

# Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa

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## Abstract

Fatty acids and plasmalogens were extracted from the phospholipids of the plasma membrane of stallion spermatozoa, to determine their relation with sperm quality after freezing and thawing. Sperm quality was rated using a quality index that combined the results of the analysis of sperm motility and velocity (CASA analysis), membrane status and mitochondrial membrane potential (flow cytometry) post thaw. Receiving operating system (ROC) curves were used to evaluate the value of specific lipid components of the sperm membrane herein studied as forecast of potential freezeability. From all parameters studied the ratio of percentage of C16 plasmalogens related to total phospholipids was the one with the better diagnostic value. For potentially bad freezers, the significant area under the ROC-curve was 0.74, with 75% sensitivity and 79.9% specificity for a cut off value of 26.9. Also the percentage of plasmalogens respect to total phospholipids gave good diagnostic value for bad freezers. On the other hand, the percentage of C18 fatty aldehydes related to total phospholipids of the sperm membrane properly forecasted freezeability with an area under the ROC curve of 0.70 with 70% sensitivity and 62.5% specificity for a cut off value of 0.32. © 2011 Elsevier Inc. All rights reserved.

*Keywords:* Stallion; Sperm; Plasmalogens; Fatty acids; Flow cytometry; Cryopreservation

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## 1. Introduction

Sperm technology has become an area of increasing interest for the equine industry [1]. One of the major problems of the equine species is the large inter-individual variability in sperm quality. Such variability is often ascribed to the fact that most stallions have been

selected by performance and phenotype, and not for sperm quality either directly or indirectly. The physiological and biochemical reasons behind this variability are being slowly disclosed [2], and recent attempts include the predictive value of several markers for successful freezability of a given ejaculate [2–7]. In many species including horses, peroxidation of lipids of the plasma membrane (lipid peroxidation, LPO) has been claimed to be a major factor involved in sperm quality after thawing [5,8,9]. The particular susceptibility of the sperm plasma membrane to peroxidative

damage is due to a high cellular content of polyunsaturated fatty acids as well as their deficiency in protective enzymes, consequence of spermatozoa losing most of their cytoplasm during spermiogenesis. Long chain polyunsaturated fatty acids (PUFAs) have been detected in the sperm membrane of humans and other mammals [10]. These unsaturated fatty acids give the plasmalemma the fluidity that the spermatozoon requires to participate in the membrane fusion events associated with fertilization. However, these molecules are also vulnerable to the attack by reactive oxygen species (ROS). Such peroxidative damage would disrupt the plasmalemmal fusogenicity and its ability to support key membrane-bound enzymes such as ATPases. Moreover, alterations in the fluidity of the sperm membrane could alter the activation of signal transduction pathways, critical for sperm function. The aim of the present study was to disclose to what extent the composition of fatty acids and plasmalogens of the membrane of ejaculated stallion spermatozoa relates to their ability to survive freezing and thawing procedures.

## 2. Material and methods

### 2.1. Semen collection and processing

Semen (four ejaculates per stallion) was obtained from 7 Andalusian stallions individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, and were collected on a regular basis (two collections/week) during the 2008 breeding season. Ejaculates were collected using a Missouri model artificial vagina with an inline filter to separate the gel fraction, lubricated and pre-warmed to 45–50 °C. The collected ejaculate was immediately transported to the laboratory for evaluation and processing. Only ejaculates with at least 60% progressive motility and intact membranes were included in the study. The filtered ejaculate was extended 1:3 (v/v) with INRA 96 (IMV, L'Aigle, France), and centrifuged at 600 g for 10 min. The resulting sperm pellet was re-extended in freezing medium (Ghent, Minitüb Ibérica, Spain) to a final concentration of  $100 \times 10^6$  spermatozoa per mL. The spermatozoa were slowly cooled to 4 °C within 1 h, loaded in 0.5 mL plastic straws and frozen horizontally in racks placed 4 cm above the surface of LN<sub>2</sub> for 10 min, after which they were directly plunged in LN<sub>2</sub>. After

at least 4 weeks of storage, straws were thawed in a water bath at 37 °C for 30 sec for analysis.

### 2.2. Sperm motility

Sperm kinematics was assessed using a CASA system (ISAS<sup>®</sup> Proiser Valencia Spain). The analysis was based on the examination of 25 consecutive, digitized images obtained from a single field using an  $\times 10$  negative phase contrast objective and a warmed (37 °C) stage. Two straws per stallion and freezing operation were thawed. Semen was loaded in a 20  $\mu\text{m}$  depth Leja chamber (Leja Amsterdam, the Netherlands). Images were taken with a time lapse of one second; the image capture speed therefore being one every 40 ms, the number of objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. For this, all the non-sperm objects present in the screen were removed from the analysis. With respect to the setting parameters for the program, spermatozoa with a VAP < 10  $\mu\text{m}/\text{s}$  were considered immotile, while spermatozoa with a velocity > 15  $\mu\text{m}/\text{s}$  were considered motile. Spermatozoa deviating < 45 % from a straight line were designated linearly motile.

### 2.3. Evaluation of mitochondrial membrane potential ( $\Delta Tm$ )

The lipophilic cationic compound 5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethylbenzimidazolyl carbocyanine iodine (JC-1, Molecular Probes Europe, Leiden, The Netherlands) has the unique ability to differentially label mitochondria with low and high membrane potential. In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. At the same excitement wavelength (488 nm), however, JC-1 forms monomers within mitochondria with low membrane potential, emitting in the green wavelength (525–530 nm). For staining, a 3mM stock solution of JC-1 was prepared in DMSO. From each sperm suspension,  $5 \times 10^6$  spermatozoa were placed in one mL of PBS and stained with 0.5  $\mu\text{L}$  JC-1 stock solution. The samples were incubated at 38 °C in the dark for 40 min before flow cytometric analysis [3].

### 2.4. Assessment of subtle sperm membrane changes and viability

Early sperm membrane changes and viability were determined as described in Peña et al [11], with modifications for adaptation to the equine species [2]. In

brief, one mL of sperm suspension ( $5 \times 10^6$  spermatozoa/mL) from freshly ejaculated or post-thawed spermatozoa was loaded with 3  $\mu$ L of YO-PRO-1 (25  $\mu$ M) and one  $\mu$ L of Ethidium Homodimer-1 (1.167 mM) (Molecular Probes Europe, Leiden, The Netherlands), which was—after thorough mixing—incubated at 37 °C

in the dark for 16 min. This staining distinguishes four sperm subpopulations. The first is the subpopulation of unstained spermatozoa. These spermatozoa are considered alive and without any membrane alteration. Another sperm subpopulation consists of YO-PRO-1 positive cells emitting green fluorescence. It has been demonstrated that in the early stages of apoptosis there is a modification of membrane permeability that selectively allows entry of some non-permeable DNA-binding molecules [12,13]. This subpopulation groups spermatozoa which may show early damage or a shift to another physiological state, since membranes become slightly permeable during the first steps of cryoinjury, enabling YO-PRO-1 but not ethidium homodimer to penetrate the plasma membrane. None of these probes enters intact cells. Finally, two subpopulations of cryo-induced necrotic spermatozoa were easily detected, early necrotic, spermatozoa stained both with YO-PRO-1 and ethidium homodimer (emitting both green and red fluorescence), and late necrotic spermatozoa, cells stained only with ethidium homodimer (emitting red fluorescence).

### 2.5. Staining for detection of lipid peroxidation

Lipid peroxidation (LPO) was measured using the probe BODIPY<sup>581/591</sup>-C<sub>11</sub> (Molecular Probes, Leiden, The Netherlands) as previously described in our laboratory [5]. In brief, a suspension of  $2 \times 10^6$  spermatozoa was loaded with the probe at a final concentration of 2  $\mu$ M. The spermatozoa were then incubated at 37 °C for 30 min, washed by centrifugation to remove the unbound probe, and analyzed using a flow cytometer and a confocal laser microscope. Positive controls were obtained after addition of 80  $\mu$ M ferrous sulfate to additional sperm suspensions. Egg yolk was removed from the FT samples by centrifugation through 35% density colloid (Pure Sperm Nidacom, Gothenburg Sweden).

### 2.6. Flow cytometry

Flow cytometry analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa

Clara, CA, USA) performing 15 mW at 488 nm and EXPO 2000 software. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Non-sperm events (debris) were gated out based on the forward scatter and side scatter dot-plot by drawing a region enclosing the cell population of interest. Events with scatter characteristics similar to spermatozoa but without reasonable DNA content were also gated out. Forward and sideways light scatter were recorded for a total of 10,000 events per sample (YO-PRO-1 and BODIPY-C11), or 30,000 events for JC-1. Samples were measured at flow rate of 200–300 cells/sec. Green fluorescence was detected in FL1 red fluorescence was detected in FL3, and orange fluorescence in FL2.

### 2.7. Fatty acid analysis

Stallion ejaculates were centrifuged at 800g for 20 min. at 4 °C. Once centrifuged, the seminal plasma was removed, the pellet resuspended in 15 mL of PBS and centrifuged again (800g  $\times$  20=at 4 °C) in order to wash the cells. The sperm pellet was used for total lipid extraction. Total lipids from the sperm membranes were extracted from the cell pellet in a suitable excess of chloroform/methanol (2:1 v/v) according to the method of Folch [14]. After solvent evaporation under nitrogen flow, the polar lipids (PL) were isolated according to the method described by Ruiz et al [15] using NH<sub>2</sub>-aminopropyl cartridges. Fatty acid methyl esters were obtained by acidic transesterification, following the method described by Ruiz et al [15] and Estevez and Cava [16]. Fatty acid methyl esters (FAMES) were analyzed using a gas chromatograph (Agilent Technologies 6890-N Network GC system), equipped with an on-column injector and a flame ionization detector (FID). Separation was carried out on a polyethylenglycol capillary column (60 m  $\times$  0.32 mm i.d.  $\times$  0.25  $\mu$ m film thickness; Supelcowax-10, Supelco, Bellafonte, PA). Oven temperature started at 190 °C and held this temperature for 5 min. Immediately, it was raised at 2 °C/min to 235 °C, held for 15 min at that temperature and increased again at 6 °C per minute until 250 °C was reached and held at this temperature for 20 min more. Carrier gas was Helium at a flow rate of 0.8 mL/min. Individual compounds were identified by comparing their retention times with those of standards (Sigma, St. Louis, MO). Tridecanoic acid was used as internal standard. Results were expressed as % of each fatty acid methyl ester.

## 2.8. Statistical analysis

The data were firstly examined using the Kolmogorov–Smirnov test to determine their distribution. In view of the non-Gaussian distribution of most of the data gathered, multivariate analysis of variance was performed, and when significant differences were found, the non-parametric Mann–Whitney *U*-test was used to directly compare pairs of values. The Spearman non-parametric test was used to study the correlation between the analysis of the lipids of the stallion spermatozoa pre-freezing and sperm quality post-thaw. For further definition of sperm quality post thaw, a score was given to all the sperm parameters obtained (sperm membrane integrity, motility, velocities, mitochondrial membrane potential), as previously described in our laboratory, [6] based in descriptive statistics to establish the medium, minimum, and maximum values for each sperm parameter PT. Later PT sperm quality was classified as poor, average and good using the 25<sup>th</sup> and 75<sup>th</sup> percentiles as thresholds, so that values equal to or below the 25<sup>th</sup> percentile were considered as poor (0), those values between the 26<sup>th</sup> and 74<sup>th</sup> percentiles as average (1) and values equal or above the 75<sup>th</sup> percentile were considered as good (2). Using these scores a final value was given for each ejaculate [6]. Receiving operating system curves (ROC) were used to investigate the value of the proposed indexes as predictors of freezability, considering good freezers those presenting the final freezeability score in the best 25% of the values, while bad freezers presented the worst 25% in the final score [6]. Analyses were performed using SPSS ver. 15.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at  $P < 0.05$ .

## 3. Results

### 3.1. Sperm quality post thaw

To assess the sperm quality post-thaw, we used an index previously developed in our laboratory [6]. This index is a linear combination of seven different indicators of sperm functionality post-thaw. This index combined the evaluation of different and objectively measured sperm parameters related to sperm membrane integrity, mitochondrial membrane potential and values of sperm motility and parameters related to characteristics of sperm movement. In this way we gathered data obtained from a combination of tests that may better reflect the number of sperm functions and attributes that a sperm needs to be fertile.

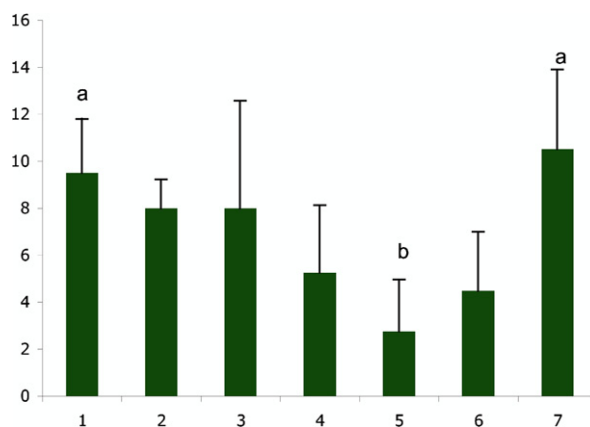


Fig. 1. Post thaw Sperm quality index in seven stallions. A score was given to all the sperm parameters obtained (sperm membrane integrity, motility, velocities, mitochondrial membrane potential), based in descriptive statistics to establish the medium, minimum, and maximum values for each sperm parameter PT. Later PT sperm quality was classified as poor and good average, using the 25<sup>th</sup> and 75<sup>th</sup> percentiles as thresholds, so that values equal to or below the 25<sup>th</sup> percentile were considered as poor (0), those values between the 26<sup>th</sup> and 74<sup>th</sup> percentiles as average (1) and values equal or above the 75<sup>th</sup> percentile were considered as good (2). Using these scores a final value was given for each stallion.

As expected, significant variations were found in sperm quality post thaw among sperm variables. Significant differences ( $P < 0.05$ ) among stallions were observed. Stallion number one and seven gave the best results, while stallion number 5 and 6 gave the poorest results (Fig. 1). Variations were greater among stallions and lower within stallions, with the percentage of sperm membrane integrity presenting coefficients of variability below 10% for all stallions here studied.

### 3.2. Variations among stallions in the fatty acid composition of phospholipids of the sperm membranes

The predominant fatty acid was Docosapentanoic acid (C22: 5 n - 6), representing on average the  $49.9 \pm 8.70$  % of all the fatty acids, followed by palmitic (C16:0) and stearic (C18:0) acids, representing respectively the  $17.6 \pm 3.65$  and  $8.7 \pm 3.05$ % of all the fatty acids. While the percentages of palmitic and stearic acid were not different among stallions, the percentage of Docosapentanoic acid varied significantly ( $P < 0.01$ ) among stallions (Fig. 2). The concentration of this fatty acid was significantly higher ( $P < 0.01$ ) in stallions number 2, 5 and 7 and lower in stallion number 6. Also differences in the percentages of highly unsaturated fatty acids were observed, being significantly higher in

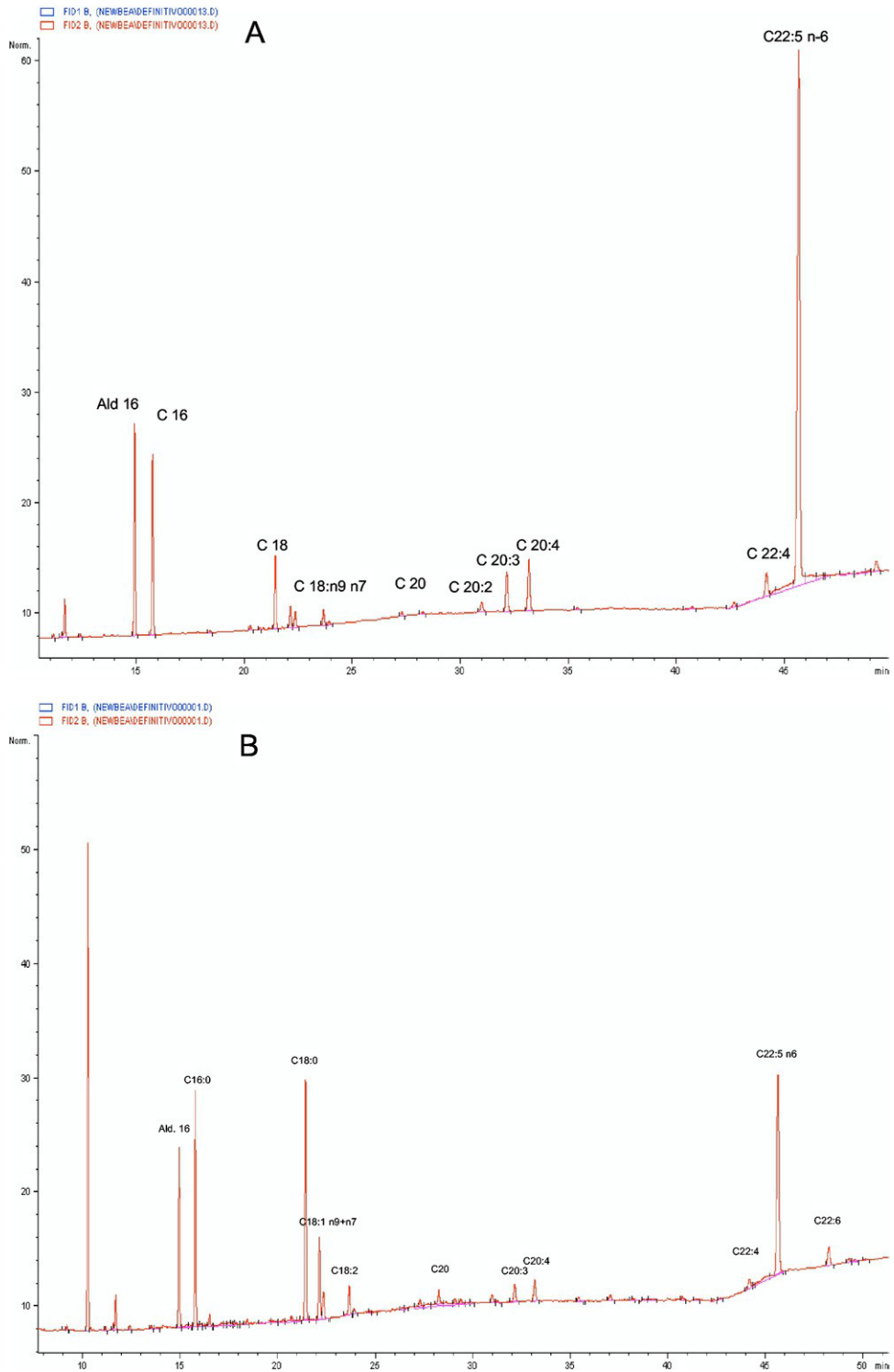


Fig. 2. Lipid profile showing the retention times for the major fatty acids and plasmalogens of the phospholipids of the sperm membrane in two stallions. A represents a good freezer, B represents a bad freezer. Ald 16 (Hexadecanal plasmalogen); C16 (Palmitic acid), C 18 (Stearic acid), C18:1 n - 9+7 (Oleic acid), C 20 (Arachidic acid), C 20:2 n - 6 (Eicosadienoic acid), C 20: 3 n - 3 n - 6 (Eicosatrienoic acid), C 20: 4 n - 6 (Arachidonic acid), C22:4 n - 6 (Docosatetraenoic), C 22:5 n - 6 (Docosapentaenoic), C 22: 6 n - 3 (Docosohexanoic).

Table 1

Non parametric correlations between the composition of fatty acids of the polar lipids of the membrane of ejaculated stallion spermatozoa with intact membranes and LPO (lipid peroxidation) post thaw.

	YoPro—/Eth—	YoPro+/Eth+	YoPro—/Eth+	LPO
C14			0.466*	
C16		−0.495**	0.531**	
C20			0.381*	
C20: 2	−0.390*			
%ALD C18/PL				−0.437*
%ALDC18/ALD			−0.437*	−0.412*
Σ SFA	−0.528**		0.468**	
Σ HUFA	0.502**		−0.446**	

\* P < 0.05, \*\* P < 0.001.

stallions numbers 1, 2, 4 and 7 and lower in stallion 6 (P < 0.01).

### 3.3. Correlations among the percentage of the different fatty acids and plasmalogens of the sperm membrane of the stallion spermatozoa, and the characteristics of the sperm membrane and lipid peroxidation after thawing

Significant positive as well as negative correlations were found among the percentage of specific fatty acids and the status of the sperm membrane post thaw (Table 1). Saturated fatty acids were related with damaged (YO-PRO-/Eth+) spermatozoa. On the contrary, percentage of highly unsaturated fatty acids were positively correlated with intact membranes post-thaw (P < 0.01). The percentage of C18 plasmalogens in relation of total plasmalogens was negatively correlated with damaged membranes (P < 0.01) and the relation C18 plasmalogens: total phospholipids was negatively (P < 0.01) correlated with the percentage of LPO after thawing.

### 3.4. Value of the presence of specific plasmalogens in fresh sperm as forecast of sperm quality post thaw

Receiving operating system (ROC) curves were used to evaluate the value of specific lipid components of the sperm membrane herein studied as predictors of potential freezeability. From all the parameters studied the percentage of C16 plasmalogens/total phospholip-

ids was the one with the highest diagnostic value. For potentially bad freezers, the significant area under the ROC-curve was 0.74, with a 75% sensitivity and 79.9% specificity for a cut off value of 26.9 (Table 2). Also the percentage of plasmalogens respect to total phospholipids gave good diagnostic value for bad freezers. On the contrary, the percentage of C18 plasmalogens:total phospholipids of the sperm membrane forecasted freezeability, with an area under de ROC curve of 0.70 with a 70% sensitivity and 62.5% specificity for a cut off value of 0.32 (Table 3).

## 4. Discussion

In the present study, we investigated the composition of fatty acids and plasmalogens of the phospholipids of the membrane of the stallion spermatozoa, focusing on their relationship with sperm quality post thaw. One of the major drawbacks for the use of frozen thawed semen in equine breeding is the large variation in freezeability among stallions. Recent research from our laboratory indicates that susceptibility to lipid peroxidation largely explains this variability [5]. However, we did not detect differences among stallions in antioxidant enzymatic systems in their seminal plasma, so a plausible explanation would be differences in composition of fatty acids and other lipids of the sperm membranes. It is clear that if the mechanisms explain-

Table 2

ROC curve analysis of the sperm lipid (polar lipids) composition as a forecast of low freezeability ("BAD" FREEZER).

Parameter	Area under ROC curve	SE	95% confidence interval	Cut off value <sup>a</sup>	Sensitivity %	Specificity %
%ALD C16/PL	0.74	0.11	0.51–0.96	26.92	75	79
%ALD/PL	0.73	0.11	0.51–0.95	27.78	75	77%

SE, standar error.

<sup>a</sup> Cut-off values for optimal sensitivity and specificity.



Table 3

ROC curve analysis of the sperm lipid (polar lipids) composition as a forecast of high freezeability (“GOOD” FREEZER).

Parameter	Area under ROC curve	SE	95% confidence interval	Cut off value <sup>a</sup>	Sensitivity %	Specificity %
ALD C18/PL	0.70	0.08	0.53–0.85	0.32	70	62.5

SE, standard error.

<sup>a</sup> Cut-off values for optimal sensitivity and specificity.

ing the difference in freezeability among stallions can be disclosed, adequate strategies to minimize it can be implemented. Apparently, the composition of fatty acids and or plasmalogens of the membrane of stallion spermatozoa explain, at least partially, this variability. The composition of fatty acids in the sperm membrane should be considered as a major factor explaining sperm quality in general, and ability to freeze in particular. As previously stated, of this composition largely depends the susceptibility to LPO, already demonstrated to be directly related to freezeability [5]. Another factor is sperm membrane fluidity, which is also directly related to lipid composition of the sperm plasmalemmae. Freezing, and probably also thawing, damages sperm membranes, and as a result of such injuries, the membrane becomes more rigid (less fluid) and thus less functional after thawing [17]. Experiments conducted to increase membrane fluidity have demonstrated a great improvement in the quality of frozen-thawed spermatozoa [18]. In the present study, the percentage of highly unsaturated fatty acids was correlated with intact sperm membranes post thaw. This finding may be related to the ability of highly unsaturated fatty acids to provide fluidity to sperm membranes. While long-chain saturated fatty acids increase the rigidity of the membrane, the polyunsaturation of the fatty acids gives a more physiological, flexible and fluid membrane [19,20].

Spermatozoa are unique among mammalian cells in that they contain high levels of ether-linked phospholipids (plasmalogens) [21]. A previous study [22] revealed that plasmalogens represented on average ~25% of the phospholipids of the membranes of stallion spermatozoa. Ether-lipids were suggested to contribute to both the formation of the macro- and microdomains required for the compartmentalization of the highly polarized sperm membrane. In the present study, we found that the percentage of plasmalogens in the phospholipids of the membranes in freshly ejaculated spermatozoa were good forecasts for both “good” and “bad” potential freezers. The percentage of C18 plasmalogens prognosed potential freezeability the best, and was negatively correlated with LPO post thaw. On the contrary, the percentage of C16 plasmalogens and

total plasmalogens were best diagnostic markers for low freezeability. At this moment, we can only speculate about the mechanisms behind these findings. However, the antioxidant properties of plasmalogens [23] could explain the predictive value of C18 plasmalogens as forecast of freezeability, a hypothesis supported by the negative correlation of this specific plasmalogen with LPO post thaw.

In sum, the specific composition of fatty acids and plasmalogens of the membranes of stallion spermatozoa may provide clues for the understanding of the mechanisms behind differences in freezeability among sires. Moreover, approaches such as antioxidant supplementation and/or nutritional strategies to modulate the lipid compositions of stallion spermatozoa may help to increase the number of potentially “good” freezer stallions.

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