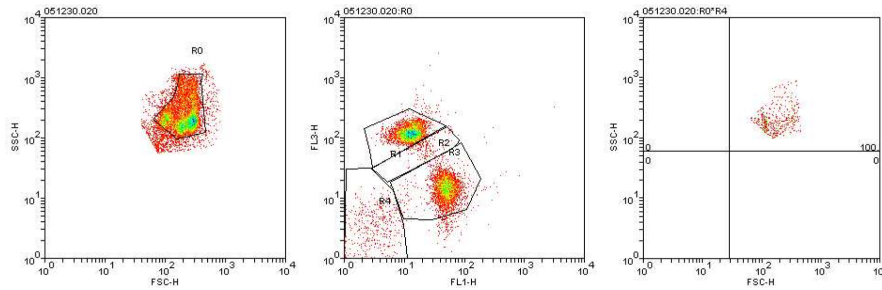

Figure 2. S2. R4=1.96%.

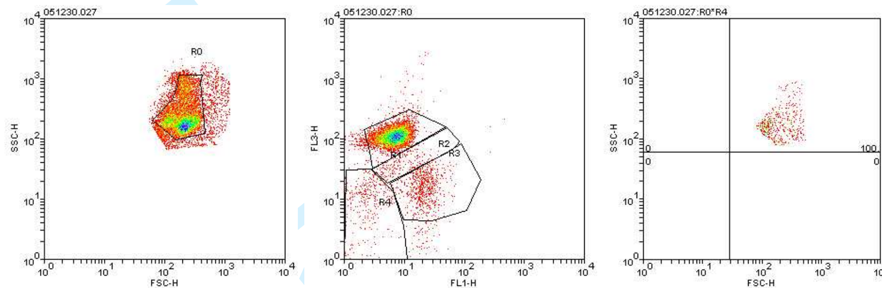

Figure 3. S3.5. R4=1.98%.

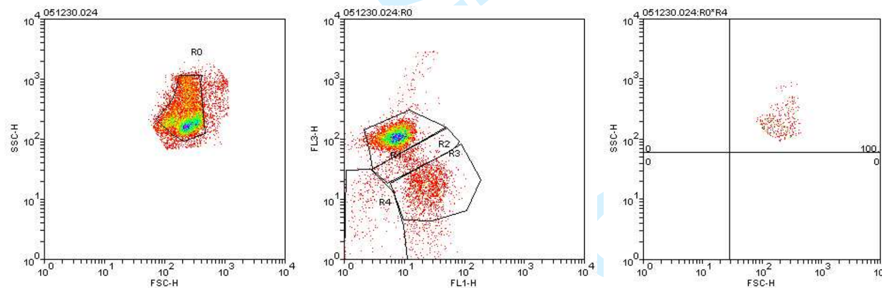

Figure 4. S5 (S5-A). R4=3.03%.

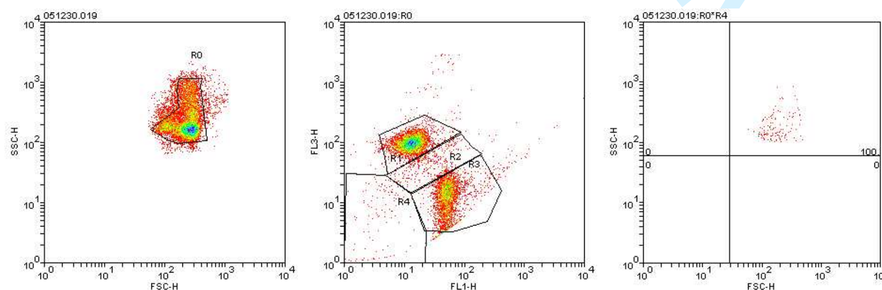

Figure 5. S5-B. R4=2.03%.

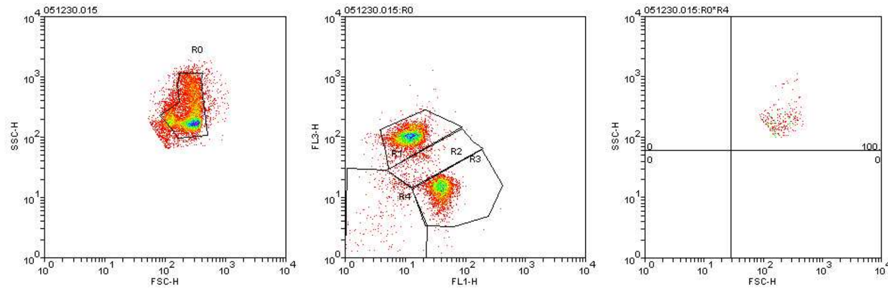

Figure 6. LDL5. R4=1.58%.

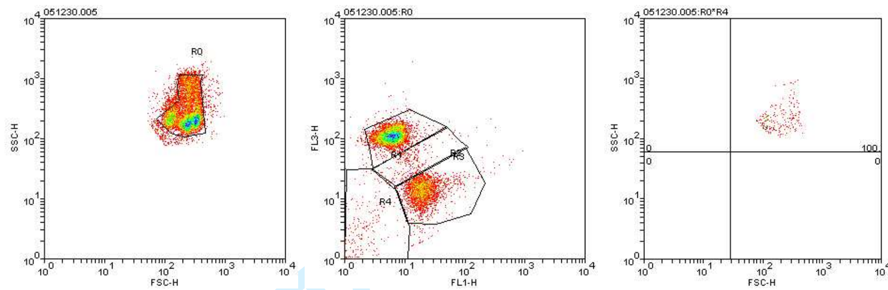

Figure 7. LDL10. R4=2.78%.

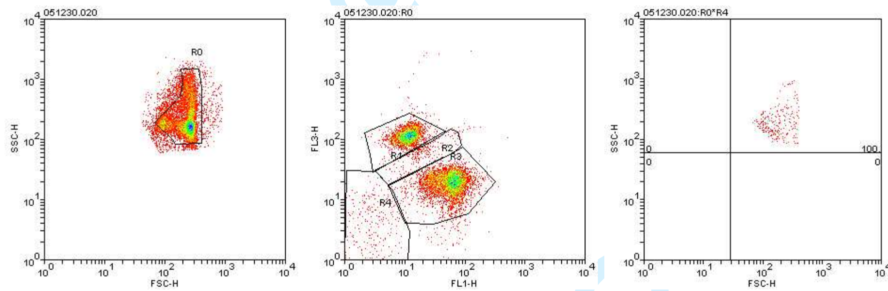

Figure 8. LDL15. 3.08%