Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXXII

**Settore Concorsuale:** 07/H5 – Clinica chirurgica e ostetrica veterinaria

**Settore Scientifico Disciplinare:** VET/10 – Clinica ostetrica e ginecologia veterinaria

# *EFFECT OF SEASON AND CRYOPRESERVATION ON OXIDATIVE STATUS OF STALLION SPERMATOZOA*

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**Esame finale anno 2020**

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# ABSTRACT

Stallion semen cryopreservation includes several steps that can separately affect semen parameters, but it is still unclear which step may increase the risk for oxidative stress, which is regarded as an important cause of sperm damage during cryopreservation. Freezing and thawing cycle represents the two major steps in sperm cryopreservation procedure. Moreover, changing in sperm quality during non breeding and breeding season have been well established before, but seasonal changes in equine sperm oxidative status in relation to semen quality and cryopreservation are not well characterized.

For these reasons, the aim of the study was to investigate if different concentrations of ROS and lipid peroxidation could be responsible of decreased sperm characteristics, comparing pre-freezing and post-thawing samples. Moreover we tested the hypothesis that these sperm ROS-related parameters 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 (January/non breeding seasons) and summer (July/ breeding season) (N = 36) and processed for freezing. After 90 min at +4°C, some straws from each ejaculate were not frozen (unfrozen group), whereas the remainder were frozen by $N_2$ vapors and plunged in $N_2$ (frozen-thawed group).

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 during incubation at +37°C at 0, 3, 6, 12 and 24h in both seasons. 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.

In the first study, conducted in winter, ROS concentrations and lipid peroxidation were higher and faster ($P < 0.0001$) in frozen-thawed than unfrozen; DFI% was similar between two treatments at 0h (11.39%±6.00% unfrozen and 12.93%±8.90% frozen; $P > 0.05$) but higher in frozen-thawed one starting from 3h ($P < 0.0001$) of incubation. Sperm quality (RAP, PMAI, Mpos and Fneg) were lower in frozen thawed compared to unfrozen group at all time points ($P < 0.0001$).

In the second study, conducted in summer, data were compared with winter results. Overall, ROS concentrations and lipid peroxidation were higher and faster ($P < 0.001$) in winter versus summer, DFI% was lower in winter versus summer ($P < 0.001$), but similar between the two groups within season immediately after thawing (0h). Differences between two treatments were found only after incubation at 3h and 12h for DFI% and for DCFH and MitoSOX at 0h and 12h in winter and summer respectively. There were no differences between seasons for RAP, PMAI, Mpos and Fneg.

ABSTRACT

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.

Therefore, according to our results, we can conclude that:

- In both studies, we showed a similar resistance to DNA damage in pre-freezing and post-thawing samples, despite a significant higher ROS-related parameters in winter, suggesting that only incubation was useful to asses sperm resistance in particular for chromatin integrity.
- ROS-related parameters (DCFH, MitoSOX, BODIPY) higher in winter than in summer, did not have a negative effect on sperm quality.
- Increased ROS concentrations were less deleterious to sperm than freezing-thawing. Furthermore, incubation at +37°C and sequential analysis should be included in the laboratory test when evaluating stallion frozen semen.

# ABBREVIATIONS

ATP (adenosine triphosphate)

BODIPY (sperm with membrane lipid peroxidation)

$Ca^{2+}$ (Calcium ion)

CPAs (cryoprotectans)

DCFH (intracellular level of $H_2O_2$, hydrogen peroxide)

DFI% (DNA fragmentation index)

Fneg (% sperm with low intracellular $Ca^{2+}$ level)

FU (fluorescence intensity)

$H_2O_2$ (hydrogen peroxide)

LPO (membrane lipid peroxidation)

MitoSOX (intracellular level of $O_2$, superoxide anion)

Mpos+ (% sperm with high mitochondrial membrane potential)

$O_2^{-\bullet}$ (superoxide anion)

OXPHOS (oxidative phosphorylation)

OS (oxidative stress)

PMAI (% sperm with plasma membrane and acrosome integrity)

RAP (% rapid sperm)

ROS (reactive oxygen species)

# INTRODUCTION

## Frozen semen in the equine industry

Currently cryopreservation is the only method for long term storage of spermatozoa, and this technology has both advantages and disadvantages. Main advantages are that it preserves the genetic material for unlimited time, and illness, injury or death of a stallion do not prevent insemination of mares with his semen, that eliminates geographic barriers making easier international trade and finally it minimizes the spread of diseases reducing mares transportations. Despite of this, the use of frozen semen is still limited because of a lower pregnancy rate compared with fresh or cooled semen, a remarkable individual variation in stallion semen freezability, and high costs for freezing and breeding management of mares (Aurich, 2012).

Horses are selected for breeding on the basis of pedigree and athletic performance as opposed to reproductive traits, and for that reason they are not subjected to selection for fertility. This has resulted in equine populations with significantly lower per-cycle conception rates than other species (Nath et al., 2010).

Artificial insemination in equine breeding industry is commonly used employing fresh, cooled and frozen-thawed semen (Aurich and Aurich, 2006).

The remarkable improvement of the cryopreservation technologies in the last few years increased the use of frozen semen, and even if standards for processing and using equine frozen semen are not clearly defined (Loomis, 2001; Samper, 1998), centralized processing of frozen semen (Loomis 2001) by specialized laboratories has resulted in a reduction of the variability in semen quality compared to cooled semen, safe and continuous availability of semen and less need for coordination with the production center.

## Evaluation of sperm function and in vitro fertility prognosis

Predicting fertility, or at least identifying subfertile stallions before the breeding season, is extremely important for the equine industry (Barrier Battut et al ., 2016).

Cryopreservation alters the physical as well as the chemical membrane architecture of cells by inducing changes and damages at different membrane and intracellular levels (James et al., 1999).

Sperm quality is typically evaluated on the basis of number, motility (total, progressive and velocity) and morphology, but these parameters, more frequently than in fresh or cooled semen, are not highly correlated with the fertilizing potential of frozen-thawed semen (Samper et al., 1991). With the

establishment of flow cytometry in spermatology, new sperm parameters could be identified and correlated to fertility (Barrier Battut et al., 2016, 2017; Love and Kenney, 1998).

Flow cytometry allows allows the rapid and objective evaluation of multiple attributes of thousands of spermatozoa in < 1 min including sperm viability, acrosomal status (Farlin et al., 1992; Graham et al., 2005), mitochondrial membrane potential (Garner et al., 2005) and chromatin structure (Evenson et al., 1980).

Other parameters can be evaluated, including intracellular calcium level (Collin et al., 2000). lipid peroxidation (Ortega Ferrusola et al., 2009), oxidative status (Aitken et al., 2013, 2016).

Regulation of calcium plays an important role in sperm function, capacitation and acrosome reaction. Calcium is a major mediator of $Ca^{2+}$ ATPase pumps, which are needed to initiate motility, hyperactivation, capacitation, acrosome reaction, and therefore fertilization in vivo (Collin et al., 2000), maintaining low ion concentration therefore allowing the correct signaling by calcium influxes.

Cryopreservation induces premature capacitation accompanied by an increase in intracellular $Ca^{2+}$ level, negatively correlated with fertility immediately after thawing in bull (Collin et al., 2000).

In stallion oxidative phosphorylation (OXPHOS) of mitochondrial spermatozoa is the main source of ATP (adenosine triphosphate), which provides the energy for supporting the key functions (Ferrusola et al., 2010; Gibb et al., 2014).

Mitochondrial functionality is especially important in stallion sperm because in that mitochondrial respiration is the most efficient metabolic process for ATP synthesis (du Plessis et al., 2015). Cryopreservation induces osmotic stress with mitochondrial swelling, resulting in the release of pro-apoptotic factors and in an increase of superoxide leakage; release of ROS (reactive oxygen species) exhaust antioxidant defences leading to OS (oxidative stress) (Peña et al., 2015). Mitochondria are more prone to damage induced by osmotic excursions in sperm plasma membrane. (García et al., 2012). These damages on mitochondria lead to loss of sperm motility via alterations in mitochondrial function through ATP depletion, since high mitochondrial membrane potential is required for mitochondrial ATP production and sperm motility.(De Lamirande and Gagnon, 1992).

Excessive production of ROS attacks membrane poly unsaturated fatty acids (PUFAs) thus determining a cascade of chemical reactions called lipid peroxidation (LPO), an autocatalytic self-propagating reaction where almost 60% of fatty acids is lost from the membrane, affecting its function by decreasing its fluidity and increasing non-specific permeability to ions (Makker et al., 2009; Tremellen, 2012).

In stallion, sperm midpiece, where mitochondria are located, is particulary sensitive to LPO (Neild et al., 2005). For this reason, LPO causes a rapid loss of intracellular ATP, axonemal damage, decreases

sperm viability, and increases midpiece sperm morphological defects, with deleterious effects on sperm capacitation, acrosome reaction and fertilizing ability (Sikka, 1996).

The relationship between motility loss and OS was demonstrated in independent studies (Aitken, 1989; Gomez et al., 1998; De Lamirande et al., 1997) and is mainly due to the LPO (Aitken, 1989; Suleiman et al., 1996) and to depletion and reduction of ATP synthesis from damaged mitochondria (du Plessis et al., 2015).

Finally, cryopreservation promotes DNA fragmentation (Lòpez-Fernàndez C. et al., 2007) and his relationship with ROS has been evaluated in equine spermatozoa (Baumber et al., 2003; Yeste et al., 2015), as in other species (Cortés-Gutiérrez et al., 2008; Gürler et al., 2016; Koderle et al., 2009; López-Fernández et al., 2008).

It has been reported that chromatin in the sperm nucleus is vulnerable to oxidative damage, leading to base modifications and DNA fragmentation (Zribi et al., 2011).

For these reasons, knowing spermatozoa oxidative status is of enormous importance and, although extensively investigated, the role of ROS in relation to male fertility is not clearly understood yet (Guthrie and Welch, 2012).

In addition, ROS levels and their role in sperm function are different in different species and more important, in fresh and cryopreserved semen as shown in human (Marques et al., 2014), bovine (Gürler et al., 2016) and stallion semen (Gibb et al., 2014).

## Seasonal changes in sperm quality

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. In stallions, increasing the daylight period results in an increase in LH and testosterone release (Gerlach and Aurich, 2000).

In the Northern hemisphere, the physiological breeding season lasts from April to October but the stallion remains fertile throughout the year.

The endocrine stimulation of testicular function is reduced in winter (Clay and Squires, 1988) and gonadotrophin, testosterone and estradiol release, testicular size, sperm production, and sexual behavior are clearly reduced outside the breeding season and increased during breeding season (Aurich et al., 1994), as the efficacy of spermatogenesis. Moreover, the efficacy of spermatogenesis changes. With a period of 58 days for spermatogenesis and 10 days for epididymal sperm maturation, spermatozoa collected in late winter and early spring have started their life at the time of lowest endocrine stimulation of reproductive function, that is, around the winter solstice.(Aurich, 2016).

Nevertheless, stallions remain fertile throughout the year, albeit with variations between non-breeding and breeding seasons in endocrine profiles (Aurich et al., 1994; Clay and Squires, 1988), scrotal thermoregulation, heat dissipation (Freidman et al., 1991) and characteristics of fresh and frozen semen, including sperm motility (Blottner et al., 2001; Magistrini et al., 1987), morphology (Blottner et al., 2001), concentration and volume (Janett, Thun, Bettschen, et al., 2003; Janett, Thun, Niederer, et al., 2003), viability and acrosomal status (Morte et al., 2008; Wach-Gygax et al., 2017), fatty acid composition of plasma membrane (Aurich et al., 2018), seminal plasma composition (Morte et al., 2008), DNA integrity (Blottner et al., 2001; Janett et al., 2014; Wach-Gygax et al., 2017) and fertility (Morte et al., 2008).

Stallion semen is often collected and cryopreserved during the non-breeding season, in autumn and winter (Blottner et al., 2001) due to higher availability, as the dogma is that differences among seasons are small and production of frozen semen is possible throughout the year (Aurich, 2016)

To our knowledge, investigation has yet to be done on seasonal changes in oxidative status and the lipid peroxidation level of the membrane, in relationship with semen quality. In fresh semen collected from stallions in breeding and non-breeding seasons, ROS concentrations never seemed to coincide with compromised sperm DNA integrity (Morte et al., 2008), although there were differences between fertile and subfertile stallions in protein and lipid oxidation. suggesting that measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells (Morte et al., 2008)In another study characterizing dismounting semen samples, more fertile stallions had higher metabolically and active sperm, generating higher ROS concentrations (Gibb et al., 2014). Under tropical condition, in *Bos indicus* and *Bos taurus*, the authors (Nichi et al., 2006) found higher lipid peroxidation in summer than in winter through the measurement of malondialdehyde (MDA) concentration (TBARS assay), an end product of LPO and moderate but significant correlation between LPO and semen primary defects.

## Sperm damages induced by cooling and freezing (structural, functional and metabolic)

The application of temperature-induced metabolic restriction by chilling or cryopreservation is finalized to semen storage. Mammalian sperm are not adapted to endure low temperatures, and are very sensitive to cooling from body temperature to near the freezing; in particular, cooling of stallion spermatozoa from 18 to 8°C and then to 4°C is a critical step.(Amann and Pickett, 1987). Reducing temperature to 4°C, reduces cellular metabolic activity and increases the lifespan of cells and, at the same time, alters membrane permeability to water and solutes with consequent ultrastructural, biochemical and functional damages, named "cold shock".

Moreover, other significant damages collectively referred to "cold shock" consist in osmotic and toxic stress derived from exposure to molar concentrations of cryoprotectants, high salt concentration and membrane shrinking and swelling, intracellular and extracellular ice formation with consequent cytoplasm fracture or even effects on the cytoskeleton or genome related structures (Medeiros et al.2002).

Sperm cells are haploid, they are unable to regulate gene expression and to synthesize new proteins or enzymes, due to the fact that they are almost devoid of cytoplasm, constituted by a large nucleus, with highly condensed chromosomes that impede transcriptional activity to replace proteins. This implies that sperm cannot modulate gene expression to face stressful environmental conditions and to regulate its physiology as other cells (Barbas and Mascarenhas, 2009). They rely on absorption of molecules from the surrounding environment to maintain functional cell membranes and to undergo the maturational changes that confer to the sperm motility and ability to fertilize (Amann et al., 1993). During the final phases of spermatogenesis, spermatozoa lose the ability to biosynthesize and repair, and consequently become extraordinarily simple in their metabolic functions (Hammerstedt, 1993).

The negative effects of cold shock and cryopreservation upon sperm membrane vary among species and is influenced by several elements such as: the chemical composition of the sperm membrane; the ionic deregulation and the increase in internal $Ca^{2+}$, $Na^{+}$ and pH, consequent to the destabilization and alteration of membrane permeability (Fraser, 1995); the generation of ROS (Aitken, 1995), that will be deeply discussed in chapter "ROS and oxidative stress".

Chemical composition of sperm membrane (cholesterol/ phospholipids ratio, content of lipids in the bilayer) plays a relevant role on membrane permeability to water and the fluidity at different temperatures. Cold shock induces membrane lipids and proteins rearrangements with alterations in the cholesterol/phospholipid ratio (Cross, 1998); the major factor predisposing stallion spermatozoa to cold shock is probably a lower ratio of cholesterol to phospholipid compared to other species

(Darin-Bennett and White, 1977). A decrease in temperature causes a thermotropic phase transition in the membrane phospholipids from a liquid-crystalline to a gel phase, resulting in more rigid (ordered) membrane structure and in an alteration of metabolic cell function (Medeiros et al., 2002). Depending on a combination of these membrane elements (unsaturated:saturated phospholipids and cholesterol:phospholipid ratios), boar sperm is the most sensitive; bull, ram and stallion are very sensitive; dog and cat are somewhat sensitive; rabbit, human, and rooster are less sensitive to cold shock (Parks, 1997).

All these changes can lead to a phenomenon known as sperm capacitation-like changes (White, 1993; Yeste, 2016) also called cryo-capacitation because it is exacerbated by cryopreservation (Naresh and Atreja, 2015a) that will be deeply discussed in chapter "Protocols for cryopreservation of stallion semen".

During freezing, cells are exposed to ice crystal formation and osmotic shock. Ice crystal formation causes the disruption of cellular structures by mechanical stress and osmotic dehydration, changes in plasma and acrosome membranes, mitochondrial sheath and axoneme (Salamon and Maxwell, 2000). A major aim during cryopreservation is to reduce the amount of intracellular ice crystal formation which can be achieved by: 1) increasing the total solute concentration with different CPAs (cryoprotectants); 2) increasing the degree of cellular dehydration; 3) changing the speed of cooling and/or warming. Treatment of the cells with CPAs forces the movement of the water by osmosis and leads to dehydration of the cell (Mazur, 1963). While shifting water out of a cell may help to reduce the amount of intracellular ice crystal formation, this may inflict a new and different problem for a cell in the form of osmotic shock (Mazur, 1963). During freezing, the extracellular environment experiences a progressive increase in osmolality as temperatures drop. Thus, cells lose water and shrink until intracellular and extracellular solute concentrations equilibrate. At thawing, spermatozoa are exposed to a hypotonic environment that results in an increased sperm volume until intracellular and extracellular volumes equilibrate; if cell volume excursions go beyond tolerance limits, cellular damage or death will occur (Mazur. P., 2004).

A cryoprotectant can be defined as a substance that, when added to cells prior to cryopreservation, improves the cryosurvival of the cells. The ideal cryoprotectant should have a low molecular weight, great water solubility and minimal toxicity.

There are two classes of cryoprotectans (CPAs):

- **Permeating CPAs** (glycerol, amides, dimethyl sulfoxide, ethylene glycol, propylene glycol): they reduce the concentration of the electrolytes and prevent cell shrinking in the hypertonic solution; they cause membrane lipid and protein rearrangement, resulting in increased

membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation because they replace water inside the cell, and increased survival to cryopreservation; moreover they are solvents that dissolve sugars and salts in the cryopreservation medium (Holt, 2000; Purdy, 2006);

- **Non-permeating CPAs** (egg yolk, milk or some sugars) do not cross plasma membrane and only act extracellularly; they activate the water loss by the cell and decrease the probability of the intracellular ice formation in the fast cooling process; they act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation (Kundu et al., 2002).

The first cryoprotectant used was glycerol (Polge et al., 1949) and despite glycerol toxicity causes injury to spermatozoa during the cryopreservation process (Fahy et al., 1990), it has been the major cryoprotectant routinely used on stallion semen for cryopreservation. Glycerol toxicity may result in protein denaturation, alteration of actin interactions and induction of protein-free membrane blisters (Demick et al., 1976) and is partly due to osmotic stress, because glycerol permeates the cell membrane more slowly than other cryoprotectants (Gilmore et al., 1995). The extent of the damage varies according to the species and depends on its concentration in the extender solution (Purdy, 2006). For these reasons it is critical to establish which cooling/thawing rate and different freezing/thawing media components can minimize unwanted effects of cryopreservation. Moreover, the addition of different CPAs (with high or low molecular weight) tries to minimize exposure to osmotic stress, stabilizing biomolecules and cellular structures and limiting the damaging effects of ROS.(Gibb and Aitken, 2016a).

Normally, 40–50% of sperm cells don't tolerate cryopreservation, and in stallions the success of cryopreservation is lower than in other farm animals (Blottner et al., 2001). Freezing and thawing reduce acrosomal integrity, viability, and motility of spermatozoa in all species examined including the horse (Bedford et al., 2000; Brum et al., 2008) and cryopreservation results in the formation of DNA lesions on genes that are essential for fertilization and normal embryonic development (Valcarce et al., 2013).

## Protocols for cryopreservation of stallion semen

In general, the cryopreservation method includes temperature reduction, cellular dehydration, freezing and thawing; this process is identified as “slow freezing”, compared to the second more rapid method, vitrification (the transition of a liquid to an ice crystal-free stable glass), unsuitable in spermatozoa for the high risks of solution effects or crystallization (Medeiros et al., 2002).

Cellular damage of different degrees of severity is induced by distinct mechanisms at each of the cryopreservation phases, and the functional state of the frozen-thawed cells is the result of the injuries accumulated throughout the cryopreservation procedure (Isachenko et al., 2003). A reasonable result of the cryopreservation process was estimated as maintenance of sperm motility around 50% of that in the fresh sample (Medeiros 2002). As previously mentioned, the occurrence of cold shock can be prevented by controlling the rate of cooling and by adding protective compounds to semen diluents.

Current techniques of cryopreservation of stallion semen processing include different processing methods; most of these freezing protocols involve a two steps dilution procedure in which semen is first diluted in an extender without cryoprotectant, seminal plasma removed, and concentrated-sperm resuspended in a second extender containing the cryoprotectant (Table1).

**Table 1**. Processing methods used for cryopreservation of stallion semen (adapted from McKinnon A.O., Squires E.L., Vaala W.E., Varner D.D.: Equine Reproduction, second edition, VOL. 2, pg 2975, 2011).

| **Source** | **Dilution** | **Centifugation extender** | **Centrifugation** | **Freezing extender** | **Glycerol conc.** | **Packaging** | **Freezing** | **Thawing** |
|---|---|---|---|---|---|---|---|---|
| *Tischner M.* (Tischner, 1979) | Collection of sperm rich fraction | | | Lactose-EDTA-EY | 3,5% | Aluminium tubes (20-25ml) | 7-9 min $N_2$ vapor | 40°C 50 sec |
| *He W-Y cited in* (Amann and Pickett, 1987) | 1:1 | 11% sucrose | 350-450g | Sucrose-milk-EY | 5% | Glass vials (1ml) | $N_2$ vapor | Sucrose-milk |
| *Martin* (Martin et al., 1979) | 1:1 | Glucose-EDTA | 1000g 10 min | Merck-Lactose-EY | 5% | Macrotub© (4.,0ml straw) | 20 min $N_2$ vapor | 50°C 40 sec |
| *Loomis* (Loomis et al., 1983) | 1:1 | Glucose-EDTA | 650g 15 min | EDTA-lactose-EY | 5% | 0,5ml straw | 10 min $N_2$ vapor | 38°C 30 sec |
| *Palmer E. cited in* (Amann and Pickett, 1987) | 1:4 | INRA82-EY »+4°C/1h | 600g 10 min | INRA82-EY | 2,5% | | +4» -140°C 60°C/min | 37°C 30 sec |
| *Vidament* (Vidament, 2005) | $2,5x10_9$sp/ 50-ml tube | UHT skim milk (37°C) | 600g 10 min | INRA82-EY | 2,5% | | » 4°C: 1h 15 min +4» -140°C: 40-60°C/min | 37°C 30 sec |
| *Loomis and Graham* (Loomis and Graham, 2008) | | Skim milk-glucose | Remove 90% seminal plasma | Four different | 1% glycerol, 4% methylformamide | | +5°C for 20 min vapor or automaticall y 20-40°C/min | 46°C for 20 sec or 37°C for 1 min |

EDTA, ethylenediaminetetraacetic acid; ext, extender; EY, egg yolk; sp, spermatozoa.

- *first dilution*: after collection, semen must be filtered to remove the gelatinous portion and debris from the ejaculate. The gel-free portion must then be diluted with skim milk base extender and evaluated. This dilution should be at minimum of 1:1 ration (vol/vol). There is a wide variety of commercial skimmed milk– or casein-based extenders for horse semen. They provide the necessary nutrients for sperm metabolism and functions, buffers to maintain the proper pH, and also protect the plasma membrane against cold shock and oxidative damage. Furthermore, because of the presence of antibiotics, these extenders play an important role in preventing bacterial growth (Alvarenga et al., 2016).

- *seminal plasma removal and sperm concentration*: the seminal plasma is mostly produced by the accessory sex glands, carries fluid and sperm and participates in the sperm capacitation process. (Aurich et al., 1996). Under natural mating, semen is ejaculated directly into the uterine lumen and exposure of spermatozoa to seminal plasma is less than after semen collection. Greater concentrations of seminal plasma in stallion semen are detrimental to motility and fertility but 5 to 20% of seminal plasma should be retained after centrifugation (Jasko et al., 1992; Loomis, 2006) because of its antioxidant properties (Kankofer et al., 2005).

  Centrifugation is the most common technique used for concentrating sperm from a stallion ejaculate and to reduce seminal plasma amount. Some studies, however, underlined the damaging effects of centrifugation on the sperm: the force and duration of centrifugation required to remove seminal plasma might negatively affect sperm motility, integrity, and recovery rate (Alvarenga et al., 2016).
  The total yield of pelleted cells is determined by the sedimentation rate and depends on:
    - relative centrifugal force;
    - centrifugation time;
    - size and shape of the cells;
    - density of the cells and medium;
    - viscosity of the medium;
    - length of the column of suspension(Katkov and Mazur, 1999).

  Semen can be centrifuged 10–15 min at 400–600×g or at 1000×g for 20 min (with the “cushion technique” ) without severe damages (Loomis, 2006).

- *second dilution (CPAs and freezing extender):* after centrifugation, the pellet is resuspended with extenders for freezing usually containing buffers, one or more sugars (glucose, lactose, raffinose,

saccharose and trehalose), salts (sodium citrate, citric acid), a non permeating cryoprotectant (milk and/or egg yolk), a penetrating cryoprotectant and antibiotics (Evans, 1991).

"Equilibration" means the time needed for an osmotic equilibrium to be reached between intra and extra-cellular compartments; it could last between 1,30-2 hours allowing temperature decreases from room to 4°C.

Because stallion sperm are quite sensitive to the osmotic effect induced by glycerol, cryopreservation extenders for stallion sperm generally contain only 2-5% glycerol.

- *packaging*: successful cryoprotection has been achieved at concentrations ranging from 20 to 1600 x $10_6$ spermatozoa/ml (Heitland et al., 1996; Leipold et al., 1998). The influence of sperm concentration on fertility has not been clearly explained for frozen sperm of stallions. In general, the survival of stallion sperm is enhanced when frozen at a low concentration, due to the greater availability of CPAs surrounding the spermatozoa. The choice for packaging stallion semen for freezing is based on quality and practicability and first choice are polypropylene or polyvinyl chloride tubular straws of different volumes (0.25, 0,5 and 4ml) (see Table 1). From a cryobiological point of view, packages that have a large surface area enable more homogeneous freezing and thawing rates (Katila, 2001).

- *cooling and freezing*: spermatozoa can endure fast cooling rates from 37°C to 20°C, but require slow cooling rates (best option 0,1°C/min by programmable devices) between 20°C and 5°C to minimize the effect of "cold shock" (Heitland et al., 1996). The addition of CPAs to the cooling medium may influence the length of the cooling phase. Depending on the method used for freezing (programmable devices or on liquid nitrogen vapour), different freezing rates could be achieved (i.e., freezing rates of 10°C/min to -15°C and 25° C/min to -150°C (Blottner et al., 2001). For stallion spermatozoa, freezing rates between -10°C/min and -60°/min work well, depending upon the cryodiluent used (Amann and Pickett, 1987).

- *thawing procedure*: the optimal warming rate is dependent upon the cooling rate used; cells that are cooled rapidly need to be warmed rapidly, while cells that are cooled slowly need to be warmed slowly. Because of the cooling rates used to cryopreserve stallion sperm are intermediate between the two extremes, thawing straws in a water bath at 37°-39°C works well (Brinsko et al., 2011). Recent evidence indicates that osmotic shock, especially at thawing, is the factor causing most of the cellular death during cryopreservation (Morris, 2006; Morris et al., 2007)

Unfortunately, damages occur to the spermatozoa in each step of the stallion semen cryopreservation, as shown by different studies.

Blach et al., (Blach et al., 1989). analyzed motility and integrity of plasma-acrosomal membranes by immunofluorescence (binding of an antibody specific to the acrosome) at four different points during equine semen cryopreservation: less than 15 min after collecting and before processing (Step 1); after centrifugation and just before freezing (Step 2); immediately after thawing less than 3 h after freezing (Step 3) and immediately after thawing, 10 to 20 d after freezing (Step 4). Most damages to sperm occurred as a result of freezing-thawing, whereas centrifugation caused only minor damage. In another study, Neild et al., investigated sperm viability and state of capacitation in six progressive stages during equine semen cryopreservation using a fluorescence microscope (Neild et al., 2003). The average data for all stallions indicated that each step resulted in increased sperm death and the last step (freeze-thawing) did not further reduce the amount of live non-capacitated cells and the amount of live capacitated sperm cells remained stable during the entire freezing-thawing procedure.

## ROS and oxidative stress

With the term "cryopreservation" we usually mean the whole procedure including dilution, seminal plasma removal, CPAs addition, equilibration temperature and time, freezing and thawing. As previously described, each of these steps affects semen quality by altering the lipid architecture of the plasma membrane, increasing osmotic stress cold shock, intracellular ice crystal formation; in addition, production of ROS is increased (Chatterjee and Gagnon, 2001; Evangelista-Vargas and Santiani, 2017).

In stallion semen, several authors consider the exposure to low temperatures and subsequent sperm thawing critical moments for oxidative stress (OS). OS is a condition that reflects an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify (antioxidant defenses) the reactive intermediates or to repair the resulting damage (Saalu, 2010). Therefore, OS results when the uncontrolled production of ROS exceeds the antioxidant capacity of the spermatozoa. Sperm are susceptible to oxidative stress because of their relative lack of antioxidant protection due to the limited distribution of cytoplasmic defensive-repair enzymes and their abundance of oxidizable substrates in the form of polyunsaturated fatty acids (PUFAs) of their membrane, proteins and DNA.(Aitken et al., 2016).

OS has been acknowledged as a cause of defective sperm function since the pioneering studies of Tosic and Walton, who demonstrated the toxic effect of endogenously generated $H_2O_2$ on bull sperm metabolism and motility (Tosic and Walton, 1946).

The term ROS covers a variety of oxygen metabolites, such as the superoxide anion ($O_2^{-\bullet}$), the hydroxyl radical (OH•), nitric oxide (NO) and hydrogen peroxide ($H_2O_2$). These are also named free radicals, containing one or more unpaired electrons that form highly reactive molecules with a wide range of cellular targets (carbohydrates, lipids, proteins, nucleic acids). Superoxide anion ($O_2^{-\bullet}$) derived by cellular respiration, exibits a short half-life but is reactive in the hydrophobic environment provided by cellular membranes; moreover, it is spontaneously converted in $H_2O_2$ by SOD (superoxide dismutase). Several others different redox entities might be involved in OS, as peroxynitrite (ONOO-), derived by the reaction of $O_2^{-\bullet}$ with the biologically active nitric oxide (•NO), another free radical species (Aitken and Nixon, 2013).

Generation of ROS in spermatozoa may occur at two levels:

1) sperm membrane level: superoxide anions ($O_2^{-\bullet}$) derived from the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, which becomes active upon removal of seminal plasma;
2) mitochondrial level: this site appears to be the main source of ROS in spermatozoa. Spermatozoa are rich in mitochondria because a constant supply of energy (ATP) is required for their motility. Therefore, the presence of dysfunctional spermatozoa significantly elevates the production of ROS, which in turn affects its mitochondrial function and subsequently motility (Henkel, 2011).

Low and controlled concentration of ROS play a physiological role in the cascade of events leading to sperm capacitation, acrosome reaction (Aitken, 1995) and sperm-oocyte fusion in order to ensure appropriate fertilization (Agarwal and Saleh, 2002); in the same way, because of their high reactivity, they are toxic for sperm, cells particularly susceptible to oxidative damages because of many polyunsaturated phospholipids in plasma membrane, inability to resynthesize membrane components and low capability anti-oxidative system (Hammerstedt, 1993). Capacitation is defined as a succession of biochemical changes necessary for sperm to develop the capability to fertilize the oocyte. These changes occur during ejaculated sperm progression through the extracellular environment of, first, the male and, second, the female reproductive tract (Austin, 2016).

Capacitation represents the penultimate modification of spermatozoa, and is required to render them competent to successfully fertilize the oocyte; when sperm comes in contact with the zona pellucida, acrosome reaction is triggered, sperm penetrate the zona pellucida and fuse with oocyte plasma membrane (Yanagimachi, 1994). During the capacitation process, controlled sperm ROS production occurs in spermatozoa, initiating various molecular modifications; once they are released from the oviductal epithelium, sperm generate high levels of ROS, just prior to their final ascent within the Fallopian tube to the site of fertilization. Under these circumstances, each spermatozoon's exposure

to ROS will be short-lived and serves to complete their preparation for imminent fertilization. If a spermatozoon fails to achieve fertilization, the self- perpetuating generation of ROS will result in over-capacitation and a state of oxidative stress and LPO, that trigger more free radical generation from the mitochondria, causing the sperm to initiate apoptosis (Aitken, 2017).

As we already mentioned, spermatozoa from many domestic species undergo capacitation-like changes as a result of the oxidative stress associated with cryopreservation, named "cryocapacitation" (Cormier et al., 1997). The cryo-capacitated spermatozoa usually show different patterns of protein tyrosine phosphorylation (PTP) than the normal in vitro capacitated spermatozoa.(Naresh and Atreja, 2015b). Instead of a true capacitation, researchers speak about an advanced stage of capacitation (Medeiros 2002) or rather a by-passing of the capacitation process (Green and Watson, 2001). In addition, $H_2O_2$ has been suggested to play a primary role in the induction of capacitation in human, hamster or bovine spermatozoa through the stimulation of tyrosine phosphorylation; this can be reversed by the addition of catalase (Aitken et al., 1998; O'Flaherty et al., 2006).

An external source of ROS is L-amino acid oxidases, a cytosolic enzyme involved in the oxidative deamination of aromatic amino acids. L-amino acid oxidases is thought to be of a practical relevance only in the cryostorage of spermatozoa, where a great number of dead sperm can release a large amount of this enzyme. The direct contact with a high concentration of free aromatic amino acids, derived from egg yolk in the freezing extenders, can activate ROS production by L-amino acid oxidases, damaging the rest of live spermatozoa in ejaculate (Aitken, 2017; Aitken, Naumovski, et al., 2015).

For all these reasons, many authors concluded that ROS are a two-edged sword for spermatozoa: a low level of ROS exposure is physiologically required to drive the signal transduction processes associated with sperm capacitation, whereas over-exposure to such metabolites leads to a state of OS that curtails the fertilizing potential, as well as the capacity of these gametes to support the initiation of normal embryonic development.(Aitken, 2017). Since both capacitation and apoptosis are redox-regulated, it is tempting to speculate that they are part of a continuous process: ROS control sperm capacitation as well as cell death! (Aitken, 2011; Aitken, Baker, et al., 2015). When spermatozoa are stressed, they default to an intrinsic apoptotic pathway characterized by mitochondrial ROS generation, loss of mitochondrial membrane potential, caspase activation, phosphatidylserine exposure, motility loss and DNA damage (Aitken et al., 2016).

However, contradictory information is beginning to emerge on the role of ROS in stallion semen quality and fertility. An interesting study done on dismounting (fresh) semen samples has shown how more fertile stallions have higher metabolically and active sperm, producing higher ROS levels. At the same time, while high rates of OXPHOS are indicative of metabolically active and therefore

highly fertile stallion spermatozoa, during storage this metabolic activity may cause spermatozoa to "live fast and die young" due to an elevated production of ROS (Gibb et al., 2014).

In addition, studies in other species have demonstrated that not only freezing–thawing process, but also suprazero temperatures, cooling phase or cryoprotectant addition promote oxidative stress OS.

Understanding which step of semen cryopreservation is more at risk for sperm OS and identifying the critical points of the process will contribute to the development of strategies for OS prevention, for example by adding antioxidants.

With this purpose, OS and the timing of its raise were separately investigated during cryopreservation process in different species [ human (Wang et al., 1997), bull (Chatterjee and Gagnon, 2001), rhesus macaque (Martorana et al., 2014), dog (Lucio et al., 2016) and alpaca (Evangelista-Vargas and Santiani, 2017)], but not in horse.

For this reason, we focused our analysis during freezing-thawing only, excluding from our variables the first part of osmotic and cold shock (the cooling-cryoprotectant exposure procedure), keeping samples under the same conditions during cooling-equilibration time, since extender type and storage temperature are two important factors affecting semen quality (Karabinus et al., 1991; Province et al., 1985).

# OBJECTIVES

The first aim of our study was to investigate oxidative stress during the two major steps in sperm cryopreservation (freezing and thawing), comparing samples immediately pre-freezing (unfrozen), after freezing/thawing cycle (frozen-thawed). Our hypothesis was that different levels of specific ROS could be responsible of a decreased sperm quality, lipid peroxidation and DNA fragmentation, during freezing- thawing and during incubation at 37°C for up to 24 h. For this reason, we focused our analysis during freezing-thawing only, excluding from our variables the first part of osmotic and cold shock (the cooling-cryoprotectant exposure procedure), keeping the two groups under the same conditions during cooling-equilibration time.

The first experiment was conducted during non breeding season (winter/January).

Moreover, our second objective was to investigate seasonal changes (winter/non breeding vs summer/breeding season) in ROS concentrations and lipid peroxidation and their effects on stallion sperm in the same laboratory condition of the first experiment.

The second experiment was conducted during breeding season (summer/July) and data were compared with ones obtained during non breeding season.

# RESULTS

To accomplish the objectives listed above, two experiments were developed. In this Chapter the main results obtained in each set of experiments were reported.
The thesis consists of a compendium of two papers.

## PAPER 1

Freezing and thawing induced higher production of hydrogen peroxide (DCFH) and mitochondrial superoxide anions (MitoSOX) and higher membrane lipid peroxidation (BODIPY) both at T0 and during incubation ($P < 0.001$).
Freezing and thawing decreased sperm quality parameters (RAP, PMAI, $M_{pos}$, $F_{neg}$) immediately after thawing (T0) and in all time points ($P < 0.01$).
In contrast, DFI% did not differ between two treatments (unfrozen and frozen-thawed) at T0 ($P > 0.05$), but was higher in frozen-thawed than unfrozen group, starting from 3h (T1) of incubation ($P < 0.001$).
Moderate positive correlations were found between DFI% and MitoSOX, DCFH, BODIPY in both groups. Furthermore, a negative correlation was evident between sperm quality (RAP, PMAI, Mpos, Fneg) and BODIPY, DCFH, MitoSOX. There were strong positive correlation between RAP, PMAI, Fneg and Mpos in both treatments.

## PAPER 2

Environmental parameters during the experimental period 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. Values for TM, PM and RAP changed over time similarly in unfrozen and frozen-thawed samples; therefore, only RAP is presented.

Lipid peroxidation, measured by BODIPY, was higher in winter than in summer, independent of group (unfrozen/frozen-thawed) ($P < 0.00$); within the same season, freezing thawing cycle induced higher lipid peroxidation ($P < 0.001$).
Hydrogen peroxide production, measured by DCFH-DA, was higher in winter than in summer until 6 h (T2) of incubation ($P < 0.001$); 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 ($P < 0.00$).

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 ($P < 0.001$); 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 ($P < 0.001$).

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 ($P < 0.001$). 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 ($P < 0.001$).

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

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.

# ARTICLES COMPENDIUM

## Paper 1

**Relationships between sperm oxidative status and semen quality in pre-freezing and post-thawing stallion spermatozoa**

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**Short title**: ROS and frozen stallion semen quality relationship

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### Introduction

Cryopreservation protocols of stallion semen include various collection procedures, sperm concentration (i.e. centrifugation), use of extenders and cryoprotectants, packaging, cooling, freezing and thawing (Antonio et al., 2016; Mari et al., 2015). Each of these steps can affects sperm motility, viability and acrosome integrity (Blach et al., 1989; Neild et al., 2003); however, it is still unclear which step may increase the risk for oxidative stress. With this purpose, oxidative stress and the timing of its raise were separately investigated in alpaca (Evangelista-Vargas and Santiani, 2017), dog (Lucio et al., 2016), macaque (Martorana et al., 2014) and bull (Chatterjee and Gagnon, 2001), but not in horse. These studied demonstrated for example that not only freezing–thawing, but also cooling phase and suprazero temperatures, promote oxidative stress in sperm with increase in ROS (Reactive Oxygen Species) (Martorana et al., 2014). In particular, they reported an increase of both superoxide anions and hydrogen peroxides (Evangelista-Vargas and Santiani, 2017) or superoxide anions but not hydrogen peroxides (Chatterjee and Gagnon, 2001) and lipid peroxidation (Lucio et al., 2016) after sperm freezing and thawing cycle, when compared to cooling or glycerolization steps. ROS play a physiological role in the signaling events controlling many sperm functions (Gibb and Aitken, 2016), but an excess in their production, generated by damaged (Ball et al., 2001) or defective (Aitken et al., 1989) spermatozoa, can be a major cause of sub-lethal damage and decreased fertilization capacity (Baumber et al., 2000, 2003). The specific cellular structure of spermatozoa (low amount of cytoplasm and antioxidant capacity, plasma membrane rich in polyunsaturated fatty acids), makes them particularly sensitive to oxidative stress and vulnerable to damage from free radicals (Bollwein et al., 2008).

We hypothesized that freezing and thawing could determine lipid peroxidation and ROS production in relation to sperm damage in stallion. Furthermore, in order to detect differences between samples pre-freezing (unfrozen group) and after thawing (frozen-thawed group) Thermal Stress Test (TST), or Thermal Stress (TS), was performed by incubating sperm samples for different time periods; in the past, this test has been already used to estimate potential fertility of semen in bull (Bacinoglu et al., 2008), stallion (Katila, 2001) and boar frozen semen (Fiser et al., 1991). Therefore the aim of our study was to investigate the occurrence of oxidative stress due to freezing procedure (comparing unfrozen and frozen sperm samples) and if incubation at 37°C can detect differences not measurable after freezing and thawing, and during incubation at +37°C up to 24 h.

## Materials and methods

### 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 581-591, SYTOX, MitoSOX) and Polysciences Europe GmbH, Eppelheim, Germany, (Acridine Orange).

### Stallions and experimental design

In January, in Northern hemisphere, 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, Istituto Nazionale Fecondazione Artificiale (Italy) and fed 15 kg of hay and 4 kg of concentrate per day, with *ad libitum* access to water. Before starting the experiment, one ejaculate was collected once daily from each stallion for 1 wk to deplete extra-gonadal sperm reserves.

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.

**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 $50\times10_6$/mL with Kenney extender pre-warmed (+37°C) (Kenney et al., 1975), 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(Mari et al., 2015). Supernatant was discarded, cushion fluid aspirated and sperm pellet was re-suspended ($200\times10_6$ mL) in Heitland extender (Heitland et al., 1996) 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 $1.2\times10_6$ sperm/mL with pre-warmed (+37°C) Tyrode's medium (310 mOsm; 7.2 pH) (Wach-Gygax et al., 2017) and kept at +37°C for 15 min until analyzed.

**Semen analysis**

***Motility***

A computer assisted sperm analyzer (IVOS vers.12, Hamilton Thorne Inc., Denvers, USA) was used to assess sperm motility. An aliquot of semen (unfrozen/frozen) was diluted with prewarmed (37°C) Kenney extender at $30\times10_6$/mL for motility evaluation; after 10 min 37°C incubation; diluted samples was loaded into pre-warmed slide (Leja Standard Count 2 Chamber Slide 20 micron; Leja Products B.V., Nieuw Vennep, The Netherlands) at stage temperature of 37°C. 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: $\mu m^2$, 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.

***Flow cytometric analysis***

Flow cytometric analyses were performed according to recommendations of the International Society for Advancement of Cytometry (Lee et al., 2009) 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. Measurements of fluorescence emission were estimated for green fluorescence (FITC Channel) emission by using a 525±40 nm filter, for orange fluorescence (PE Channel) emission by a 585±42 nm filter, for red fluorescence (ECD Channel) emissions by 610/20±20 nm filter and far red fluorescence (APC Channel) emission by a 660/10 nm filter. 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.

*Plasma membrane and acrosome integrity*

Membrane integrity and acrosomal status of spermatozoa were evaluated after staining with propidium iodide (PI) and peanut agglutinin conjugated with fluorescin isothiocyanate (FITC-PNA) (Nagy et al., 2003; Rathi et al., 2001) Five µL of semen, previously diluted in 238.5 µL Tyrode's solution (Wach-Gygax et al., 2017), were stained by adding 1.5 µL of PI (2.99 mM) and 5 µL FITC-PNA (100 mg/mL). Four sperm subpopulations were detected: 1) PI- and FITC-PNA-negative cells, with intact plasma membrane and acrosome (PMAI); 2) PI-positive and FITC-PNA-negative cells, with defective plasma membrane and intact acrosome; 3) PI-negative and FITC-PNA-positive cells, with intact plasma membrane and defective acrosome; 4) PI- and FITC-PNA-positive cells, with defective plasma membrane and acrosome.

*Membrane lipid peroxidation*

Bodipy$_{581\text{-}591}$C11 is a lipid-based fluorophore that 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.

*Intracellular ROS concentrations: $H_2O_2$ and $O_2^-$*

DCFH-DA (dichlorofluorescein-diacetate) is a non-fluorescent agent, converted by $H_2O_2$ into DCFH (2′,7′-Dichlorofluorescin diacetate), which has a green fluorescence (LeBel et al., 1992). 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 (Aitken et al., 2006). 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).

*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.

*Sperm Chromatin Structure Assay (SCSA™)*

The susceptibility of sperm to acid-induced DNA fragmentation was assessed performing the SCSA® (Evenson and Jost, 2001). Briefly, 400 µL of acid detergent solution were added to 200 µL of semen previously diluted with TNE buffer to a final concentration of 1 to 2 $x10_6$ sperm/mL. Following the mixing of the sample for 30 s, 1.2 mL of Acridine orange (AO) staining solution (6.0 µg AO/mL AO staining buffer) were added and stained samples were flow cytometrically assessed after exactly 3 min. Cell gating and quantification of the percentage of cells with high DNA fragmentation index (%DFI) were performed as previously described by (Evenson and Jost, 2001).

The sperm chromatin structure assay (SCSA) was performed to assess sperm DNA integrity using a Coulter EPICS XL flow cytometer driven by EXPO32 ADC XL 4 Color software (Beckman Coulter Inc., Krefeld, Germany). Cells were excited by a 488-nm Argon LASER and the emitted green (FL1), orange (FL2), or red fluorescence (FL3) was captured at 525, 575 or 620 nm, respectively. A total of 10,000 events were analyzed for each sample at a flow rate of 200 cells/s. Data analysis and computation of SCSA parameters were performed using the 4.07.0005 version of FCS EXPRESS Flow Cytometry Research Edition software.

After every 10th sample. Goal: adjust the **x** (130) and **y** (500) Mean Value.

**Statistical analyses**

Mean values (±SD) of sperm parameters, conditional on type of preservation (unfrozen, frozen) and incubation time (T0, T1, T2, T3, T4), were calculated as measures of central tendency and data dispersion. Correlations between sperm parameters were quantified using the Spearman's correlation coefficient ($r_s$; at 0.05 significance level). The strength of the correlation was considered weak, moderate or strong based on the absolute value of $r_s$ ($|r_s|<0.25$, $0.25\leq|r_s|<0.75$, $|r_s|>0.75$, respectively).

Growth curve analysis was used to analyze sperm parameters over the course of a 24 h incubation. The relation of the outcome variables (RAP, PMAI, $M_{pos}$, $F_{neg}$, MitoSOX, DCFH, BODIPY, DFI%) to incubation time was assumed to be linear, quadratic or cubic; thus, time curves were modeled using first-, second- and third-order orthogonal polynomials of incubation time. The fixed effect of *freezing-thawing* was added to all incubation times. Unfrozen sperm was set as the baseline condition (unfrozen vs frozen-thawed) and model parameters were estimated to determine the effects of *freezing-thawing* on the rate of change of sperm parameters. Within-stallion variability was included as a random effect in a mixed-effects model structure; the intercept of the model curve was allowed to vary across ejaculates nested within stallion. The random effect of the *ejaculate* (nested within stallion) as well as the fixed effects of incubation (linear, quadratic and cubic time polynomials), *freezing-thawing* and their interaction term were added in a hierarchical order in the model structure. The subsequent improvement of model fit was evaluated by model comparisons based on the -2 log-likelihood ratio criterion (at 0.05 significance level). Parameter-specific P-values were estimated using the normal approximation. The *nlme* (Pinheiro et al., 2017), *lattice* (Sarkar and SpringerLink (Online service), 2008) and *ggplot2* (Software, 2009) statistical packages in R version 3.1.3 (Language et al., 2015) were used. Spearman's rank test was used to calculate correlations between sperm functional and ROS-related parameters.

## Results

Descriptive statistics (mean ± SD) for sperm endpoints [i.e., 0h (T0), 3h (T1), 6h (T2), 12h (T4), 24h (T5)] of CASA and flow cytometrically assessed sperm parameters are presented related to semen storage and incubation time (Table 1, Figs 1a-b). 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 samples) are presented in Table 2.

Freezing and thawing induced higher production of hydrogen peroxide (DCFH) and mitochondrial superoxide anions (MitoSOX) and higher membrane lipid peroxidation (BODIPY) both at T0 and during incubation ($P < 0.001$; Table 1, Fig 1a).

Freezing and thawing decreased sperm quality parameters (RAP, PMAI, $M_{pos}$, $F_{neg}$) immediately after thawing (T0) and in all time points ($P < 0.01$; Table 1, Fig 1b).

In contrast, the DFI% did not differ between two treatments (unfrozen and frozen-thawed) at T0 ($P > 0.05$), but DFI% was higher in frozen-thawed than unfrozen group, starting from 3h (T1) of incubation ($P < 0.001$; Table 1, Fig 1a).

Moderate positive correlations were found between DFI% and MitoSOX, DCFH, BODIPY in both groups. Furthermore, a negative correlation was evident between sperm quality (RAP, PMAI, Mpos, Fneg) and BODIPY, DCFH, MitoSOX. There were strong positive correlation between RAP, PMAI, Fneg and Mpos in both treatments (Table 2).

## Discussion and conclusions

The aim of the present work was to investigate the occurrence of oxidative stress due to freezing procedure (comparing unfrozen and frozen sperm samples) and if incubation at 37°C can detect differences not measurable after freezing and thawing. In the present study freezing-thawing significantly increased all ROS-related parameters (DCFH, MitoSOX and BODIPY) and %DFI was similar before and after freezing-thawing and significantly increased in frozen group only after 3 h incubation, in contrast with all the others quality sperm parameters, worst at 0 h in frozen than unfrozen samples. In the present study, the increase of peroxides (DCFH), superoxides (MitoSOX) and membrane lipid peroxidation (BODIPY) in frozen-thawed compared to unfrozen samples was in agreement with findings in alpaca semen, where both $O_2^-$ and $H_2O_2$ increased significantly after thawing compared with "equilibration period"; moreover, the increase of lipid peroxidation (BODIPY) observed in frozen-thawed compared to unfrozen samples is in agreement with results reported in dog (Lucio et al., 2016), but not in alpaca (Evangelista-Vargas and Santiani, 2017), where a higher but not significant increase of lipid peroxidation after thawing was found comparing pre-

freezing and post-thawing analyses, as in our study. On the contrary, our results were in contrast with what reported in bull, where authors observed a steady rise of $O_2^-$, but not of $H_2O_2$, comparing samples in the same conditions (Chatterjee and Gagnon (2001). However, all these studies did not include incubation after thawing in their protocols.

In bull semen, incubation for up to 24 h allowed to show changes of superoxide anions (detected by MitoSOX) and hydrogen peroxides (detected by DCFDA) (Gürler et al., 2016): authors observed a rise of $O_2^-$, but not of $H_2O_2$, comparing samples before and after freezing as in our study. They reported that changes of DNA integrity in frozen sperm seem to be related to synthesis of $H_2O_2$ but not to synthesis of other reactive oxygen species.

In our study, MitoSOX and DCFDA detect ROS in live sperm and the synthesis of superoxide anions and hydrogen peroxide begins immediately after thawing. In general, superoxide anion ($O_2^-$) appears to be one of the primary free radicals products, located into the mitochondria of the sperm, where it can be detected by MitoSOX probe (Gibb et al., 2014; Marques et al., 2014). Most $O_2^-$ is converted to $H_2O_2$ by superoxide dismutases inside and outside of the mitochondrial matrix (Koppers et al., 2008; Peña et al., 2015).

Higher level of ROS in frozen samples could be attributed to dead or damaged sperm; dead or damaged spermatozoa release substances, including ROS, and induce an excessive endogenous generation of ROS in the cryosuviving sperm (Agarwal et al., 2008; Tatone et al., 2010); moreover, they have negative influence on functions and nuclear DNA integrity of frozen-thawed surviving sperm, as showed in boar sperm (Roca et al., 2013).

In our study DFI% was similar before and after freezing-thawing and significantly increased in frozen group only after 3 h incubation, and this is one of the most interesting result.

In agreement with previous results, even if obtained with different techniques in boar (Evenson et al., 1994), bull (van der Schans et al., 2000) and human (Duru et al., 2001), we found the same values of DFI% at 0 h in both unfrozen and frozen samples, but a faster increase of DFI% in frozen sperm was detected after 3 h incubation.

Different incubation times have been used by several Authors to investigate sperm DNA dynamics (Lòpez-Fernàndez C. et al., 2007), % DFI and ROS level changes (Yeste et al., 2015) in stallion spermatozoa or in other species (Gürler et al., 2016). Lopez-Fernandez et al. (2007) analysed stallion semen at different time point (0, 4, 6, 24, 48 h) of incubation at 37° C to investigate the dynamic changes of DNA fragmentation (detected by Sperm-Halomax ® kit), and they found that the most remarkable increase in sperm DNA damage of frozen-thawed samples occurred between 1 and 6 h of incubation at 37° C, and not immediately after thawing.

In order to study relationship between ROS production and DNA changes, an interesting work has been performed by Yeste et al (2015). These Authors compared good (GFE) and poor (PFE) freezability stallion ejaculates, before (fresh) and after cryopreservation (thawed) and after 1 h of post-thawing incubation; DNA damages (detected by SCSA and free thiol groups in sperm head protein) were increased by cryopreservation, but were similar between 0 and 1 h incubation, despite a significantly higher $H_2O_2$ concentration in GFE than in PFE compared to $O_2$ (HE); these data suggested that GFE and PFE differ in their ROS levels, but not in their chromatin integrity before and after cryopreservation and 1 h incubation (Yeste et al., 2015).

Likewise, our results indicated that chromatin integrity is not susceptible to a particular ROS produced or to lipid peroxidation immediately after thawing, in agreement with Yeste et al (2015); in addition post-thawing incubating for 3 h in our study allowed to detected different DNA integrity between samples and an equal increase of both mitochondrial (MITOSOX) and cellular ROS production (DCFH) during incubation.

On the contrary, the study performed on bull semen (Gürler et al., 2016) suggested that most likely $H_2O_2$ is responsible for further damage of DNA after cryopreservation, showing a time dependent similar changes between %DFI and $H_20_2$ before and after freezing-thawing.

In fact, in agreement with our results, %DFI, O2- and $H_2O_2$ increased immediately after thawing, but, different from ours findings, in non frozen group only O2- increased, whereas %DFI and $H_2O_2$ remained almost unchanged during 24 h incubation.

Whether cryopreservation causes nuclear DNA damage in sperm is controversial: some studies (Di Santo et al., 2012; Tatone et al., 2010) shown contrasting results, while others demonstrated a ROS-associated mechanism in the DNA cryo-damage (Aitken, 2010; Baumber et al., 2003; Rath et al., 2009). The pathways of nuclear DNA damage in spermatozoa are undoubtedly complex, but excessive mitochondrial ROS generation appears to be involved in early events and leads to irreversible oxidative damage and impaired DNA integrity (Ball et al., 2001)(Gibb and Aitken, 2016). Our results suggest that DNA damage following freezing-thawing is likely to be an indirect consequence not only of the stressors associated with changes in temperature, osmolality and plasma membrane instability, but also of ROS during in vitro storage. Our data confirmed that only incubation at 3 h was able to show differences in sperm chromatin integrity resistance related to ROS increases after freezing-thawing.

Probably, frozen-thawed sperm are more susceptible to oxidative attack compared to other cells because of lack an effective DNA-repair capacity (Ahmadi and Ng, 1999; Rath et al., 2009).

In the present study, sperm parameters such as RAP, PMAI, Fneg and Mpos at 0 hours were worst in frozen compared to unfrozen samples. This is not surprising, and it could be strictly ascribed to

freezing-thawing process, as already demonstrated in many other works (Ahmadi and Ng, 1999; García et al., 2012)

On the contrary, chromatin integrity (%DFI) was initially independent from freezing cycle and, in frozen group, from higher concentration of superoxides and peroxides compared to unfrozen one, but it increased faster during time in frozen samples probably because of a higher oxidated environment. Due to the fact that the nuclear DNA in the sperm head, is physically separated from the mitochondria and most of the cytoplasm in the sperm midpiece, ROS increasing could take some time to damage DNA. As a consequence of their structure, the only product of apoptosis that can move from the sperm midpiece to the nucleus in the sperm head is the $H_2O_2$ generated following the dismutation of mitochondrial superoxide anion (Aitken et al., 2015).

Finally, BODIPY, MitoSOX and DCFH showed negative correlation with sperm quality parameters (RAP, PMAI, Mpos, Fneg) and positive correlation with DFI% in both samples and at all time points (Table 2). This finding corroborates our hypothesis that an increase in sperm oxidative status during freezing-thawing is in accordance with an increase in membrane injury, and with an inverse relationship between ROS production during freezing-thawing and the main sperm damages. However, further researches are needed for better understanding the exact role of ROS levels in relationship to fertility, both in fresh and frozen stallion sperm.

In conclusion, the results of our study clearly showed a correlation between lipid peroxidation, ROS generation and decrease of sperm quality during freezing-thawing step of equine cryopreservation process. Interestingly, despite an higher concentration of all ROS-related parameters after thawing, chromatin integrity, in contrast with all the other sperm characteristics, did not differ between unfrozen and frozen-thawed samples at Time 0, but only after 3 h of incubation at +37 °C. Moreover, our results suggested that incubation has to be included in the laboratory tests when evaluating stallion frozen semen because it was useful to asses sperm resistance in particular for chromatin integrity.

## Conflict of interest

None of the authors have conflict of interest to declare

## Author contribution

All authors contributed equally.

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**Table 1.** Mean values (SD) of functional and ROS-related parameters in unfrozen and frozen-thawed aliquots of 18 ejaculates collected from six stallions (three ejaculates per stallion), during a 24-h incubation at +37°C.

| 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) |

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.

458 **Table 2.** Spearman's correlation coefficients ($r_s$) between the functional and ROS-related parameters of unfrozen and frozen-thawed aliquots of 18
459 equine ejaculates. Sperm samples of both groups (unfrozen, frozen-thawed) were incubated at +37 °C for 24h and sperm parameters assessed at 0, 3,
460 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* |

461 BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular $H_2O_2$ concentration; MitoSOX, intracellular $O_{2-}$ concentration; DFI%, sperm
462 with high DNA fragmentation index; PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; $F_{neg}$, sperm with low intracellular
463 calcium concentration; $M_{pos}$, sperm with high mitochondrial membrane potential.
464 *$P < 0.01$

**Figure 1a**. Descriptive statistics of observed and model-predicted values of 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); FU, fluorescence intensity. Sperm parameters over a 24-hour incubation period, conditional on two storage groups: unfrozen (black dots solid line), frozen-thawed (red triangles dashed line). Points and error bars represent the mean values and 95% CI of the observed data, while lines reflect the incubation curve of model-predicted data. Parameters were determined for 18 ejaculates collected from 6 stallions in total.

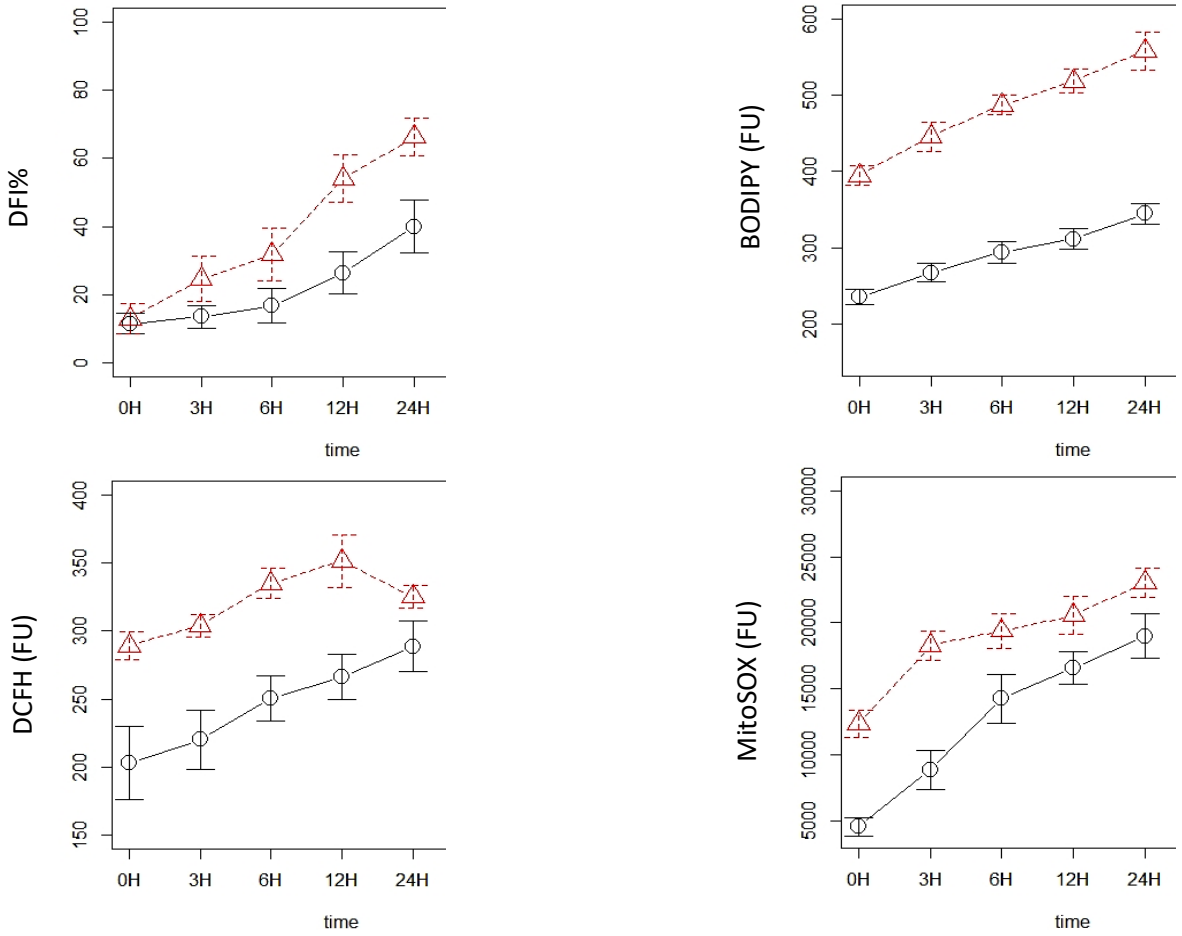
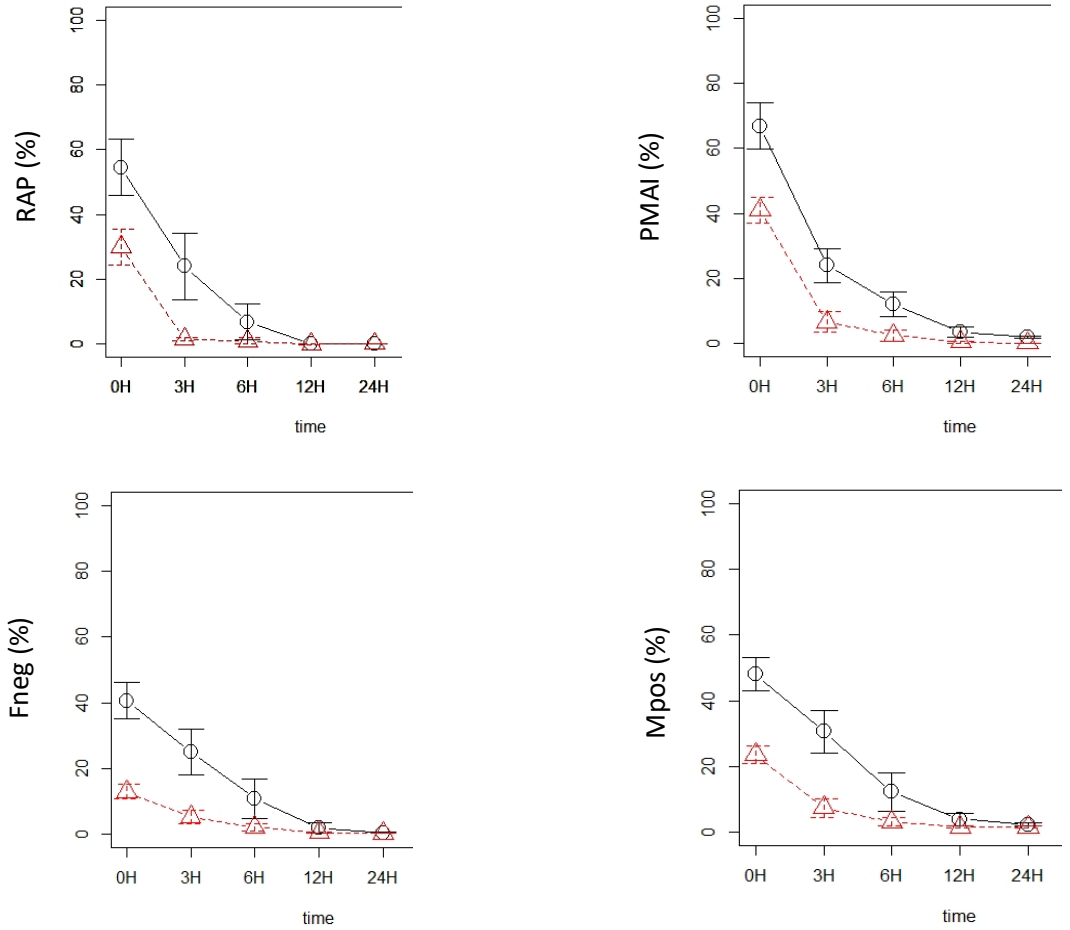


**Figure 1b**. Descriptive statistics of observed and model-predicted values of PMAI (sperm with intact plasma membrane and acrosome); RAP (rapid motility); $F_{neg}$ (sperm with low intracellular calcium level); $M_{pos}$ (sperm with high mitochondrial membrane potential).
Sperm parameters over a 24-hour incubation period, conditional on two storage groups: unfrozen (black dots solid line), frozen-thawed (red triangles dashed line). Points and error bars represent the mean values and 95% CI of the observed data, while lines reflect the incubation curve of model-predicted data. Parameters were determined for 18 ejaculates collected from 6 stallions in total.



## Paper 2

This is the final peer-reviewed accepted manuscript of:

Seasonal changes in ROS concentrations and sperm quality in unfrozen and frozen-thawed stallion semen. B. Mislei, D. Bucci, E. Malama, H. Bollwein, G. Mari, Theriogenology 2020, Vol. 144, 1 March 2020, Pages 89-97. The final published version is available at: **https://doi.org/10.1016/j.theriogenology.2019.12.016**



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

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## 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 581-591, 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 $50\times10_6$/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 ($200\times10_6$ 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 $1.2\times10_6$ 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*

MitoprobeTM 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.

145 *2.6. Statistical analysis*

146 Mean (±SD) values of sperm parameters, conditional on type of preservation (unfrozen, frozen-
147 thawed) and duration of incubation (0, 3, 6, 12 and 24 h), were calculated as measures of central
148 tendency and data dispersion. Growth curve analysis was used to analyze sperm parameters over the
149 24 h incubation. Sperm parameters (RAP, PMAI, $M_{pos}$, $F_{neg}$, MitoSOX, DCFH, BODIPY, DFI%)
150 were modelled using orthogonal polynomials of incubation time. Based on preceding analyses of
151 experimental data, the incubation time curve of most sperm parameters followed a cubic form;
152 therefore, they were modelled as a function of a third-order orthogonal polynomial of incubation time.
153 However, as DCFH values were best fit to a quadratic-shaped curve, a second-order orthogonal
154 polynomial of incubation was employed. Using the lmList function of the nlme statistical package
155 for R, values of sperm parameters were partitioned in four groups in total, conditional on two grouping
156 factors: *storage group* (unfrozen vs frozen-thawed) and *season* (winter vs summer). The above-
157 described growth curves were individually fit for each partition (winter/unfrozen, summer/unfrozen,
158 winter/unfrozen-frozen-thawed, summer/unfrozen-frozen-thawed) of sperm parameters. Using the F
159 statistic test, an analysis of variance table was computed to compare fit of the growth curves, with
160 and without grouping factors *season* and *storage group* (at 0.05 significance level). Model parameters
161 (b coefficients estimates±SEM, 95% CI of coefficients and parameter-specific P values) were
162 estimated using the normal approximation. Data processing and statistical analysis of the results were
163 done using *nlme* and *lattice* statistical packages, whereas graphical illustration of observed and
164 model-predicted sperm parameter values were done with *ggplot2* statistical packages in R version
165 3.1.3. Spearman's rank test was used to calculate correlations between sperm functional and ROS-
166 related parameters.
167

168 **3. Results**

169 Environmental parameters were as follows: winter temperature: 1.4°C mean, 7.4°C max; -3.9°C min;
170 summer temperature: 24.9°C mean; 32.9°C max; 16.8 C min. winter humidity (%): 73.5 mean; 94.8
171 max; 50.5 min; summer: 54.7 mean; 89.1 max; 24.5 min. Descriptive statistics (mean±SD) of CASA
172 and flow cytometrically assessed sperm parameters are presented related to the season at Time 0
173 (Table 1 and Fig. 1 to 4) and during incubation (Tables 2 and 3 and Fig. 1 to 4). Values for TM, PM
174 and RAP changed over time similarly in unfrozen and frozen-thawed samples; therefore, only RAP
175 is presented. Spearman's correlations coefficients $r_s$ between variables of both groups (unfrozen and
176 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.

## Acknowledgments

The authors thank Prof John P. Kastelic for revising the paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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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 ($P < 0.001$).

403 **Table 2.** Mean values (SD) of functional and ROS-related parameters in unfrozen and frozen-thawed aliquots of 18 ejaculates collected in *Winter*
404 from six stallions (three ejaculates per stallion), during a 24-h incubation at +37°C.
405

| 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) |

406

407 BODIPY, sperm with membrane lipid peroxidation; DCFH, intracellular level of $H_2O_2$; MitoSOX, intracellular level of $O_{2-}$; DFI%, sperm with high
408 DNA fragmentation index; PMAI, sperm with intact plasma membrane and acrosome; RAP, rapid motility; $F_{neg}$, sperm with low intracellular calcium
409 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.

| 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) |

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.

**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.

**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).

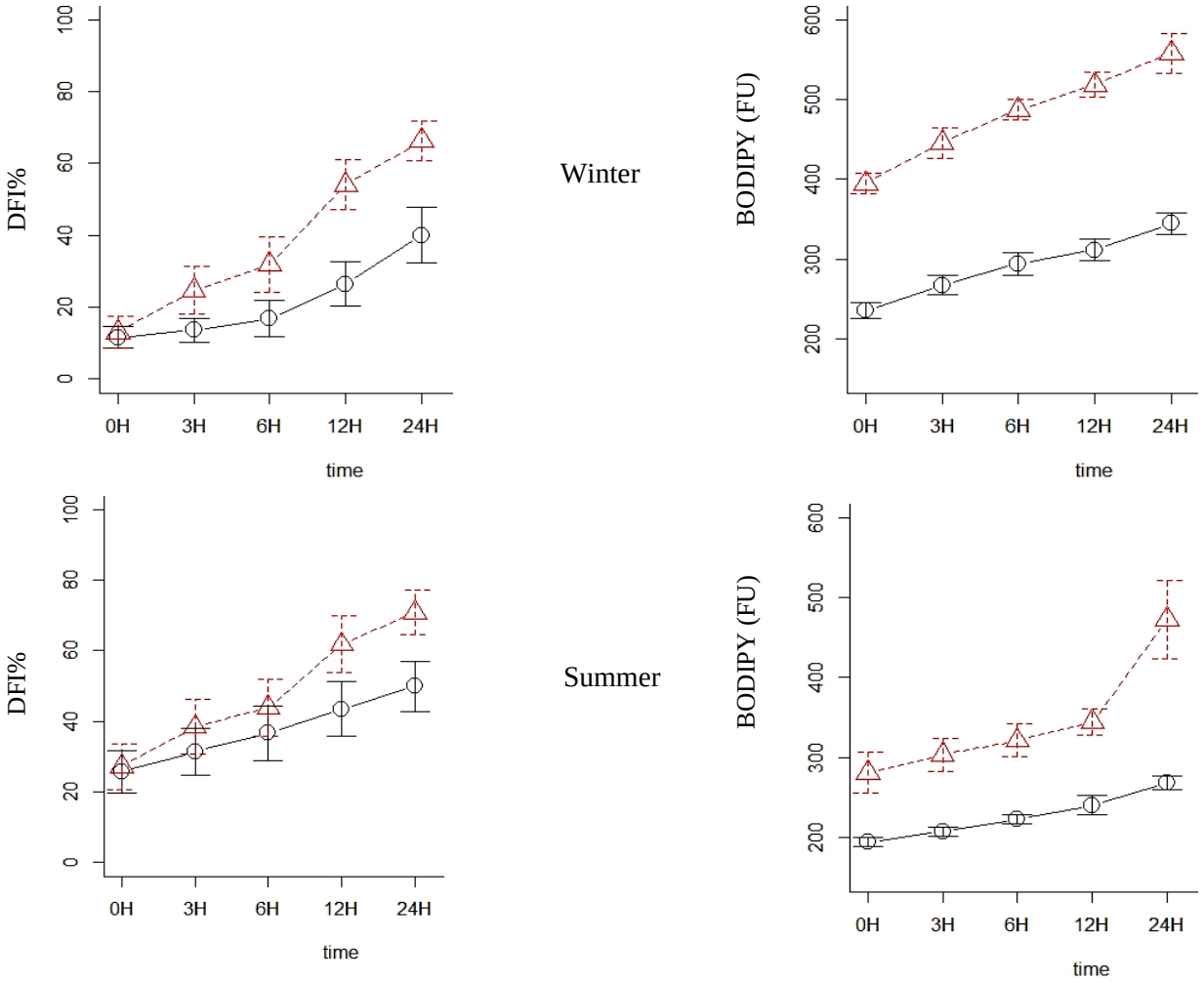


**Fig. 2. Descriptive statistics of observed and model-predicted values of DCFH and MitoSOX.** DCFH, (intracellular level of $H_2O_2$); MitoSOX, (intracellular level of O2-) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed 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).

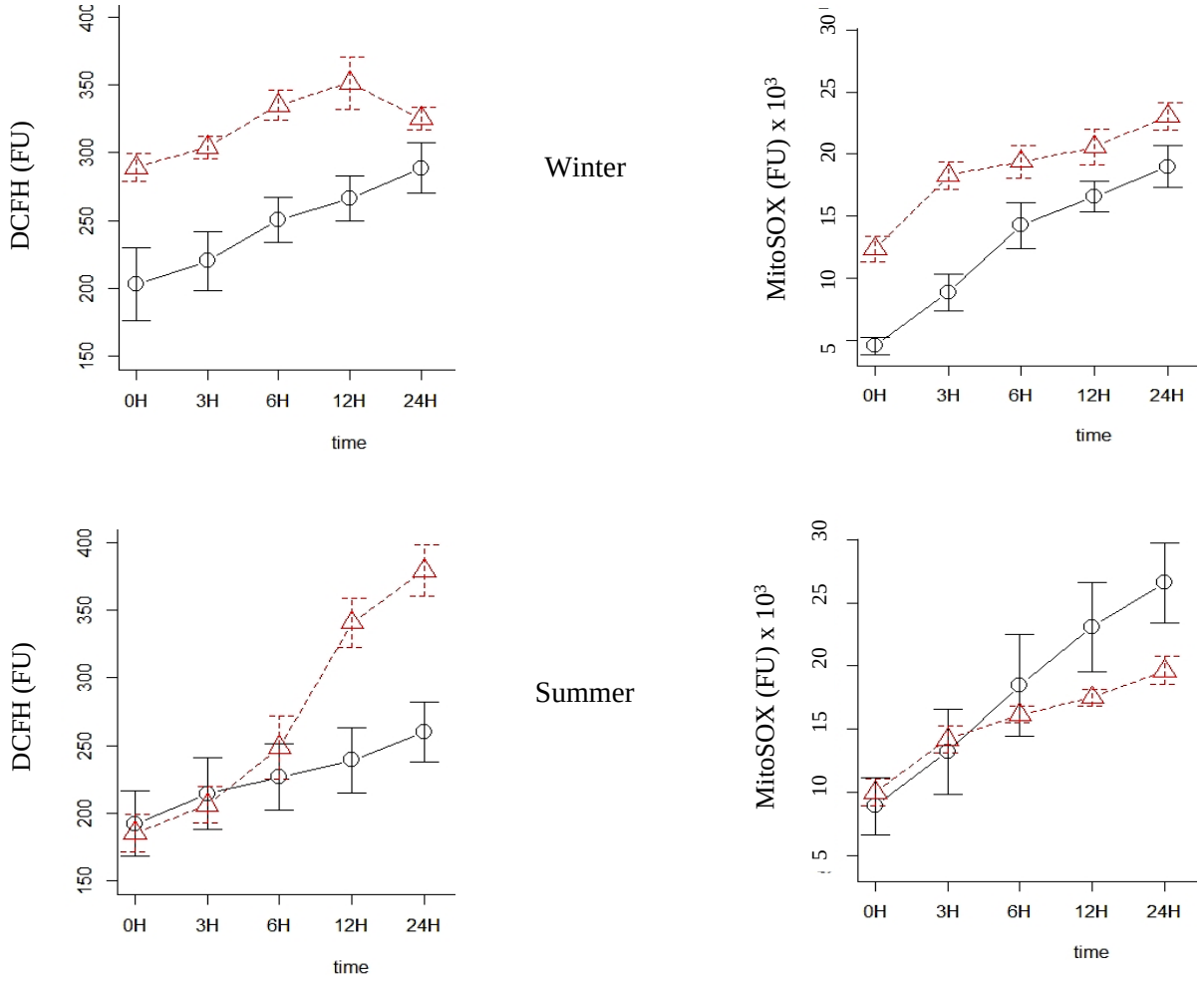


**Fig. 3. Descriptive statistics of observed and model-predicted values of PMAI and RAP.** PMAI (sperm with intact plasma membrane and acrosome); RAP (rapid motility) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (red triangles, dashed line) 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).

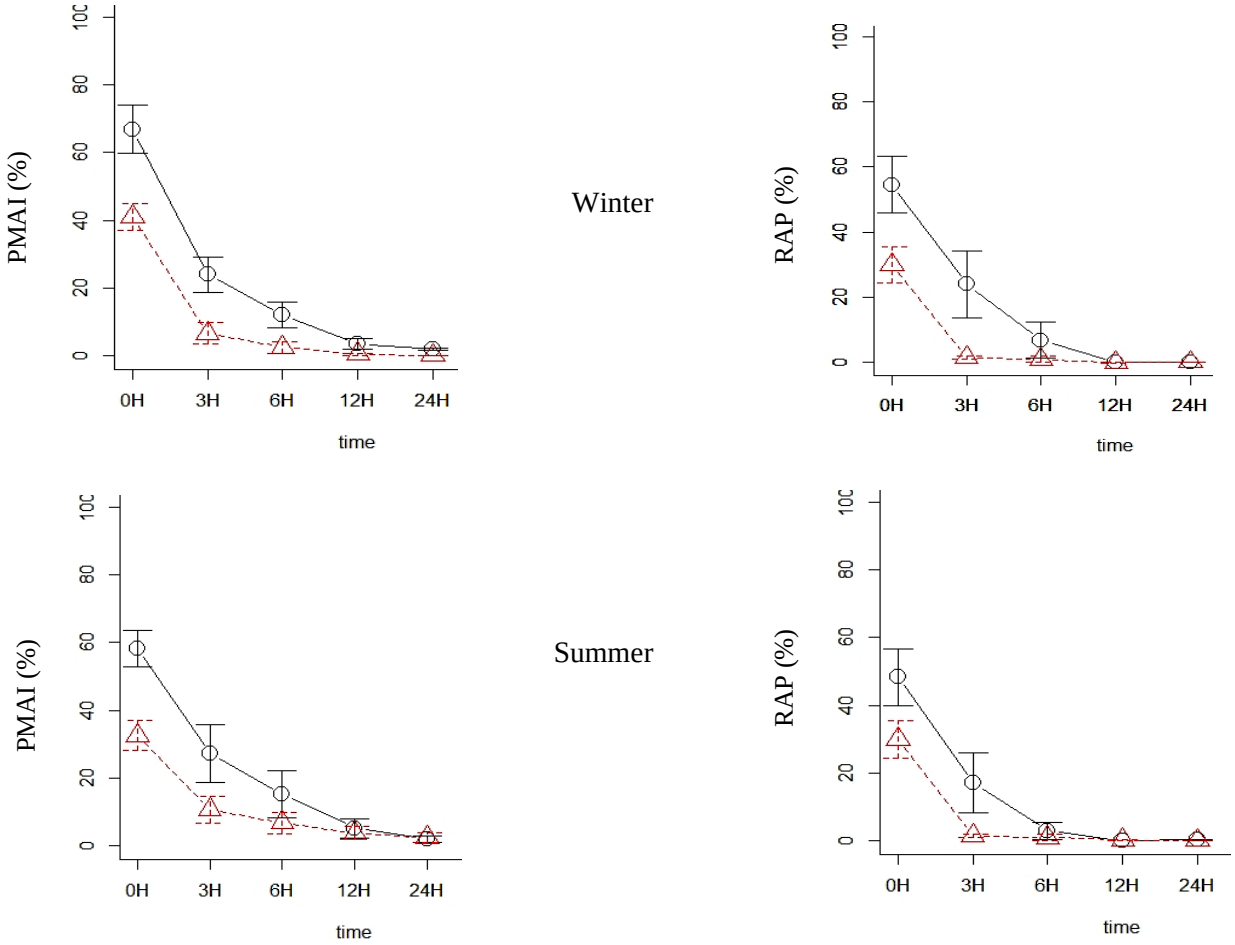
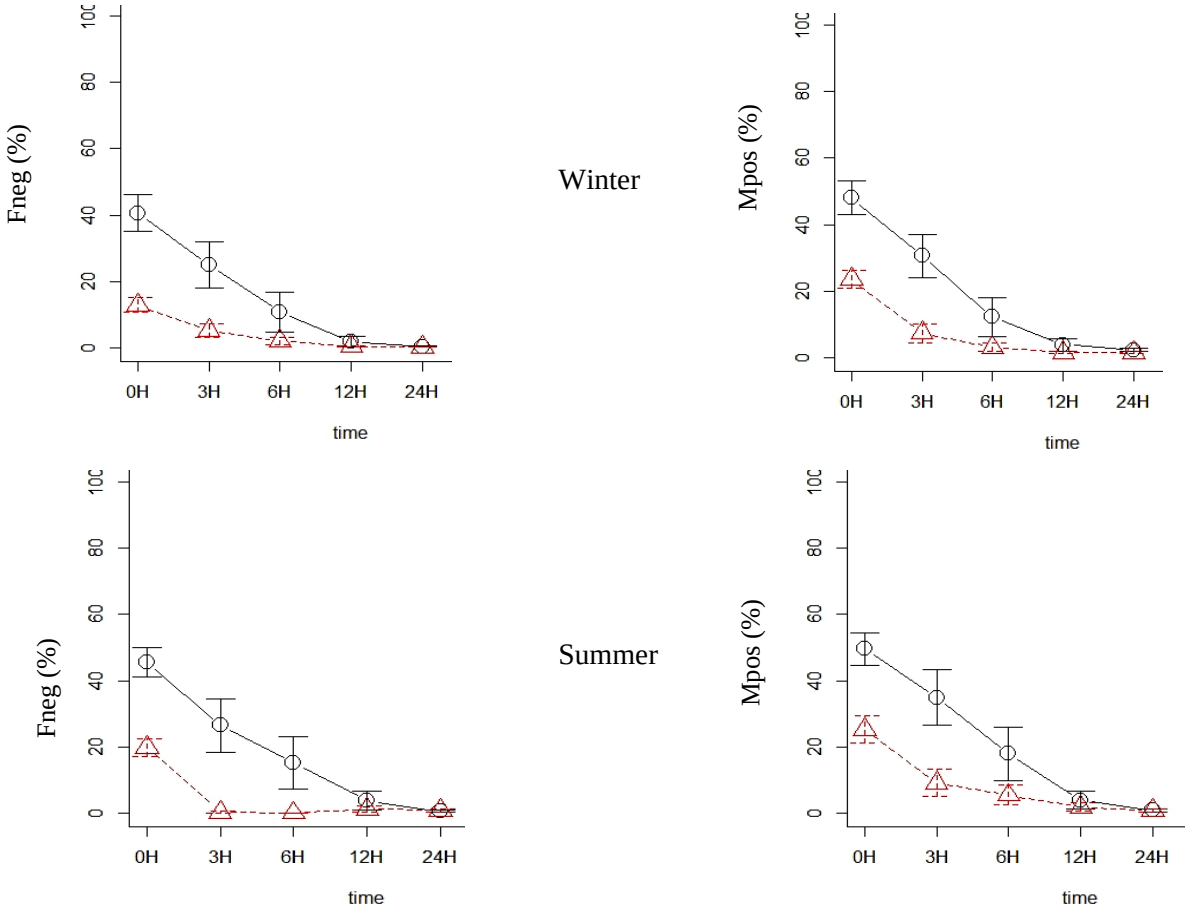


**Fig. 4. Descriptive statistics of observed and model-predicted values of Fneg and Mpos.** Fneg (sperm with low intracellular calcium level); Mpos (sperm with high mitochondrial membrane potential) over 24-h incubation, conditional on two storage groups: unfrozen (black circles, solid line), frozen-thawed (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).



# GENERAL DISCUSSION

In the first study, our hypothesis was that ROS concentration and LPO represented one of the main factors of injury for sperm quality during freezing and thawing. The aim of the work was to investigate the occurrence of oxidative stress due to freezing procedure (comparing unfrozen and frozen sperm samples) and if incubation at 37°C can detect differences not measurable after freezing and thawing. In the second one, we investigated seasonal changes (winter/non breeding vs summer/breeding season) in sperm oxidative status and their effects on sperm quality after freezing-thawing cycle and during incubation up to 24 h.

The results of our study confirmed our hypothesis only in part, because we showed that freezing-thawing promotes ROS production and LPO immediately after thawing and during incubation, with significant decrease in sperm quality. On the contrary, when the same study was performed comparing non breeding (winter) and breeding (summer) season, we showed that sperm oxidative status, induced by freezing-thawing, depends on the season: freezing thawing did not increase ROS levels until 12h of incubation in summer, and we did not find significant differences in sperm parameters between seasons. Interestingly, despite higher ROS levels and LPO detected in winter, chromatin integrity showed significantly better overall values in both samples in this season.

This was apparently the first report to definitively investigate in particular freezing-thawing and the seasonal fluctuations of oxidative status of the sperm in stallion related to this important step. It's worth to underline that in our experiment, the starting point for all analyses (Time 0) represents the moment immediately before freezing (unfrozen samples) and immediately after thawing (frozen-thawed samples), in order to investigate specifically this step of cryopreservation procedure.

In the first study (winter), freezing-thawing significantly increased all ROS-related parameters (DCFH, MitoSOX and BODIPY), but %DFI was similar before and after freezing-thawing and significantly increased in frozen group only after 3 h incubation.

The second study detected seasonal differences in ROS increase and LPO. Peroxides ($H_2O_2$) and superoxides ($O_2^{-\bullet}$) concentrations were higher in frozen samples compared to unfrozen ones at T0 (0h) in winter, but not in summer, where this difference was detected only 12h (T4) of incubation at +37°C, indicating that ROS production is faster in winter frozen-thawed samples. DFI% was higher in summer than in winter, in both unfrozen and frozen groups.

Previous studies demonstrated that superoxide anion and hydrogen peroxide showed different increase after freezing-thawing depending on the species [bovine (Chatterjee and Gagnon, 2001),

human (Wang et al., 1997), macaque (Martorana et al., 2014), dog (Lucio et al., 2016), alpaca (Evangelista-Vargas and Santiani, 2017)], on fertility (Yeste et al., 2015), but not on the seasons.
Comparing samples in the same conditions, some authors observed a steady rise of $O_2^{-\bullet}$ in bull spermatozoa (Chatterjee and Gagnon, 2001), but not of $H_2O_2$; even in alpaca semen, both $O_2^{-\bullet}$ and $H_2O_2$ increased significantly after thawing compared with "stabilization period", in agreement with our results; this effect is probably due to the viable cells continuous production of ROS.
In bull semen, incubation for up to 24 h allowed to show changes of superoxide anions (detected by MitoSOX) and hydrogen peroxides (detected by DCFDA); authors observed a rise of $O_2^-$, but not of $H_2O_2$, comparing samples before and after freezing as in our study. They reported that changes of DNA integrity in frozen sperm seem to be related to synthesis of $H_2O_2$ but not to synthesis of other reactive oxygen species (Gürler et al., 2016).

On the contrary, an interesting work performed by Yeste et al (Yeste et al., 2015), indicated that chromatin integrity is not susceptible to a particular ROS produced by stallion sperm cells. These Authors compared good (GFE) and poor (PFE) freezability stallion ejaculates, before (fresh) and after cryopreservation (thawed) and after 1 h of post-thawing incubation; DNA damages (detected by SCSA and free thiol groups in sperm head protein) were increased by cryopreservation, but were similar between 0 and 1 h incubation, despite a significantly higher $H_2O_2$ concentration in GFE than in PFE compared to $O_2$ (HE); these data suggested that GFE and PFE differ in their ROS levels, but not in their chromatin integrity before and after cryopreservation and 1 h incubation.
In our first study, MitoSOX and DCFDA detect ROS in live sperm and their values were increased soon after cryopreservation. In our study, the synthesis of superoxide anions and hydrogen peroxide begins immediately after thawing. In general, superoxide anion ($O_2^-$) appears to be one of the primary free radicals products, located both in the head (Macías-García et al., 2012)and into the mitochondria of the sperm, where it can be detected by MitoSOX probe (Gibb et al., 2014; Marques et al., 2014). Most $O_2^-$ is converted to $H_2O_2$ by superoxide dismutases inside and outside of the mitochondrial matrix (Koppers et al., 2008; Peña et al., 2015).
Higher level of ROS in frozen samples in our results could be attributed to dead or damaged sperm. Dead or damaged spermatozoa release substances, including ROS, and induce an excessive endogenous generation of ROS in the cryosuviving sperm (Agarwal et al., 2008; Tatone et al., 2010); moreover, they have negative influence on functions and nuclear DNA integrity of frozen-thawed surviving sperm, as showed in boar sperm (Roca et al., 2013).
Although seasonal variations in relation to quality of fresh, cold-stored and cryopreserved semen have been reported (Blottner et al., 2001; Janett et al., 2014; Wrench et al., 2010), seasonal changes in

sperm oxidative status and its association with sperm quality and fertility were investigated only in fresh semen in one study (Morte et al., 2008). 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.

Seasonal differences were detected in DFI%, but not in others sperm quality parameters (RAP, PMAI, Fneg, Mpos) in our study; RAP, PMAI, Fneg, Mpos have been significantly decreased after freezing thawing and during incubation independently from the season. In contrast with this, chromatin integrity (DFI%) in winter was significantly lower than in summer in both unfrozen and frozen-thawed groups, even thought was not damaged by freezing-thawing at Time 0 (T0) in both seasons and showed a significant difference between two groups at T1 (3h) and only at T4 (12h) of incubation in winter and summer respectively. These findings indicated that the two samples differed in their resistance during incubation depending on the season and ROS level and that frozen-thawed spermatozoa suffered the higher levels of oxidation in winter, but only after incubation.

Whether cryopreservation causes nuclear DNA damage in sperm is controversial: some studies (Di Santo et al., 2012; Tatone et al., 2010) shown contrasting results, while others demonstrated a ROS-associated mechanism in the DNA cryo-damage (Aitken, 2010; Baumber et al., 2003; Rath et al., 2009).

The mechanism by which chromatin damage is related to increasing ROS-related parameter is probably a prolonged generation of ROS, due to the dead spermatozoa in absence of extracellular free radical and lipid aldehyde scavengers from seminal plasma, that lead to irreversible oxidative damage and impaired DNA integrity, as reported by other Authors (Ball et al., 2001; Gibb and Aitken, 2016b). Our results suggest that DNA damage following freezing-thawing is likely to be an indirect consequence not only of the stressors associated with changes in temperature, osmolality and plasma membrane instability, but also of ROS during in vitro storage. Our data confirmed that only incubation was able to show differences in sperm chromatin integrity resistance related to ROS increases after freezing-thawing.

In any case, DFI% in winter was significantly lower than in summer in both treatments, showing that not only ROS nor LPO higher levels, but also heat stress during breeding season, promote higher chromatin fragmentation. DFI% values were probably within physiological levels and/or that there

was efficient antioxidant activity in stallion semen, as all the others quality parameters do not differ between seasons.

Moreover, our results suggest that incubation has to be included in the laboratory test when evaluating stallion frozen semen because samples differed mainly in their resistance only after incubation. Incubating sperm samples at different temperatures and for different time periods (Thermal Stress Test), has been already used for estimation of potential fertility of bull (Bacinoglu et al., 2008), stallion (Katila, 2001) and boar frozen semen (Fiser et al., 1991); moreover, exposure of frozen-thawed sperm to the "thermal stress test" revealed latent damage which is not apparent immediately after ejaculation and processing (Fiser et al., 1991).

Unfrozen semen in summer had significantly higher MitoSOX values than in winter at Time 0 (T0) and than in frozen only starting from 12 h (T4) of incubation.

This was unexpected and may have been due to seasonal fluctuations in antioxidants in seminal plasma components and sperm quality as reported in stallion (Gebauer et al., 1976; Morte et al., 2008) and in others species: ram (Marti et al., 2007; Smith et al., 1999), bull (Asadpour and Tayefi-Nasrabadi, 2012; Lone et al., 2018), boar (Lasota et al., 2004) and dog (Vieira et al., 2018). 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 (Gibb et al., 2014) and in frozen bovine sperm (Gürler et al., 2016). During the physiologic breeding season for horses, there are higher peripheral concentrations of LH and testosterone (Gerlach and Aurich, 2000). 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 both seasons, membrane lipid peroxidation (BODIPY) significantly increased after freezing-thawing confirming findings in dog (Lucio et al., 2016) and bull (Chatterjee and Gagnon, 2001) in similar experimental conditions, but in contrast with alpaca, where a higher, but not significant increase in LPO after thawing was found. Moreover, we showed that BODIPY was significantly higher in winter than in summer in both groups. Seasonal effects on sperm lipid composition can compromise semen quality in stallions (Aurich et al., 2018) as plasma membrane PUFA (poly unsaturated fatty acids) are vulnerable to ROS and peroxidative damage. Furthermore, high PUFA content in boar semen has been associated with improved membrane fluidity, and with increased resistance of spermatozoa to cooling and freezing, but also with a high risk of oxidative damage (Peña et al., 2004).

Our findings are in agreement with Aurich et al (Aurich et al., 2018), 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 quality semen (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 explain results in seasonal differences in resistance of equine sperm to cryopreservation, and are 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 (Blottner et al., 2001). It was reported by Blottner et al. 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 (T0), a significant difference between seasons after 3 or 12 h of incubation at 37°C in winter and summer, respectively, was detected in our study. 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 (Magistrini et al., 1987) demonstrated that frozen semen motility is higher if the ejaculate is collected and frozen in winter, while autumn is reported to be a better season than early spring or late winter (Janett, Thun, Bettschen, et al., 2003; Janett, Thun, Niederer, et al., 2003), and March to June (Wrench et al., 2010). Anyway, there is a general agreement that the production of frozen semen is possible throughout the year (Aurich, 2016; Blottner et al., 2001).

From our results, 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.

Finally, we detected strong positive relations between RAP, PMAI, Fneg and Mpos in both seasons, since mitochondrial function, ion exchange and cell signalling are related to development and maintenance of sperm motility (Turner, 2006). Moreover, they drive important sperm functions such as hyperactivation, capacitation, acrosome reaction, and ultimately fertilization (Moraes and Meyers, 2018).

Despite the correlation study showed some interesting significant correlations between oxidative stress related parameters and DFI%, as well as some other negative correlations between sperm quality parameters (RAP, PMAI, Fneg, Mpos) and oxidative stress related parameters in both seasons, sperm quality was not negatively influenced by ROS production, LPO and season, except for DFI%. This aspect is indicative of a relation between ROS production and decrease in sperm quality that

should be further investigated. Furthermore, we showed significantly better values of chromatin integrity when overall sperm oxidative status was higher.

# CONCLUSIONS

The results of our study showed an inverse relationship between ROS production and LPO with the main sperm damages during freezing-thawing, even though the biological role of ROS on fertility should be further investigated.

In fact, ROS-related parameters are higher in winter than in summer, without negative effects on sperm quality. Despite a high concentration of all ROS-related parameters in winter, chromatin integrity showed significantly better values both in unfrozen and frozen-thawed samples in this season. Furthermore, only incubation at +37°C and sequential analysis were useful to assess sperm resistance for DNA and changes for ROS concentrations, indicating that incubation has to be included in the laboratory test when evaluating stallion frozen semen.

These results suggest that ROS increase and LPO seemed to be less dangerous than other stresses (cooling/osmotic) to which sperm are exposed during freezing-thawing. Higher DFI% recorded in summer suggests that the quality of frozen-thawed semen could be higher when collected in winter, probably because in this season there is no heat stress during spermatogenesis.

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.

Further researches are needed for better understanding the exact role of ROS levels in relationship to fertility, both in fresh and frozen stallion sperm. Identifying the critical points of cryopreservation procedure will contribute to the development of strategies for oxidative stress prevention.

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