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Seasonal changes in ROS concentrations and sperm quality in unfrozen and frozen-thawed stallion semen

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13

14 Seasonal changes in ROS concentrations and sperm quality in unfrozen and frozen-thawed stallion
15 semen

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17

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21

22 **Abstract**

23 Oxidative stress is regarded as an important cause of sperm damage during cryopreservation.

24 However, seasonal changes in oxidative status in unfrozen and frozen-thawed stallion sperm have

25 not been well established. We tested the hypothesis that sperm ROS concentrations and lipid

26 peroxidation change between breeding and non-breeding seasons and influence quality of unfrozen

27 and frozen-thawed sperm. Eighteen ejaculates from six Warmblood stallions (8 to 21 y) known to

28 be fertile, were collected in winter and summer and processed for freezing. After 90 min at +4°C,

29 some straws from each ejaculate were not frozen (unfrozen), whereas the remainder were frozen by

30 N₂ vapors and plunged in N₂ (frozen). Rapid cells (RAP; determined by CASA), plasma membrane-

31 acrosome integrity (PMAI), high mitochondrial membrane potential (Mpos), low intracellular Ca²⁺

32 concentration (Fneg), membrane lipid peroxidation (BODIPY), intracellular ROS concentrations

33 (DCFH, MitoSOX) and chromatin fragmentation (DFI%) were evaluated by flow cytometry in both

34 groups and at intervals during incubation at +37°C for 24 h. Overall, ROS concentrations and lipid

35 peroxidation were higher and faster ($P < 0.0001$) in winter versus summer, DFI% was lower in

36 winter versus summer ($P < 0.0001$), but similar between the two groups within season. There were

37 moderate positive correlations in both seasons between DFI% and MitoSOX, DCFH, BODIPY in

38 both groups, whereas a negative correlation, stronger in winter, was evident between sperm quality

39 (RAP, PMAI, Mpos, Fneg) and BODIPY, DCFH, MitoSOX. There were no differences between

40 seasons for RAP, PMAI, Mpos and Fneg. In conclusion, ROS-related parameters were higher in
41 winter than in summer, without a negative effect on sperm quality. We concluded that increased
42 ROS concentrations were less deleterious to sperm than freezing-thawing. Furthermore, incubation
43 at +37°C and sequential analysis were useful to assess sperm resistance.

44
45 **Keywords:** sperm, horse, seasonality, oxidative status, freezing-thawing.

46

47

48 **1. Introduction**

49 Stallions are seasonal “long day” breeders, with maximal reproductive activity occurring in
50 late spring and summer, when increasing day length stimulates the hypothalamic-pituitary axis [1].
51 Regardless, stallions remain fertile throughout the year, albeit with variations between non-breeding
52 and breeding seasons in endocrine profiles [2,3], scrotal thermoregulation, heat dissipation [4] and
53 characteristics of fresh and frozen semen, including sperm motility [5,6], morphology [6],
54 concentration and volume [7,8], viability and acrosomal status [9,10], fatty acid composition of
55 plasma membrane [11], seminal plasma composition [9], DNA integrity [6,10,12] and fertility [9].
56 Stallion semen is often collected and cryopreserved during the non-breeding season, in autumn and
57 winter [6] due to greater availability, as the dogma is that differences among seasons are small and
58 production of frozen semen is possible throughout the year [13].

59 Reactive oxygen species (ROS) have physiologically relevant roles [14,15] in controlling sperm
60 capacitation, acrosome reaction, hyperactivation and sperm-oocyte binding. However, uncontrolled
61 (i.e. excessive) ROS generation can have detrimental effects on sperm functions [16] and DNA
62 integrity [17]. In fresh semen collected from stallions in breeding and non-breeding seasons, ROS
63 concentrations never seemed to account for compromised sperm DNA integrity [9], although there
64 were differences between fertile and subfertile stallions in protein and lipid oxidation. In another
65 study characterizing dismounting semen samples, more fertile stallions had higher metabolically and
66 active sperm, generating higher ROS concentrations [18]. Notwithstanding, oxidative stress is an

67 important cause of sperm damage during cryopreservation in horses [17] and various other species,
68 including cattle [19], swine [20] and humans [21].

69 To our knowledge, seasonal changes in equine sperm oxidative status in relation to semen quality and
70 cryopreservation are not well characterized. Therefore, our objectives were to investigate seasonal
71 changes (winter/non breeding vs summer/breeding season) in ROS concentrations and lipid
72 peroxidation and their effects on stallion sperm immediately pre-freezing (unfrozen), after
73 freezing/thawing cycle (frozen-thawed) and during incubation at +37°C for up to 24 h.

74

75 **2. Materials and methods**

76 *2.1. Chemicals*

77 Chemicals were obtained from Sigma-Aldrich Co., Steinheim, Germany (propidium iodide,
78 FITC/PNA, DCFH), Thermo Fisher Scientific, Inchinnan Business Park, United Kingdom
79 (Fluo4AM, Mitoprobe, BODIPY⁵⁸¹⁻⁵⁹¹, SYTOX, MitoSOX) and Polysciences Europe GmbH,
80 Eppelheim, Germany, (Acridine Orange).

81

82 *2.2. Stallions and experimental design*

83 In Northern hemisphere, in January (winter) and July (summer), three ejaculates were
84 collected from each of six Warmblood stallions of proven fertility (successfully sired multiple
85 pregnancies during the previous 2 y), ranging in age from 8 to 21 y (mean 12.5±4.7 y). These stallions
86 were housed at INFA (Italy) and fed 15 kg of hay and 4 kg of concentrate per day, with *ad libitum*
87 access to water. Before starting the experiment in January, one ejaculate was collected once daily
88 from all stallions for 1 wk to deplete extra-gonadal sperm reserves. In the subsequent breeding season,
89 all stallions were collected on a regular basis (thrice weekly). The starting point for all analyses was
90 designated Time 0 (T0). For unfrozen samples, Time 0 was 90 min after incubation at +4°C
91 (equilibration time), whereas for frozen-thawed samples, it was immediately after thawing. Unfrozen
92 and frozen-thawed samples were evaluated after incubation in a water bath at +37°C for 3 h (T1), 6

93 h (T2), 12 h (T3) and 24 h (T4). This temperature and incubation times were chosen to detect
94 differences between unfrozen and frozen-thawed samples in sperm resistance. For each sample at
95 each time point, a 100 μ L sample was frozen in liquid nitrogen and stored at -80°C for DNA analysis.

96

97 *2.3. Semen collection and freezing*

98 Semen was collected on a phantom using an artificial vagina (Missouri, IMV) with an inner
99 liner and in-line filter (Hamilton Thorne Research, Denver, MA, USA). Ejaculates were weighed (1
100 g = 1 mL) and sperm concentration determined (NucleoCounter® SP-100™, Chemotec, Denmark).
101 Then semen was diluted at $50 \times 10^6/\text{mL}$ with Kenney extender pre-warmed ($+37^{\circ}\text{C}$) [22] and
102 centrifuged in 50-mL glass conical tubes (Schott Duran® GmbH, Germany) at $600 \times g$ for 20 min
103 with 1.0 mL of cushion fluid (Cushion Fluid Minitube-Minitube GmbH, Germany) added (with a
104 spinal needle) to the bottom of the tubes [23]. Supernatant was discarded, cushion fluid aspirated and
105 sperm pellet was re-suspended (200×10^6 mL) in Heitland extender [24] with 2% egg yolk and 3%
106 glycerol v/v. For each ejaculate, 14 straws (0.5 mL) were loaded at room temperature using a fully
107 automatic straw filling and sealing machine (IMV Technologies; L'Aigle, France) and kept
108 horizontally at $+4^{\circ}\text{C}$ for 90 min (equilibration time). Then, seven straws were frozen on a floating
109 system 6 cm above liquid nitrogen for 20 min before being plunged into liquid nitrogen; the remaining
110 seven straws were placed at $+37^{\circ}\text{C}$ and incubated for analysis. Frozen samples were thawed in a water
111 bath ($+37^{\circ}\text{C}$ for 30 s). Finally, both aliquots were diluted to a concentration of 1.2×10^6 sperm/mL
112 with pre-warmed ($+37^{\circ}\text{C}$) Tyrode's medium (310 mOsm; 7.2 pH) [10] and kept at $+37^{\circ}\text{C}$ for 15 min
113 until analyzed.

114

115 *2.4. Semen analyses*

116 *2.4.1. Motility*

117 Total motility (TM), progressive motility (PM) and rapid cells (RAP) were assessed with a
118 computer system assisted sperm analyzer (IVOS Vers.12, Hamilton Thorne Inc., Denver, MA,

119 USA), using the following settings: recording rate at 60 frames/s, minimum contrast of 70 pixels,
120 minimum cell size of 10 unit: μm^2 , slow cells velocity (VSL) threshold of 30 $\mu\text{m/s}$, slow cell threshold
121 of 20 $\mu\text{m/s}$, minimum average path velocity (VAP) $>30 \mu\text{m/s}$ and threshold straightness (STR) of
122 80% for progressive cells. Sperm with VAP $\geq 30 \mu\text{m/s}$ were classified as rapid cells. A minimum of
123 1000 cells were analyzed in at least eight randomly selected fields. All end point values changed over
124 time similarly in unfrozen and frozen samples; therefore, only RAP is presented.

125

126 2.4.2. *Flow cytometric analysis*

127 Flow cytometric analyses were performed according to recommendations of the International
128 Society for Advancement of Cytometry [25] with a CytoFlex (Beckman Coulter, Fullerton, CA, USA)
129 using two solid state laser beams generated by 488 (50 mW laser output) and 638 nm (45 mW laser
130 output) lasers. Debris (non-sperm events) was gated out on the basis of forward scatter and side scatter
131 dot plot by drawing a region enclosing the cell population of interest. Flow rate (60 $\mu\text{l/min}$) was set
132 to 500-1000 events/s and for each sample, 10000 sperm were analyzed. Agglutination was gated
133 (hierarchical) out in the basis of FSC (H) vs FSC (A) + SSC (H) vs SSC (A). After addition of
134 fluorescence dyes, all sperm samples were incubated at $+37^\circ\text{C}$ for 15 min and mixed just before
135 measurement.

136

137 2.4.2.1. *Membrane lipid peroxidation*

138 This lipid-based fluorophore readily integrates into biological membranes and reacts to free
139 radical attack with a spectral emission shift from red to green that can be quantified with flow
140 cytometry. Red fluorescence represents overall incorporation of non-oxidized probe into the cell,
141 whereas green fluorescence represents oxidization of membrane-incorporated probe. To quantify
142 lipid peroxidation of plasma membrane intact sperm (BODIPY), Bodipy⁵⁸¹⁻⁵⁹¹ C11 (5 mM) and PI
143 (2.99 mM) were added to 246 μL of diluted sperm suspension.

144

145 *2.4.2.2. Intracellular ROS concentrations: H₂O₂ and O₂⁻*

146 DCFH-DA is a non-fluorescent agent, converted by H₂O₂ into DCFH, which has a green
147 fluorescence [26]. To conduct this assessment, DCFH-DA (10 μM) and PI (2.99 μM) were added to
148 246 μL of diluted semen. The percentage plasma membrane intact sperm with intracellular hydrogen
149 peroxide (H₂O₂) was gated (DCFH). Intracellular generation of superoxide radicals (O₂⁻) was
150 estimated using MitoSOX Red, a lipid- soluble, cell-permeable cation that selectively targets
151 mitochondrial matrix [27]. The SYTOX Green stain only penetrates sperm with damaged plasma
152 membranes and fluoresces green on binding DNA. For this assay, MitoSOX Red (2 μM) and SYTOX
153 Green (0.05 μM) were added to 246 μL of diluted sperm suspension. The percentage of plasma
154 membrane intact sperm with intracellular superoxide radicals (O₂⁻) was gated (MitoSOX).

155

156 *2.4.2.3. Sperm Chromatin Structure Assay (SCSATM)*

157 Susceptibility of sperm to acid-induced DNA fragmentation was assessed performing SCSA[®]
158 as described [19]. The DNA fragmentation index (DFI%) was determined.

159

160 *2.4.2.4. Plasma membrane and acrosome integrity*

161 Membrane integrity and acrosomal status of sperm were evaluated after staining with propidium
162 iodide (PI) and peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA) [28]. For
163 this, 5 μL of semen, previously diluted in 238.5 μL Tyrode's solution, were stained by adding PI
164 (2.99 mM) and FITC-PNA (100 mg/mL). Percentage intact plasma membrane and acrosome sperm
165 was gated (PMAI).

166

167 *2.4.2.5. Intracellular Ca⁺² concentrations and mitochondrial membrane potential*

168 MitoprobeTM DiIC1(5), a cationic cyanine dye, was used to detect sperm that differ in regard
169 to mitochondria membrane potential. Percentage of sperm subpopulation with outstanding red
170 fluorescence (most mitochondria had high membrane potential) and low red fluorescence

171 (predominantly low mitochondrial membrane potential) was determined (APC Channel). Percentage
172 of sperm with high mitochondrial membrane potential was gated (M_{pos}). Fluo4 AM stain distinguishes
173 sperm according to intracellular Ca^{+2} concentrations. Percentages of sperm subpopulations that
174 emitted low or high green fluorescence (indicating low or high intracellular Ca^{+2} concentrations,
175 respectively) were captured by the FITC filter. Propidium Iodide (PI), was used to distinguish
176 between two subpopulations of cells with intact and damaged cell plasma membranes, based on
177 presence or absence of red fluorescence reaction (detected by the filter ECD). Percentage of viable
178 sperm with low intracellular Ca^{+2} concentrations was gated (F_{neg}). Each reaction, (final volume of
179 250 μL), consisted of 2.5 μL Fluo4 AM, 1.25 μL DiLC1 and 1.5 μL PI diluted with 240.75 μL and 4
180 μL sperm.

181

182 *2.5. Environmental factors*

183 Environmental parameters considered were temperature and humidity (mean, maximum and
184 minimum) in the two seasons (January and July for winter and summer seasons, respectively); these
185 data were obtained from Meteorological Station of Mezzolara, Budrio, ARPAE, Italy.

186

187 *2.6. Statistical analysis*

188 Mean ($\pm\text{SD}$) values of sperm parameters, conditional on type of preservation (unfrozen,
189 frozen-thawed) and duration of incubation (0, 3, 6, 12 and 24 h), were calculated as measures of
190 central tendency and data dispersion. Growth curve analysis was used to analyze sperm parameters
191 over the 24 h incubation. Sperm parameters (RAP, PMAI, M_{pos} , F_{neg} , MitoSOX, DCFH, BODIPY,
192 DFI%) were modelled using orthogonal polynomials of incubation time. Based on preceding analyses
193 of experimental data, the incubation time curve of most sperm parameters followed a cubic form;
194 therefore, they were modelled as a function of a third-order orthogonal polynomial of incubation time.
195 However, as DCFH values were best fit to a quadratic-shaped curve, a second-order orthogonal
196 polynomial of incubation was employed. Using the `lmList` function of the `nlme` statistical package

197 for R, values of sperm parameters were partitioned in four groups in total, conditional on two grouping
198 factors: *storage group* (unfrozen vs frozen-thawed) and *season* (winter vs summer). The above-
199 described growth curves were individually fit for each partition (winter/unfrozen, summer/unfrozen,
200 winter/unfrozen-frozen-thawed, summer/unfrozen-frozen-thawed) of sperm parameters. Using the F
201 statistic test, an analysis of variance table was computed to compare fit of the growth curves, with
202 and without grouping factors *season* and *storage group* (at 0.05 significance level). Model parameters
203 (b coefficients estimates \pm SEM, 95% CI of coefficients and parameter-specific P values) were
204 estimated using the normal approximation. Data processing and statistical analysis of the results were
205 done using *nlme* and *lattice* statistical packages, whereas graphical illustration of observed and
206 model-predicted sperm parameter values were done with *ggplot2* statistical packages in R version
207 3.1.3. Spearman's rank test was used to calculate correlations between sperm functional and ROS-
208 related parameters.

209

210 **3. Results**

211 Environmental parameters were as follows: winter temperature: 1.4°C mean, 7.4°C max; -
212 3.9°C min; summer temperature: 24.9°C mean; 32.9°C max; 16.8 C min. winter humidity (%): 73.5
213 mean; 94.8 max; 50.5 min; summer: 54.7 mean; 89.1 max; 24.5 min. Descriptive statistics (mean \pm SD)
214 of CASA and flow cytometrically assessed sperm parameters are presented related to the season at
215 Time 0 (Table 1 and Fig. 1 to 4) and during incubation (Tables 2 and 3 and Fig. 1 to 4). Values for
216 TM, PM and RAP changed over time similarly in unfrozen and frozen-thawed samples; therefore,
217 only RAP is presented. Spearman's correlations coefficients r_s between variables of both groups
218 (unfrozen and frozen-thawed semen) in each season are presented in Tables 4 and 5.

219 Lipid peroxidation, measured by BODIPY, was higher in winter than in summer, independent of
220 group (unfrozen/frozen-thawed) ($P < 0.001$; Fig. 1; Tables 1 to 3); within the same season, freezing
221 thawing cycle induced higher lipid peroxidation ($P < 0.001$; Fig. 1 and Tables 1 to 3).

222 Hydrogen peroxide production, measured by DCFH-DA, was higher in winter than in summer until
223 6 h (T2) of incubation ($P < 0.001$; Fig. 2 and Tables 1 to 3); within the same season, freezing thawing
224 induced higher production of hydrogen peroxide in all time points in winter and starting from 12 h
225 (T3) of incubation in summer ($P < 0.001$; Fig. 2 and Tables 1 to 3).

226 Mitochondrial superoxide anions production, assessed by MITOSOX, was higher in winter than in
227 summer only in frozen-thawed group, whereas in summer, unfrozen group was higher than winter at
228 Time 0 and during all time points of incubation ($P < 0.001$, Fig. 2 and Tables 1 to 3); within the same
229 season, mitochondrial superoxide anions production was higher from Time 0 in frozen-thawed
230 samples in winter, but higher in unfrozen sample from 12 h (T4) incubation in summer ($P < 0.001$;
231 Fig. 2 and Tables 1 to 3)

232 DFI% was higher in summer (25.61 ± 12 unfrozen; 27.11 ± 13 frozen-thawed) than in winter (11.39 ± 6
233 unfrozen; 12.93 ± 9 frozen-thawed) in both samples at Time 0 and until 6 h (T2) of incubation ($P <$
234 0.001 ; Fig. 1 and Tables 1 to 3). In addition, DFI% values did not differ between two treatments
235 (unfrozen vs frozen-thawed) in both seasons at Time 0, but was higher in frozen-thawed than unfrozen
236 group, starting from 3 h (T1) of incubation in winter and 12 h (T3) of incubation in summer ($P <$
237 0.001 ; Fig. 1 and Tables 1 to 3).

238 Sperm quality parameters (i.e RAP, PMAI, Mpos, Fneg) did not differ between seasons ($P > 0.05$,
239 Figs. 3 and 4 and Tables 1 to 3). However, these parameters were lower in frozen-thawed semen,
240 independent of season ($P < 0.01$, Figs. 3 and 4 and Tables 1 to 3).

241 In both seasons, there were moderate positive correlations between DFI% and MitoSOX, DCFH,
242 BODIPY in both groups. Furthermore, a negative correlation, stronger in winter, was evident between
243 sperm quality (RAP, PMAI, Mpos, Fneg) and BODIPY, DCFH, MitoSOX. There were strong
244 positive correlations between RAP, PMAI, Fneg and Mpos in both treatments and in both seasons
245 (Tables 4 and 5).

246

247 **4. Discussion**

248 There were significant seasonal changes in ROS-related parameters and chromatin integrity
249 in stallion sperm before and after freezing-thawing; therefore, our hypothesis was supported.
250 However, there were no detectable seasonal variations in sperm motility, plasma membrane or
251 acrosome integrity, intracellular calcium concentration nor mitochondrial membrane potential in
252 either unfrozen or frozen-thawed samples. Despite higher ROS concentrations and lipid peroxidation
253 detected in winter, chromatin integrity had significantly better overall values in both unfrozen and
254 frozen-thawed samples.

255 This was apparently the first report to definitively investigate seasonal fluctuations of oxidative status
256 of sperm related to freezing-thawing in stallion. Although seasonal variations in relation to quality of
257 fresh, cold-stored and cryopreserved semen have been reported [6,12,29], seasonal changes in sperm
258 oxidative status and its association with sperm quality and fertility were investigated only in fresh
259 semen in one study [9]. In that study, during the breeding season, fertile stallions had better sperm
260 quality and a higher level of oxidation in sperm proteins compared to sub-fertile stallions, suggesting
261 that measurements were within physiological ranges and/or that there was efficient antioxidant
262 activity in stallion semen (although sperm oxidative status was only partially analyzed). Similarly, in
263 our study, chromatin integrity was not compromised by higher ROS concentrations and lipid
264 peroxidation in winter.

265 In the present study, MitoSOX and DCFH were significantly higher in frozen samples compared to
266 unfrozen ones at Time 0 in winter, whereas in summer, this difference was detected at T4 (12 h) of
267 incubation (Fig. 2), indicating that ROS production was faster in winter frozen-thawed samples.
268 Despite this, chromatin integrity was not damaged by freezing-thawing at Time 0 in both seasons.
269 Interestingly, DFI% was significantly different between unfrozen and frozen-thawed groups at T1 (3
270 h) and T4 (12 h) of incubation in winter and summer respectively (Fig.1), indicating that the two
271 samples differed in their resistance during incubation, depending on season and ROS level. In that
272 regard, frozen-thawed sperm had highest levels of oxidation in winter, but only after incubation.
273 Unfrozen sperm in summer had significantly higher MitoSOX values than in winter at Time 0 and

274 than in frozen-thawed sperm, only starting from 12 h (T4) of incubation (Fig. 2). This was unexpected
275 and may have been due to seasonal fluctuations in antioxidants in seminal plasma components and
276 semen quality as reported in stallions [9,30] and other species. Another possible explanation could be
277 the positive correlation between levels of superoxide anions (detected by MitoSOX), sperm
278 metabolism and fertility reported in fresh stallion sperm [18] and in frozen bovine sperm [19]. During
279 the physiologic breeding season for horses, there are higher peripheral concentrations of LH and
280 testosterone [1]. Consequently, sperm may be more metabolically active and sperm mitochondria, not
281 damaged by freezing-thawing, might produce more superoxide anions than those that are frozen-
282 thawed.

283 In our study, membrane lipid peroxidation (BODIPY) was significantly higher in winter than in
284 summer in both groups (Fig.1). Seasonal effects on sperm lipid composition can compromise semen
285 quality in stallions [11] as plasma membrane PUFA (poly unsaturated fatty acids) are vulnerable to
286 ROS and peroxidative damage. Furthermore, high PUFA content in boar sperm has been associated
287 with improved membrane fluidity and with increased resistance of sperm to cooling and freezing, but
288 also with a high risk of oxidative damage [31]. Our findings were in agreement with Aurich et al [11],
289 who reported better, albeit not significant, motility and membrane integrity in winter, with the lowest
290 PUFA membrane content, which increased from the non-breeding (winter) into the breeding season
291 (summer). Similarly, our better sperm quality (albeit not significant) after thawing and higher lipid
292 peroxidation were also in agreement with Aurich et al, despite the lower PUFA content in winter
293 could be in contrast with higher BODIPY values registered in the same season in our study. Aurich
294 et al noted that positive correlations between the content of individual fatty acids and motility and
295 membrane integrity, although statistically significant for some comparisons, were relatively low and
296 that seasonal differences in sperm membrane fatty acids in part explained results in seasonal
297 differences in resistance of equine sperm to cryopreservation, and were associated with season-
298 dependent metabolic changes.

299 In the present study, cryopreservation, more than the season, influenced sperm quality, only partially
300 in agreement with previous studies. It was reported [6] that influence of factors on sperm quality had
301 the following rank order: cryopreservation > stallion > season. On the contrary, although there was
302 no difference in DFI% between unfrozen and frozen-thawed samples at Time 0, there was a
303 significant difference between seasons after 3 or 12 h of incubation at +37°C in winter and summer,
304 respectively. Irrespective of season, freezing-thawing cycle had the same deleterious effects on RAP,
305 PMAI, Fneg and Mpos. Furthermore, chromatin integrity was better in winter, in both samples.
306 Optimal time of the year for freezing stallion semen has been widely studied. Magistrini et al [5]
307 reported motility of frozen-thawed sperm was higher if the ejaculate was collected and frozen in
308 winter, although autumn was better than early spring or late winter [7,8] or March to June [29].
309 Notwithstanding, there is a general agreement that production of frozen semen is possible throughout
310 the year [6,13].
311 We detected strong positive relations between RAP, PMAI, Fneg and Mpos in both seasons (Tables
312 4,5), since mitochondrial function, ion exchange and cell signalling are related to development and
313 maintenance of sperm motility [32]. Moreover, they drive important sperm functions such as
314 hyperactivation, capacitation, acrosome reaction, and ultimately fertilization [33]. There were
315 interesting significant correlations between oxidative stress related parameters and DFI%, as well as
316 negative correlations between sperm quality parameters and oxidative stress related parameters
317 (Table 4,5), although sperm quality was not negatively influenced by ROS production, LPO and
318 season, except for DFI%.

319

320 **5. Conclusions**

321 In conclusion, our hypothesis was supported; ROS-related parameters were higher in winter than in
322 summer, without negative effects on sperm quality. However, ROS increase and lipid peroxidation
323 seemed to be less deleterious than other stresses (cooling/osmotic) to which sperm are exposed during
324 freezing-thawing. Moreover, due to higher DFI% in summer, quality of frozen-thawed sperm could

325 be higher when cryopreservation is done during winter, probably due to an absence of heat stress
326 during spermatogenesis. Incubation for 24 h with analysis at various times was useful for detecting
327 specific changes in both ROS related parameters and DFI%. Finally, although sperm quality
328 parameters differed slightly between seasons, with highest ROS in winter, we suggest choosing winter
329 as the best period for freezing stallion semen in the Northern hemisphere because DFI% was best in
330 this season.

331

332 **Declarations of interest**

333 None.

334

335 **Author contribution**

336 G.M. and H.B. were responsible for the main experimental concept and design; E.M. performed the
337 data analyses; B.M. and D.B. performed the experiments and wrote the manuscript.

338

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342

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345

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435 **Table 1.** Mean (\pm SD) functional and ROS-related parameters in unfrozen and frozen-thawed
 436 aliquots at *Time 0 (T0)* of 18 ejaculates collected from six stallions (three ejaculates per stallion) for
 437 each season.

438

Winter

Summer

	Unfrozen	Frozen-thawed	Unfrozen	Frozen-thawed
BODIPY (FU)	235.3 \pm 21.1 ^{a1}	394.3 \pm 25.9 ^{b1}	193.8 \pm 10.7 ^{a2}	281.0 \pm 51.5 ^{b2}
DCFH (FU)	202.6 \pm 54.1 ^a	289.2 \pm 20.3 ^{b1}	192.1 \pm 48.2	185.1 \pm 27.4 ²
MITOSOX (FU) x 10³	4.5 \pm 1.36 ^{a1}	12.3 \pm 2.02 ^{b1}	8.9 \pm 0.45 ²	10.0 \pm 2.17 ²
DFI%	11.3 \pm 6.01 ¹	12.9 \pm 8.8 ¹	25.6 \pm 12.3 ²	27.1 \pm 12.9 ²
PMAI (%)	66.7 \pm 14.2 ^a	40.9 \pm 7.7 ^b	58.2 \pm 10.6 ^a	32.5 \pm 8.4 ^b
RAP (%)	54.5 \pm 17.6 ^a	29.9 \pm 10.9 ^b	48.2 \pm 16.8 ^a	29.2 \pm 9.9 ^b
Fneg (%)	40.6 \pm 11.3 ^a	12.9 \pm 4.2 ^b	45.4 \pm 8.8 ^a	19.7 \pm 5.2 ^b
Mpos (%)	73.2 \pm 6.7 ^a	43.5 \pm 8.5 ^b	65.5 \pm 12.6 ^a	37.0 \pm 9.6 ^b

439

440 BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular level of H₂O₂; MitoSOX,
 441 intracellular level of O₂⁻; DFI%, sperm with high DNA fragmentation index; PMAI, sperm with intact
 442 plasma membrane and acrosome; RAP, rapid motility; F_{neg}, sperm with low intracellular calcium level; M_{pos},
 443 sperm with high mitochondrial membrane potential; FU, fluorescence intensity.

444 ^{a,b}Within a season, means without a common superscript indicate a difference between unfrozen versus
 445 frozen-thawed semen (P < 0.001).

446 ^{1,2}Within a treatment, means without a common superscript indicate a difference between seasons (P <
 447 0.001).

448

Table 2. Mean values (SD) of functional and ROS-related parameters in unfrozen and frozen-thawed aliquots of 18 ejaculates collected in *Winter* from six stallions (three ejaculates per stallion), during a 24-h incubation at +37°C.

BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular level of H₂O₂; MitoSOX, intracellular level of O₂⁻; DFI%, sperm with high DNA fragmentation index;

Sperm parameter	Unfrozen (h)					Frozen-thawed (h)				
	0	3	6	12	24	0	3	6	12	24
BODIPY (FU)	235.3 (21.1)	267.2 (23.9)	294.1 (28.7)	311.3 (27.0)	344.4 (27.4)	394.3 (25.9)	445.5 (38.6)	487.1 (25.4)	518.7 (32.4)	558.2 (50.1)
DCFH (FU)	202.6 (54.1)	220.0 (43.9)	250.1 (33.3)	266.5 (33.4)	288.6 (37.6)	289.2 (20.3)	303.7 (16.4)	335.0 (22.0)	351.2 (39.0)	325.3 (16.4)
MitoSOX (FU) x 10 ³	4.5 (1.36)	8.9 (3.00)	1.4 (3.72)	1.7 (2.47)	1.9 (3.38)	1.2 (2.01)	1.8 (2.22)	19.4 (2.64)	20.6 (2.83)	23.0 (2.17)
DFI% (%)	11.3 (6.0)	13.4 (6.6)	16.7 (10.2)	26.3 (12.4)	39.9 (15.6)	12.9 (8.8)	24.6 (13.1)	31.7 (15.7)	54.0 (14.0)	66.2 (11.3)
PMAI (%)	66.7 (14.2)	23.9 (10.5)	11.9 (7.4)	3.4 (3.0)	1.7 (0.5)	40.9 (7.7)	6.5 (6.3)	2.4 (3.6)	0.4 (0.6)	0.06 (0.07)
RAP (%)	54.5 (17.6)	23.9 (20.1)	6.2 (10.9)	0.0 (0.0)	0.0 (0.0)	29.9 (10.9)	1.4 (0.9)	0.9 (2.0)	0.1 (0.4)	0.0 (0.1)
F_{neg} (%)	40.6 (11.3)	24.9 (13.9)	10.7 (11.8)	1.9 (3.2)	0.3 (0.3)	12.9 (4.21)	5.2 (4.1)	1.9 (2.2)	0.4 (0.4)	0.2 (0.0)
M_{pos} (%)	73.2 (6.7)	44.8 (18.9)	20.4 (16.9)	4.8 (5.2)	2.4 (0.8)	43.5 (8.5)	10.0 (8.2)	4.2 (4.4)	1.6 (1.0)	1.5 (0.8)

PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; F_{neg}, sperm with low intracellular calcium level; M_{pos}, sperm with high mitochondrial membrane potential; FU, fluorescence intensity

Table 3. Mean values (SD) of functional and ROS-related parameters in unfrozen and frozen-thawed aliquots of 18 ejaculates collected in *Summer* from six stallions (three ejaculates per stallion), during a 24-h incubation at +37°C.

BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular level of H₂O₂; MitoSOX, intracellular level of O₂⁻; DFI%, sperm with high DNA fragmentation index;

Sperm parameter	Unfrozen (h)					Frozen-thawed (h)				
	0	3	6	12	24	0	3	6	12	24
BODIPY (FU)	193.8 (10.7)	207.1 (11.4)	222.6 (10.6)	240.1 (23.7)	268.1 (17.7)	281.0 (51.5)	303.2 (41.6)	321.1 (41.3)	344.1 (32.5)	472.2 (98.1)
DCFH (FU)	192.1 (48.2)	214.3 (52.7)	226.5 (49.4)	239.0 (48.1)	260.0 (44.6)	185.1 (27.4)	206.1 (27.6)	248.2 (47.3)	340.6 (36.2)	379.2 (37.9)
MitoSOX (FU) x 10 ³	8.9 (0.45)	13.2 (6.78)	18.5 (8.07)	23.1 (7.12)	26.6 (6.4)	9.9 (2.16)	14.2 (2.15)	16.2 (1.33)	17.5 (1.32)	19.6 (2.2)
DFI% (%)	25.6 (12.3)	31.3 (13.1)	36.5 (15.5)	43.4 (15.6)	49.8 (14.1)	27.1 (12.9)	38.2 (15.5)	43.7 (16.2)	61.7 (16.1)	70.8 (12.9)
PMAI (%)	58.2 (10.6)	27.1 (17.0)	15.2 (14.1)	5.1 (3.6)	1.8 (1.7)	32.5 (8.4)	10.5 (7.7)	6.7 (6.3)	3.6 (3.8)	2.3 (2.6)
RAP (%)	48.2 (16.8)	17.0 (18.0)	2.8 (5.2)	0.0 (0.0)	0.0 (0.0)	29.2 (9.9)	1.4 (0.9)	0.9 (2.0)	0.0 (0.1)	0.0 (0.0)
F_{neg} (%)	45.4 (8.8)	26.4 (16.3)	15.2 (16.0)	3.7 (5.5)	0.5 (0.7)	19.7 (5.2)	0.3 (0.6)	0.0 (0.0)	1.2 (1.9)	0.7 (0.8)
M_{pos} (%)	65.5 (12.6)	41.3 (18.8)	21.5 (17.9)	4.9 (7.2)	0.9 (1.6)	37.0 (9.6)	11.8 (10.1)	6.6 (7.6)	2.3 (3.4)	0.7 (0.8)

PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; F_{neg}, sperm with low intracellular calcium level; M_{pos}, sperm with high mitochondrial membrane potential; FU, fluorescence intensity

Table 4. Spearman's correlation coefficients (r_s) between the functional and ROS-related parameters of unfrozen and frozen-thawed aliquots of 18 equine ejaculates (*Winter*). Sperm samples of both groups (unfrozen, frozen-thawed) were incubated at +37 °C for 24h and sperm parameters assessed at 0, 3, 6, 12 and 24 h.

	PMAI	M_{pos}	F_{neg}	BODIPY	DCFH	MitoSOX	DFI%
Unfrozen							
M _{pos}	0.95*						
F _{neg}	0.89*	0.93*					
BODIPY	-0.77*	-0.73*	-0.67*				
DCFH	-0.47*	-0.52*	-0.65*	0.24*			
MitoSOX	-0.86*	-0.89*	-0.92*	0.69*	0.64*		
DFI%	-0.69*	-0.69*	-0.69*	0.66*	0.34*	0.66*	
RAP	0.89*	0.90*	0.88*	-0.71*	-0.61*	-0.86*	-0.68*
Frozen-thawed							
M _{pos}	0.89*						
F _{neg}	0.94*	0.91*					
BODIPY	-0.79*	-0.69*	-0.78*				
DCFH	-0.60*	-0.60*	-0.61*	0.66*			
MitoSOX	-0.70*	-0.57*	-0.67*	0.76*	0.37*		
DFI%	-0.73*	-0.68*	-0.72*	0.70*	0.43*	0.61*	
RAP	0.84*	0.80*	0.81*	-0.74*	-0.63*	-0.67*	-0.71*

BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular H₂O₂ concentration; MitoSOX, intracellular O₂⁻ concentration; DFI%, sperm with high DNA fragmentation index; PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; F_{neg}, sperm with low intracellular calcium concentration; M_{pos}, sperm with high mitochondrial membrane potential.

*P < 0.01

Table 5 Spearman's correlation coefficients (r_s) between the functional and ROS-related parameters of unfrozen and frozen-thawed aliquots of 18 equine ejaculates (*Summer*). Sperm samples of both groups (unfrozen, frozen-thawed) were incubated at +37 °C for 24h and sperm parameters assessed at 0, 3, 6, 12 and 24h.

	PMAI	M_{pos}	F_{neg}	BODIPY	DCFH	MitoSOX	DFI%
Unfrozen							
M _{pos}	0.97*						
F _{neg}	0.94*	0.94*					
BODIPY	-0.64*	-0.68*	-0.58*				
DCFH	-0.41*	-0.37*	-0.54*	0.18			
MitoSOX	-0.72*	-0.75*	-0.78*	0.57*	0.54*		
DFI%	-0.46*	-0.52*	-0.50*	0.33*	0.18	0.28	
RAP	0.91*	0.88*	0.87*	-0.53*	-0.42*	-0.65*	-0.47*
Frozen-thawed							
M _{pos}	0.98*						
F _{neg}	0.83*	0.83*					
BODIPY	-0.47*	-0.50*	-0.37*				
DCFH	-0.59*	-0.60*	-0.45*	0.57*			
MitoSOX	-0.77*	-0.78*	-0.74*	0.58*	0.65*		
DFI%	-0.57*	-0.65*	-0.49*	0.44*	0.64*	0.66*	
RAP	0.89*	0.90*	0.93*	-0.41*	-0.49*	-0.78*	-0.57*

BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular H₂O₂ concentration; MitoSOX, intracellular O₂⁻ concentration; DFI%, sperm with high DNA fragmentation index; PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; F_{neg}, sperm with low intracellular calcium concentration; M_{pos}, sperm with high mitochondrial membrane potential.

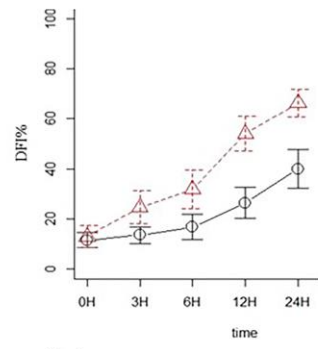
*P < 0.01

Fig. 1. Descriptive statistics of observed and model-predicted values of DFI and BODIPY. DFI% (sperm with high DNA fragmentation index); BODIPY (sperm with membrane lipid peroxidation) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed line) and two seasons (winter vs summer). Points and error bars represent mean values and 95% CI (confidence interval) of observed data, whereas lines reflect the incubation curve of model-predicted data. Parameters were determined for 36 ejaculates collected from six stallions in total. FU (fluorescence intensity).

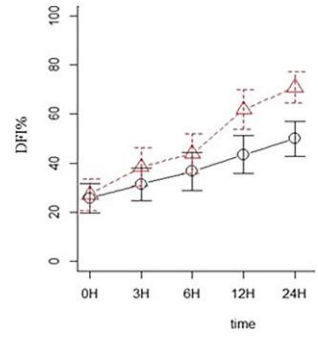
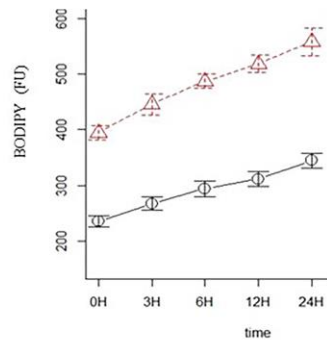
Fig. 2. Descriptive statistics of observed and model-predicted values of DCFH and MitoSOX. DCFH, (intracellular level of H_2O_2); MitoSOX, (intracellular level of O_2^-) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed line) and two seasons (winter vs summer). Points and error bars represent mean values and 95% CI (confidence interval) of observed data, whereas lines reflect the incubation curve of model-predicted data. Parameters were determined for 36 ejaculates collected from six stallions in total. FU (fluorescence intensity).

Fig. 3. Descriptive statistics of observed and model-predicted values of PMAI and RAP. PMAI (sperm with intact plasma membrane and acrosome); RAP (rapid motility) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed line) and two seasons (winter vs summer). Points and error bars represent mean values and 95% CI (confidence interval) of observed data, whereas lines reflect the incubation curve of model-predicted data. Parameters were determined for 36 ejaculates collected from six stallions in total. FU (fluorescence intensity).

Fig. 4. Descriptive statistics of observed and model-predicted values of Fneg and Mpos. Fneg (sperm with low intracellular calcium level); Mpos (sperm with high mitochondrial membrane potential) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed line) and two seasons (winter vs summer). Points and error bars represent mean values and 95% CI (confidence interval) of observed data, whereas lines reflect the incubation curve of model-predicted data. Parameters were determined for 36 ejaculates collected from six stallions in total. FU (fluorescence intensity).



Winter



Summer

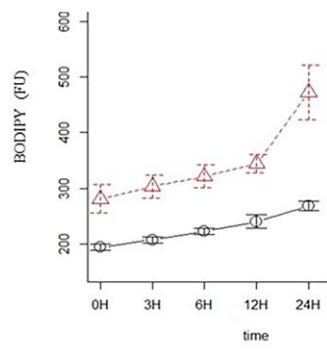


Fig1

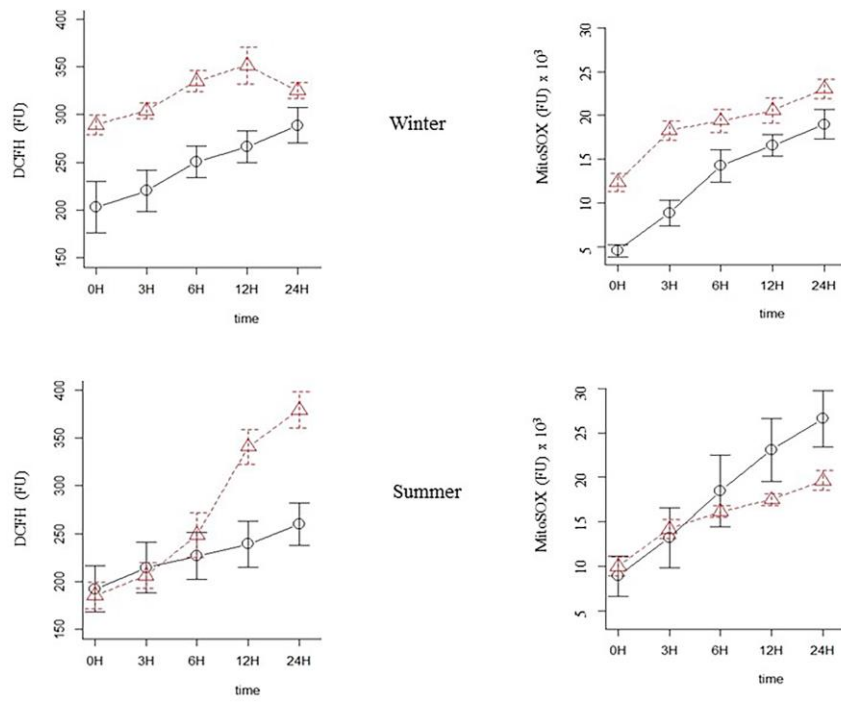


Fig 2

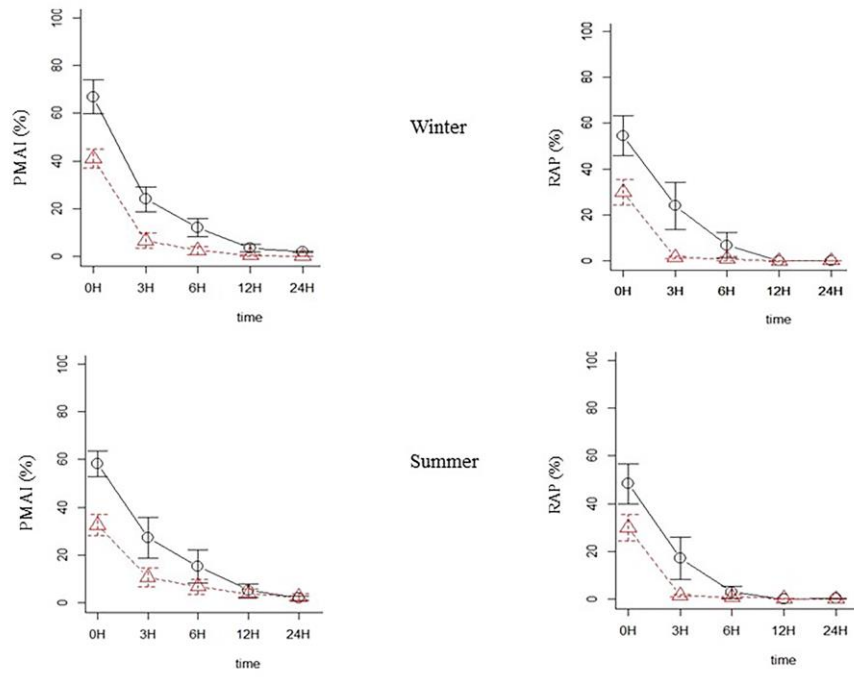


Fig 3

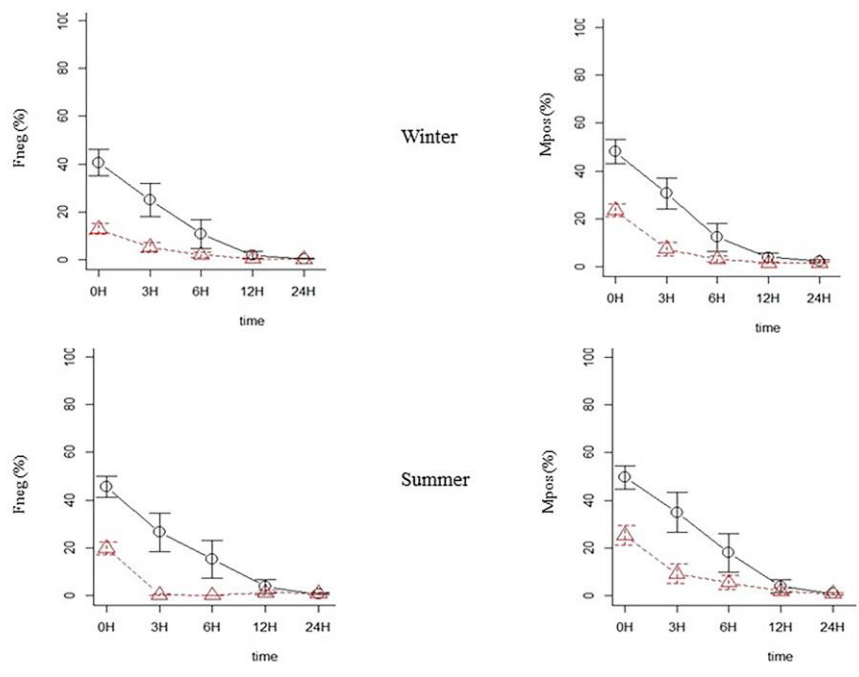


Fig 4