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

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Abstract

Oxidative stress is regarded as an important cause of sperm damage during cryopreservation. However, seasonal changes in oxidative status in unfrozen and frozen-thawed stallion sperm have not been well established. We tested the hypothesis that sperm ROS concentrations and lipid peroxidation change between breeding and non-breeding seasons and influence quality of unfrozen and frozen-thawed sperm. Eighteen ejaculates from six Warmblood stallions (8 to 21 y) known to be fertile, were collected in winter and summer and processed for freezing. After 90 min at +4°C, some straws from each ejaculate were not frozen (unfrozen), whereas the remainder were frozen by N_2 vapors and plunged in N_2 (frozen). Rapid cells (RAP; determined by CASA), plasma membrane-acrosome integrity (PMAI), high mitochondrial membrane potential (Mpos), low intracellular Ca^{2+} concentration (Fneg), membrane lipid peroxidation (BODIPY), intracellular ROS concentrations (DCFH, MitoSOX) and chromatin fragmentation (DFI%) were evaluated by flow cytometry in both groups and at intervals during incubation at +37°C for 24 h. Overall, ROS concentrations and lipid peroxidation were higher and faster ($P < 0.0001$) in winter versus summer, DFI% was lower in winter versus summer ($P < 0.0001$), but similar between the two groups within season. There were moderate positive correlations in both seasons between DFI% and MitoSOX, DCFH, BODIPY in both groups, whereas a negative correlation, stronger in winter, was evident between sperm quality (RAP, PMAI, Mpos, Fneg) and BODIPY, DCFH, MitoSOX. There were no differences between

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Chemicals

Chemicals were obtained from Sigma-Aldrich Co., Steinheim, Germany (propidium iodide, FITC/PNA, DCFH), Thermo Fisher Scientific, Inchinnan Business Park, United Kingdom (Fluo4AM, Mitoprobe, BODIPY $^{581\text{-}591}$, SYTOX, MitoSOX) and Polysciences Europe GmbH, Eppelheim, Germany, (Acridine Orange).

2.2. Stallions and experimental design

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

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

2.3. Semen collection and freezing

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

2.4. Semen analyses

2.4.1. Motility

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

USA), using the following settings: recording rate at 60 frames/s, minimum contrast of 70 pixels, minimum cell size of 10 unit: µm², slow cells velocity (VSL) threshold of 30 µm/s, slow cell threshold of 20 µm/s, minimum average path velocity (VAP) >30 µm/s and threshold straightness (STR) of 80% for progressive cells. Sperm with VAP ≥30 µm/s were classified as rapid cells. A minimum of 1000 cells were analyzed in at least eight randomly selected fields. All end point values changed over time similarly in unfrozen and frozen samples; therefore, only RAP is presented.

2.4.2. Flow cytometric analysis

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

2.4.2.1. Membrane lipid peroxidation

This lipid-based fluorophore readily integrates into biological membranes and reacts to free radical attack with a spectral emission shift from red to green that can be quantified with flow cytometry. Red fluorescence represents overall incorporation of non-oxidized probe into the cell, whereas green fluorescence represents oxidization of membrane-incorporated probe. To quantify lipid peroxidation of plasma membrane intact sperm (BODIPY), Bodipy $^{581\text{-}591}$ C11 (5 mM) and PI (2.99 mM) were added to 246 µL of diluted sperm suspension.

2.4.2.2. Intracellular ROS concentrations: H_2O_2 and O_2^-

DCFH-DA is a non-fluorescent agent, converted by H_2O_2 into DCFH, which has a green fluorescence [26]. To conduct this assessment, DCFH-DA (10 µM) and PI (2.99 µM) were added to 246 µL of diluted semen. The percentage plasma membrane intact sperm with intracellular hydrogen peroxide (H_2O_2) was gated (DCFH). Intracellular generation of superoxide radicals (O_2^-) was estimated using MitoSOX Red, a lipid- soluble, cell-permeable cation that selectively targets mitochondrial matrix [27]. The SYTOX Green stain only penetrates sperm with damaged plasma membranes and fluoresces green on binding DNA. For this assay, MitoSOX Red (2 µM) and SYTOX Green (0.05 µM) were added to 246 µL of diluted sperm suspension. The percentage of plasma membrane intact sperm with intracellular superoxide radicals (O_2^-) was gated (MitoSOX).

2.4.2.3. Sperm Chromatin Structure Assay (SCSA™)

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

2.4.2.4. Plasma membrane and acrosome integrity

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

2.4.2.5. Intracellular Ca^{+2} concentrations and mitochondrial membrane potential

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

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

2.5. Environmental factors

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

2.6. Statistical analysis

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

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

3. Results

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

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

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

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

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

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

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

4. Discussion

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

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

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

Unfrozen sperm in summer had significantly higher MitoSOX values than in winter at Time 0 and

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

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

In the present study, cryopreservation, more than the season, influenced sperm quality, only partially in agreement with previous studies. It was reported [6] that influence of factors on sperm quality had the following rank order: cryopreservation > stallion > season. On the contrary, although there was no difference in DFI% between unfrozen and frozen-thawed samples at Time 0, there was a significant difference between seasons after 3 or 12 h of incubation at +37°C in winter and summer, respectively. Irrespective of season, freezing-thawing cycle had the same deleterious effects on RAP, PMAI, Fneg and Mpos. Furthermore, chromatin integrity was better in winter, in both samples.

Optimal time of the year for freezing stallion semen has been widely studied. Magistrini et al [5] reported motility of frozen-thawed sperm was higher if the ejaculate was collected and frozen in winter, although autumn was better than early spring or late winter [7,8] or March to June [29]. Notwithstanding, there is a general agreement that production of frozen semen is possible throughout the year [6,13].

We detected strong positive relations between RAP, PMAI, Fneg and Mpos in both seasons (Tables 4,5), since mitochondrial function, ion exchange and cell signalling are related to development and maintenance of sperm motility [32]. Moreover, they drive important sperm functions such as hyperactivation, capacitation, acrosome reaction, and ultimately fertilization [33]. There were interesting significant correlations between oxidative stress related parameters and DFI%, as well as negative correlations between sperm quality parameters and oxidative stress related parameters (Table 4,5), although sperm quality was not negatively influenced by ROS production, LPO and season, except for DFI%.

5. Conclusions

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

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

Declarations of interest

None.

Author contribution

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

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

	Winter		Summer	
	Unfrozen	Frozen-thawed	Unfrozen	Frozen-thawed
BODIPY (FU)	235.3±21.1[a1]	394.3±25.9[b1]	193.8±10.7[a2]	281.0±51.5[b2]
DCFH (FU)	202.6±54.1[a]	289.2±20.3[b1]	192.1±48.2	185.1±27.4[2]
MITOSOX (FU) x 10^3	4.5±1.36[a1]	12.3±2.02[b1]	8.9±0.45[2]	10.0±2.17[2]
DFI%	11.3±6.01[1]	12.9±8.8 [1]	25.6±12.3[2]	27.1±12.9[2]
PMAI (%)	66.7±14.2[a]	40.9±7.7[b]	58.2±10.6[a]	32.5±8.4[b]
RAP (%)	54.5±17.6[a]	29,9±10.9[b]	48.2±16.8[a]	29.2±9.9[b]
Fneg (%)	40.6±11.3[a]	12.9±4.2[b]	45.4±8.8[a]	19.7±5.2[b]
Mpos (%)	73.2±6.7[a]	43.5±8.5[b]	65.5±12.6[a]	37.0±9.6[b]

BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular level of H_2O_2; MitoSOX, intracellular level of O_2^-; 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 level; M_{pos}, sperm with high mitochondrial membrane potential; FU, fluorescence intensity.

[a,b]Within a season, means without a common superscript indicate a difference between unfrozen versus frozen-thawed semen ($P < 0.001$).

[1,2]Within a treatment, means without a common superscript indicate a difference between seasons ($P < 0.001$).

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 H_2O_2; MitoSOX, intracellular level of O_2^-; 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)
$\mathbf{F_{neg}}$ (%)	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)
$\mathbf{M_{pos}}$ (%)	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 H_2O_2; MitoSOX, intracellular level of O_2^-; 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 H_2O_2 concentration; MitoSOX, intracellular O_2^- 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	-064*	-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 H_2O_2 concentration; MitoSOX, intracellular O_2^- 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.

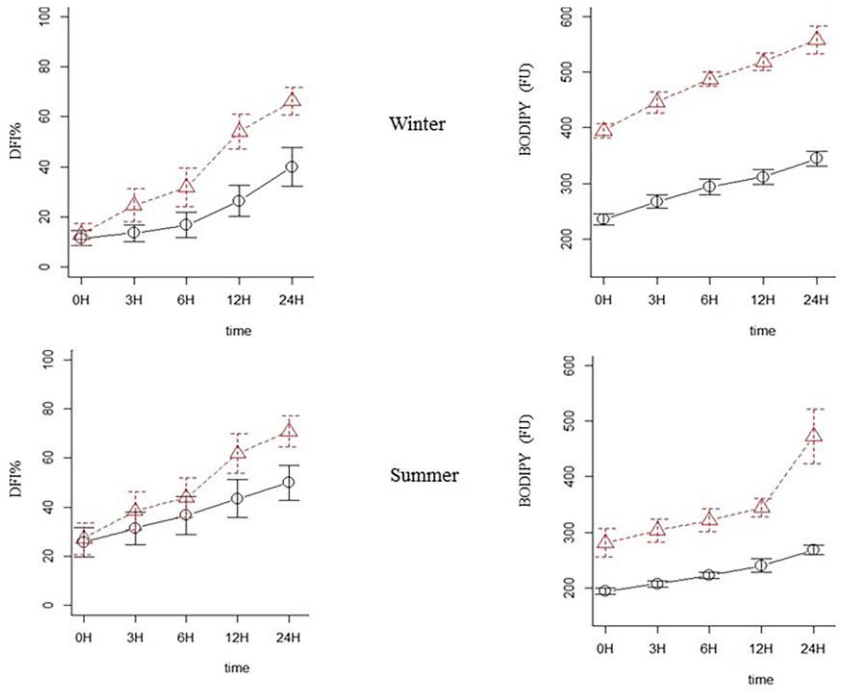
*$P < 0.01$

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 O2-) 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).


DFI%
0
20
40
60
80
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3H
6H
12H
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time
Winter
BODIPY (FU)
200
300
400
500
600
0H
3H
6H
12H
24H
time
DFI%
0
20
40
60
80
100
0H
3H
6H
12H
24H
time
Summer
BODIPY (FU)
200
300
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3H
6H
12H
24H
time

Fig1

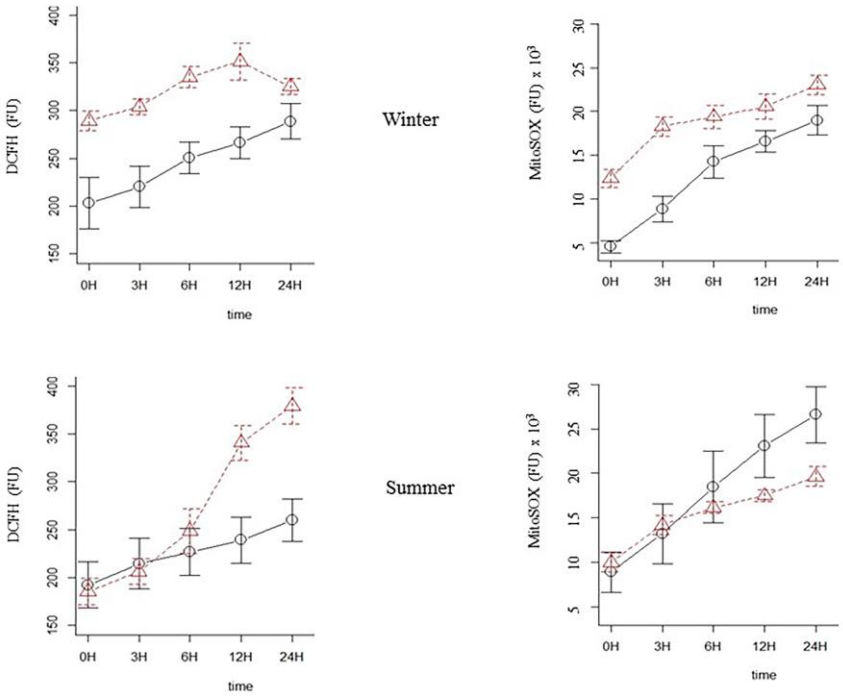

Fig 2

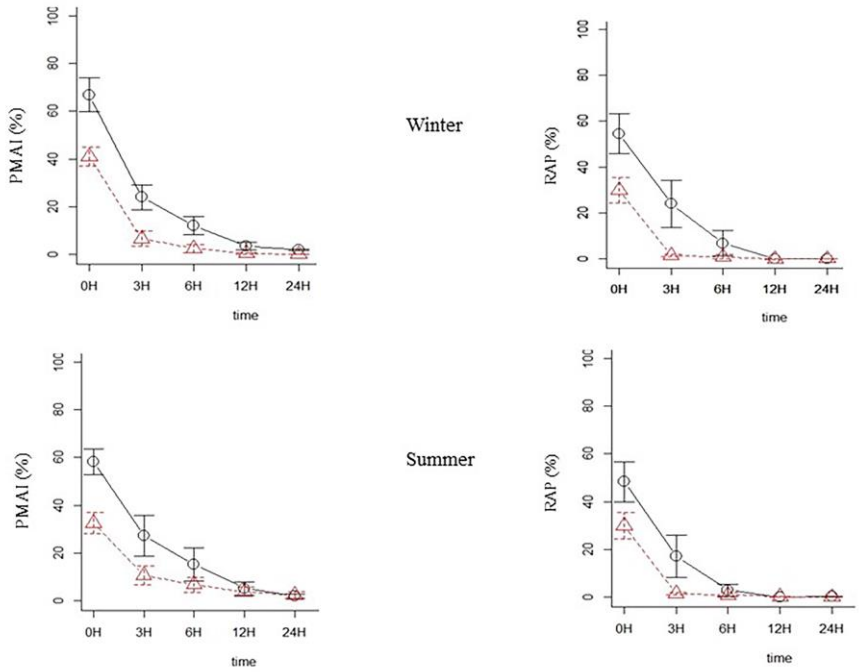

Fig 3

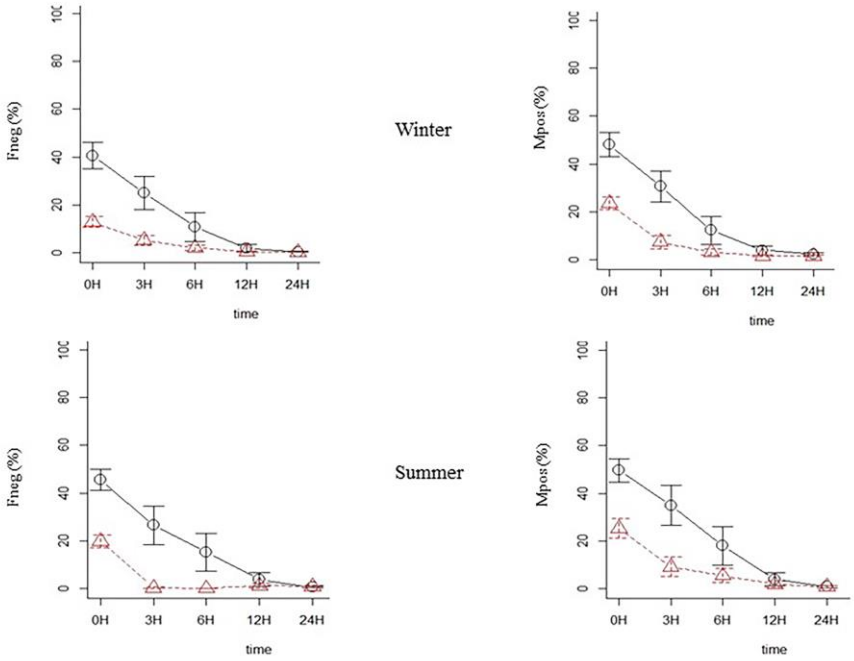

Fig 4