Theriogenology 172 (2021) 88–94

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Cryotolerance of equine spermatozoa correlates with specific fatty acid pattern: A pilot study

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ARTICLE INFO

Article history:
Received 7 December 2020
Received in revised form
28 May 2021
Accepted 6 June 2021
Available online 8 June 2021

Keywords:
Sperm cryotolerance
Principal components analysis
Fatty acids
Horse

ABSTRACT

Sperm cryopreservation represents a powerful tool for horse breeding. To improve the efficiency of artificial insemination in the horse using cryopreserved spermatozoa, an adequate understanding of the underlying biophysical properties that affect sperm cryosurvival needs to be reached yet. In this pilot study, we described isolation and analysis of the main fatty acids from sperms of stallions classified as good and poor freezers (7 GF and 5 PF, according to sperm motility and viability, before and after cryopreservation). Fatty acid profiles were only assessed in pre-thaw sperms. Eight main fatty acids were identified, using gas chromatography, and their contents were expressed as percentage of the total lipid content. We found that lauric, myristic and oleic acid (C12:0, C14:0 and C18:1n9c) turned out to be about 2-fold more abundant in the sperm cells of the GFs compared with PFs. Moreover, we described for the first time the presence of a very high amount of a *trans* geometrical isomer of linoleic acid, linolelaidic acid (C18:2n6t), in pre-thaw PF spermatozoa. Notably, we found in fresh sperms of PF stallions a ratio of unsaturated fatty acids to saturated fatty acids which was twice that of those of GF group, suggesting a positive effect of a high saturated-to-unsaturated fatty acid ratio for the "freezability" of equine spermatozoa. Finally, principal component analysis (PCA) confirmed the relationships between specific fatty acids and cryotolerance of equine spermatozoa, also providing a graphical classification and additional information about the dominant variables governing the classification process.

1. Introduction

The biological response of sperm cells to cryopreservation and the fertility of frozen-thawed spermatozoa varies between species [1–3]. Even within a given species, it varies from individual to individual, especially in the horse [4–8]. The sources of male-to-male variability in the context of equine sperm cryotolerance are not entirely identified, but it seems that the composition of the fatty acids of the spermatic membrane may have a crucial role [9].

Indeed, the specific fatty acid composition and the ratio of saturated to unsaturated fatty acids appear to be particularly relevant for the "freezability" of spermatozoa in horses, as well as in other mammals [9,10]. However, several studies have produced complex, and somewhat contradictory, results regarding the protective and/or detrimental value of saturated and unsaturated fatty acids. In particular, there is no unanimous agreement on the effect of unsaturated and polyunsaturated fatty acid levels on post-thaw viability and motility of sperm cells [11]. Unsaturated fatty acids seem to be essential to ensure the correct fluidity of the sperm membrane, a property which is necessary to withstand thermal shock [12,13]. Moreover, the presence of carbon-carbon double bonds makes unsaturated fatty acids more vulnerable than saturated ones to the attack by freeze/thaw-induced reactive oxygen species, thus compromising the integrity of the plasma membrane [14–17].

Abbreviations: GF, good freezer; PF, poor freezer; PCA, principal component analysis.

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https://doi.org/10.1016/j.theriogenology.2021.06.004
0093-691X/

In the present study, we investigated possible relationships between post-thaw survival rate and fatty acid composition of the sperm plasma membranes by using both univariate and principal component analysis (PCA), a multi-variate statistical method widely exploited for: (*i*) reducing the data size removing noise and redundancy in the data, (*ii*) identifying related variables, and (*iii*) revealing hidden patterns in the dataset, even in the presence of a rather limited sample size [18–20].

2. Materials and methods

2.1. Stallions

A total of 12 stallions were used throughout the experiment. The age of stallions varied from 4 to 8 years, while all were of proven fertility and were subjected to semen collection regimen before the experiment. Based on previously performed freezing trials for stallions breeding evaluation, the animals were classified as "good freezer" (GF) when post-thaw progressive motility was >30%, and "poor freezer" (PF) when post-thaw progressive motility was <30% [21]. All the stallions included in this research project were approved for assisted reproduction and involved in breed mating programs. For this reason, the animals were trained for semen collection procedures and were all in competitive sports activities. Semen collection, sperm analysis and freezing trials performed for this project were also integral parts of the regular andrological visit and were performed with the informed consent of the owner.

2.2. Isolation, analysis and freezing/thawing of sperm cells

Semen collection was carried out with a Missouri artificial vagina. The seminal material was collected from each subject twice, 3 days apart. The first collection was not examined, while the second was used for the purpose of the study. Semen was analyzed in terms of gel-free volume and concentration (Bürker's counting chamber). Progressive motility was assessed by the computerized image analyzer IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA, USA). The cell membrane integrity/cell viability was determined by using propidium iodide and SYBR-14 fluorescent staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probe, Eugene, OR, USA). For fatty acid analysis, samples of freshly isolated spermatozoa (100×10^6) were resuspended in PBS, centrifuged twice at $500 \times g$ for 10 min, and the semen-free sperm cells were directly used as described in 2.3.

For cryopreservation, gel-free semen diluted with a centrifugation extender (INRA Freeze, IMV Tech, France) to a concentration of 20×10^6 sperm/mL was centrifuged ($500 \times g$ for 10 min) to separate sperms from seminal plasma. Then, an aliquot of freezing extender was used to resuspend the sperm-rich pellet obtained to reach a final concentration of 100×10^6 sperm/mL. Finally, straws of 0.5 mL were filled with seminal material, closed with PVC and frozen on nitrogen vapor for 20 min, then kept in nitrogen (−196 °C) until thawing for analysis [8,22]. After a period of storage in liquid nitrogen of at least 48 h, it was thawed in water at 38 °C for 30 s and kept at this temperature for at least 10 min. Spermatozoa were washed by centrifugation in PBS to remove extender. Subsequently, progressive motility and vitality were assessed as described above.

2.3. Lipid extraction

Total lipids were extracted using a modification of the procedure of Folch et al. [23] as described by Bitman et al. [24]. The method was further modified using more extractions to improve the recovery.

Briefly, after the washing step with PBS described in 2.2, fresh semen-free spermatozoa were resuspended in 6 mL 0.7% aqueous sodium chloride and 18 mL chloroform-methanol (2:1, v/v). The lipids on the cells were extracted with homogenizer Ultraturrax (16,000 turn/min for 10 min). After removal of the lower phase (first chloroform extract), 6 mL chloroform were added to the remaining aqueous phase and the resulting mixture was homogenized to obtain a second organic extract. Finally, the aqueous phase was acidified with 0.1 M hydrochloric acid up to pH = 2 and re-extracted with an additional 6 mL chloroform (third extract). The organic extracts were dried over anhydrous sodium sulphate. Recovery tests on standard solutions showed that two extractions were needed to recover 98–99% of total lipids. The volume of the first extract was at best 11.5 mL and allows recovery of about 93%: if a smaller chloroform volume was obtained, a corresponding decrease in the recovery was observed but, in such case, the recovery of the second extraction increased.

2.4. Preparation of fatty acid methyl esters

A 2-mL aliquot of each chloroform extracts (containing the free acid C11:0 as internal standard) or a 3-mL aliquot of the mixture of the first two chloroform extracts were dry evaporated under a cold gentle nitrogen flow. The residue was treated with 1 mL of 0.5 M methanolic potassium hydroxide solution and heated in boiling water under reflux for 20 min followed by addition of 1.5 ml BF3-methanol (14:86, w/v) and finally heated for 15 min according to the AOAC-IUPAC method [25]. After cooling, 2 mL of a saturated sodium chloride solution was added and the methyl esters were extracted twice with 2 mL hexane. The unified extracts were dried on anhydrous sodium sulphate and analyzed by gas chromatography. Whenever the fat content was very low, the hexane extracts were reduced to 0.5 mL under nitrogen flow: air from methanolic potassium hydroxide solution and from the tube was removed by passing in a stream of nitrogen for a few minutes. The preparation and extraction of fatty acid methyl esters were done in the same conical tube (12 mL) with a ground-glass joint.

2.5. GC analyses

The gas chromatographic analysis was performed by a GC-Autosystem XL (PerkinElmer, Norwalk, USA) gas chromatograph equipped with a flame ionization detector. The injection was made using a split 100:1 injector according to the following temperature: 220 °C. The column temperature program is the following: starting period at 140 °C for 5 min, linear increase from 140 °C to 240 °C at 4 °C/min and finally a period at 240 °C for 10 min. The methyl esters were separated with a capillary column Supelco SPT-2560 (100 m × 0.25 mm I.D., 0.25 μm thickness); carrier gas: helium. The chromatographic peaks were analyzed with GC-Autosystem workstation (Turbo GC Lite, PerkinElmer Nelson). Results were expressed as percentage of each fatty acid methyl ester.

2.6. Statistical analysis

Data were elaborated and analyzed statistically using the R Statistical Package [26] within RStudio software (version February 1, 5033; https://rstudio.com/). The data were firstly tested for normality (Wilk-Shapiro's test), and then analyzed by unpaired Welch's *t*-test with Holm-Sidak correction for multiple comparisons. Correlation coefficients between fatty acids and post-thaw sperm vitality were determined by the Pearson correlation test using "FactoMineR" and "Hmisc" packages [27,28]. Principal component analysis (PCA) was performed to reduce and group 8 individual fatty acids (expressed as % total fatty acid) and post-thaw sperm vitality to more concisely explain and visualize overall

variation. Prior to the PCA, data were standardized (variable deviations homogenized) to prevent the abundant components with large variance from dominating the analysis. PCA was performed using the "FactoMineR" package. Biplot graph was prepared using the "ggplot2" package [29]. PCA was performed on the 9 variables (*i.e.*, eight fatty acids and post-thaw sperm vitality), which had highly significant correlations. The assumptions for sphericity (or the existence of the identity matrix) and sample adequacy [30] were assessed by performing Bartlett chi-square and Kaiser-Meyer-Olkin (KMO) tests, respectively, by using the "rela" package. The Bartlett chi-square value of 88.43 was statistically significant ($P < 0.0001$), indicating that sufficient correlations were present in the matrix for analysis. The KMO value was close to 1.0 (KMO = 0.75), indicating that n = 12 with 9 variables was an adequate sample size for the analysis. Finally, the determinant of the correlation matrix was positive and extremely low (4.4×10^{-6}), indicating that groups of three or more variables had high intercorrelations. Differences were considered to be significant at the $P < 0.05$ level.

3. Results and discussion

Five stallions displayed semen with post-thaw viability/motility lower than 30% over multiple collections and were considered as PF (n = 5); instead, seven stallions reported a post-thaw viability/motility higher than 30% and were classified as GF (n = 7). The main semen parameters of the stallions are summarized in Table 1. Notably, spermatozoa isolated from PF stallions exhibited less sperm viability and motility than those of GF stallions even before freezing, suggesting that less vital and mobile spermatozoa are also more sensitive to cold shock. However, in PF sperms, freezing caused reductions in vitality (from 40% to 8%, with a 32% reduction, $P < 0.001$) and in motility (from 45% to 13%, with a 32% reduction, $P < 0.001$) that were ~ two-fold higher than vitality (from 64% to 46%, with a 18% reduction, $P < 0.001$) and motility (from 68% to 51%, with a 16% reduction, $P < 0.001$) observed in GF sperms.

3.1. Fatty acid profiles

The analysis of sperm membrane lipid composition identified a total of 8 main fatty acids (Table 2). The lipid profiles of the two groups appeared clearly different for half of the recognized fatty acids. Lauric, myristic and oleic acid (C12:0, C14:0 and C18:1n9c) turned out to be about 2-fold more abundant in the sperm membranes of the GFs compared with PFs. Interestingly, a high amount of trans fatty acid C18:2n6t (linolelaidic acid) was massively detected in PF spermatozoa. The palmitic (C16:0) and stearic (C18:0) saturated fatty acids and the palmitoleic (C16:1) and oledaidic (C18:1n9t) monounsaturated fatty acids were instead present in the same percentage between the two groups. Incidentally, the sperm lipid composition reported here was consistent with that of the neutral lipid fraction described in another study [10], in which C14:0, C16:0, C18:0 and C18:1n9 were the predominant fatty acids of equine spermatozoa. In addition, we also found a significant amount of lauric acid (C12:0). Notably, our study reports in PF stallions a ratio of unsaturated fatty acids to saturated fatty acids which was twice that of the GF group. This finding suggests that the negative impact of higher amounts of unsaturated fatty acids in the plasma membrane on the resistance of stallion spermatozoa could be due to cryopreservation-driven oxidative stress and lipid peroxidation damage, as it has been described in human studies [16,31,32].

Table 1
Main parameters of semen/spermatozoa before (pre-thawing) and after (post-thawing) cryopreservation in samples from stallions classified as good and poor freezers.

Parameter	Good freezer (n = 7)	Poor freezer (n = 5)
Age	5.9 ± 1.1	6.2 ± 2.0
pH	7.10 ± 0.21	7.14 ± 0.19
Volume (mL)	55.9 ± 18.4	76.3 ± 37.0
Concentration (x10^6 spz/mL)	215 ± 81	151 ± 73
Motility pre-thawing (%)	67.9 ± 6.9	45 ± 5*
Vitality pre-thawing (%)	63.9 ± 7.9	39.8 ± 4.8*
Motility post-thawing (%)	51.4 ± 5.6	13 ± 10*
Vitality post-thawing (%)	45.9 ± 6.4	7.4 ± 7.8*

The results represent the mean ± SD of five different samples from subjects analyzed in duplicate. *$P < 0.001$.

Table 2
Main fatty acids isolated from sperm cell membranes of good and poor freezers stallions.

Fatty acid	Good freezer (n = 7)	Poor freezer (n = 5)
C12:0 (lauric acid)	**20.7 ± 20**	**13.6 ± 1.0***
C14:0 (miristic acid)	21.8 ± 1.0	13.7 ± 2.0#
C16:0 (palmitic acid)	23.6 ± 3.0	24.1 ± 3.0
C16:1n7c (palmitoleic acid)	5.0 ± 0.5	5.4 ± 0.7
C18:0 (stearic acid)	12.1 ± 0.7	11.9 ± 0.5
C18:1n9t (elaidic acid)	3.5 ± 0.5	3.2 ± 0.3
C18:1n9c (oleic acid)	**11.4 ± 0.7**	**6.0 ± 0.5***
C18:2n6t (linolelaidic acid)	**1.6 ± 2.0**	**22.0 ± 2.0***
Saturated	78 ± 2.9	63.3 ± 4.7#
Unsaturated	**21.5 ± 1.8**	**36.6 ± 2.0***
Ratio unsaturated/saturated	**0.28 ± 0.03**	**0.58 ± 0.04***

Each fatty acid is given as a percentage of the total. The results represent the mean ± SD of five different samples from subjects analyzed in duplicate. #$P < 0.01$, *$P < 0.001$. The most significant correlations are marked in boldface ($P < 0.001$).

The correlation analysis between the main fatty acids was carried out with the aim of identifying those most correlated with the post-thawing viability of the spermatozoa (Table 3). The 12:0, 14:0, and 18:1n9c fatty acids were highly correlated among them and with the percentage of live sperms after freezing and thawing. Conversely, a nearly perfect negative correlation of *trans*-18:2n6 fatty acid with sperm post-thaw vitality was observed ($r = -0.946$; $P < 0.0001$). Notably, Macias et al. [12] reported a significant negative correlation of C16:0 and C16:1 with the percentage of intact sperm membranes. Here, we observed a similar relationship, which however did not reach significance, between the same fatty acids with the post-thaw vitality of spermatozoa (Table 3).

The complex and strong correlation among some of the analyzed fatty acids indicated that there was strong redundancy in the dataset and supported the opportunity of using PCA to reduce their dimensionality and complexity.

3.2. Principal component analysis

The whole dataset of the main 8 fatty acids isolated from the stallion spermatozoa, along with their post-thaw vitality and motility, was subjected to PCA to decrease the number of descriptors associated with the initial variables, while still explaining the maximum amount of variability present in the original data. A new set of nine orthogonal variables (PCs) was generated by PCA. The first and second principal components (*i.e.*, PC1 and PC2) accounted for 56% and 20%, of the variability in the data set, respectively (Table 4). The remaining six generated PCs (*i.e.*, PC3 to PC9 did not explain significant variability in the data. Thus, the most part of whole information of the data matrix could be graphically represented by a biplot showing both PC1 and PC2 scores of individual stallion (as dots) and loadings (eigenvalues) of the different variables (fatty acids and post/thaw vitality shown as vectors or arrows) (Fig. 1). Each vector represents a variable, and the

Table 3
Pearson correlation coefficients among percentage levels of the main fatty acids from sperm membrane and their post-thaw vitality.

Variables	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1n9t	C18:1n9c	C18:2n6t	Vitality
C12:0	1	**0.888**	−0.039	−0.459	0.065	0.239	**0.936**	**−0.898**	**0.874**
C14:0		1	−0.071	−0.433	0.051	0.255	**0.927**	**−0.920**	**0.909**
C16:0			1	0.301	−0.401	0.391	−0.206	0.046	−0.188
C16:1				1	−0.154	0.354	−0.388	0.307	−0.418
C18:0					1	0.101	0.161	−0.122	0.250
C18:1n9t						1	0.278	−0.456	0.389
C18:1n9c							1	**−0.956**	**0.947**
C18:2n6t								1	**−0.946**
Vitality									1

The most significant correlations are marked in boldface ($P < 0.001$).

correlation of two variables is reflected by the angle between the two corresponding vectors (numerically, is defined as the cosine of the angle between their respective vectors on the plot). Variables closest to one another and far from the plot origin are positively correlated, while variables opposite one another on the plot are negatively correlated. Moreover, the color scale and the length of each vector are related to the contribution to the total variance, namely, the longer/darker, the line, the higher is the variance.

From this graph it is apparent that C12:0, C14:0 and C18:1n9c highly correlated to post-thaw sperm viability (acute angles between the respective vectors of similar size), whereas C16:1, C16:0, and C18:1n9t showed good correlations to each other, but were unrelated to sperm viability (orthogonal arrows). C18:0 contributed weakly to PC2 (light gray small vector) and was uncorrelated to sperm viability. Negative correlation between C18:2n6t and post-thaw sperm viability can be seen by the opposite direction of the corresponding variable vectors (Fig. 1).

Incidentally, we also verified that viability and motility (both in fresh and in post-thaw sperms) were strongly correlated each other by including motility in the PCA of fatty acid profile, and obtaining very similar segregation of GFs and PFs (data not shown). This was not surprising, since vitality and motility of spermatozoa are strongly related parameters of the sperm functionality.

The biplot also revealed a clear clustering of stallions according to their sperm cryotolerance. Indeed, the horses were assigned to GF and PF groups, on the basis of the values of PC1 (the abscissa). The PF stallions, represented by green dots in Fig. 1, were exclusively concentrated in the left half-plane of the biplot (PC1 values < 0), while the GF stallions, represented by red dots, were entirely clustered in the right half-plane of the biplot (PC1 values > 0). Therefore, PC1 clearly discriminated the stallions with good from poor sperm quality, allowing to make accurate predictions of sperm cryotolerance. Since negative PC1 values correspond to low values of the C12:0, C14:0 and C18:1n9c and to high values of C18:2n6t, it is arguable that these fatty acids may be used as valuable predictors for the health of spermatozoa and their ability to survive freezing-thawing process. These findings confirmed PCA as a robust multivariate technique for predicting the classification of equine sperms as PFs and GFs, by using the levels of the main fatty acids isolated from fresh spermatozoa. Although, in this case, the traditional univariate statistical analysis was more than enough to highlight the significant variables involved in the sperm cryotolerance, it is noteworthy that PCA is a useful statistical approach for predicting patterns and providing a graphical read-out to capture the whole information (*i.e.*, significant variables, their relative importance and their relationships) related to sperm "cryopreservability".

Table 4
Eigen analysis of the correlation matrix loadings of the significant principal components (PCs).

Variables	PC1	PC2	PC3
C12:0	**0.94***	0.04	−0.17
C14:0	**0.95***	0.04	−0.15
C16:0	−0.15	0.8$^{\#}$	−0.29
C16:1	−0.47	0.62§	0.35
C18:0	0.20	−0.40	0.81$^{\#}$
C18:1n9t	0.34	0.75$^{\#}$	0.46
C18:1n9c	**0.98***	−0.01	−0.01
C18:2n6t	**−0.97***	−0.17	−0.03
Vitality	**0.98***	0.01	0.10
SS loadings (Eigenvalue)	5.03	1.78	1.14
Variance (%)	55.91	19.83	12.66
Cumulative Variance (%)	55.91	75.74	88.40

$^{\S}P < 0.05$, $^{\#}P < 0.01$, $^{*}P < 0.001$. The most significant correlations are marked in boldface ($P < 0.001$).

This study suffers from a number of important limitations: (*i*) the reduced number of samples; (*ii*) the lack of isolation of healthy spermatozoa from damaged spermatozoa; (*iii*) the lack of analysis of the fatty acid profile of frozen-thawed sperm cells.

Regarding the first point, the small size sample design of our study did not allow us to fully evaluate the intra- and inter-individual variability in the fatty acid composition of spermatozoa. In fact, ours was a pilot study, and for this reason, we could use one sample per stallion. To confirm the role of the bulk composition in fatty acids in sperm functionality, it will be necessary to enrol more animals.

Concerning the second point, we did not investigate the reason for the differences in the number of damaged/dead spermatozoa both in fresh semen of GFs and PFs. Nor did we try to isolate viable cells from less viable ones to reduce possible artefacts due to the different number of damaged cells in GF and PF stallions. In fact, the study was conducted on all spermatozoa. Although the reason for this lower vitality/motility was not studied, it still seems to be associated with a particular profile of total fatty acids, and in particular with high levels of linolelaidic acid, whose origin and role will need to be further investigated.

In relation to the third point, having not analyzed the fatty acids before and after freezing, we cannot establish whether, and how, the freezing/thawing cycle can alter their profile. Although it is known that freezing induces changes in the composition of membrane lipids, due to the activation of phospholipases, as well as other enzymes capable of hydrolyzing membrane lipids, such as phospholipids and sphingolipids, the total fatty acid content is unlikely to be affected by a physical process or hydrolytic enzymes. On the other hand, the role of freezing on the possible change in the ROS-induced peroxidation of the double bonds of mono- and bi-unsaturated fatty acids that we described in our samples (*i.e.*, acid C16:1; C18:1n9t; C18:1n9c; C18:2n6t) is more likely. Based on this aspect, the higher unsaturated/saturated ratio we found in fresh sperms of PFs seems to suggest that their membrane lipids are much more easily attacked by freezing/thawing-induced radicals than those in the GFs. This could explain, at least in part, the

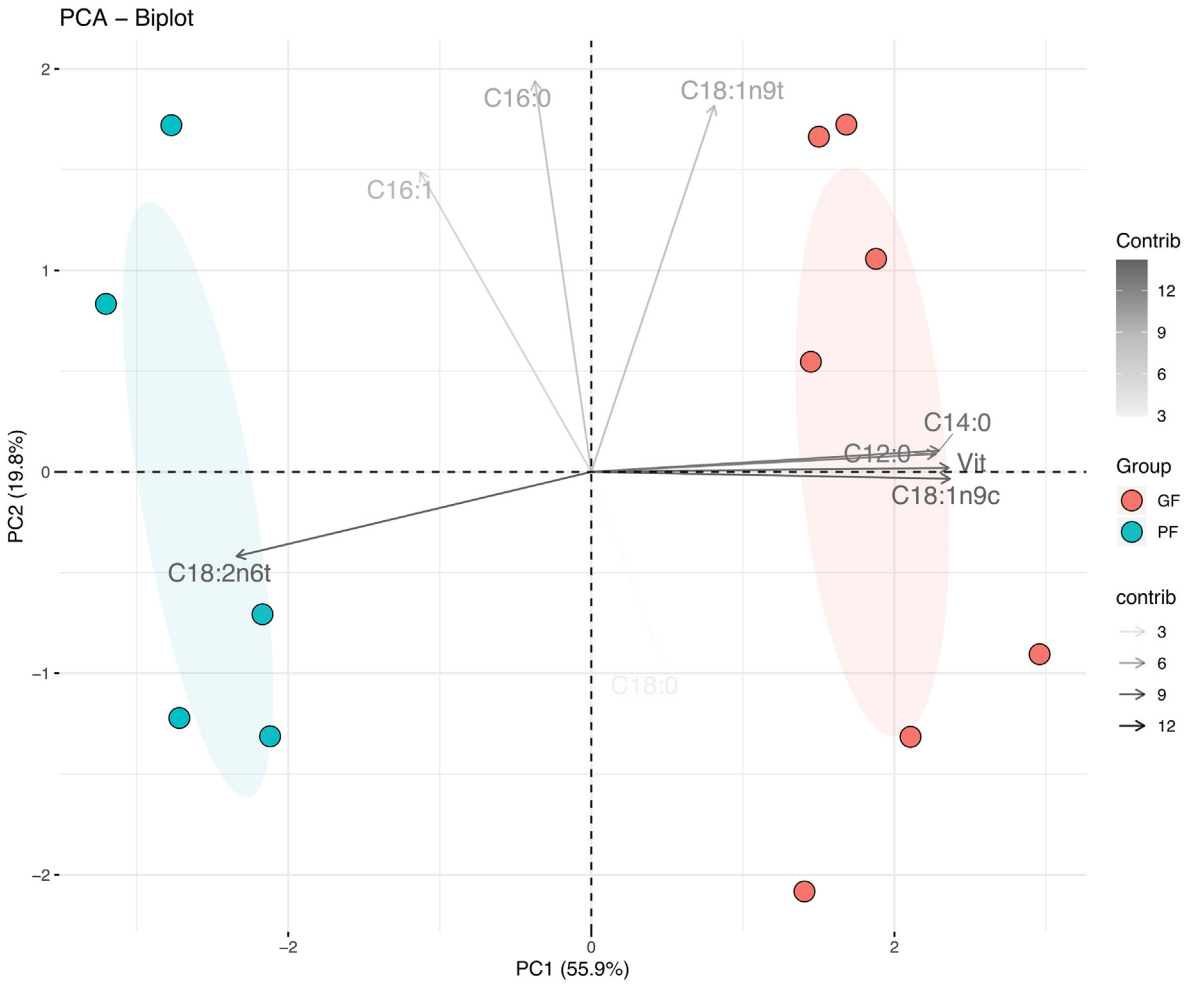

Fig. 1. Biplot of stallions and the main variables for the first (PC1) and second (PC2) principal components. Each vector represents a variable (fatty acid or post-thaw sperm vitality), and the correlation of two variables is reflected by the angle between the two corresponding vectors. The color scale and the length of each vector are related to the influence —or loading— of the variable into that component. Ellipses and shapes (enveloping 95% confidence intervals) show two distinct groups of the stallions (GF, good freezer and PF, poor freezer, as red and green dots, respectively). The percent variance captured by each PC is shown in parenthesis along each axis. Vit = post-thaw sperm vitality. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reduced vitality of frozen/thawed spermatozoa isolated from PF stallions.

Finally, the predictive value of the relative abundance of C12:0, C14:0, C18:1n9c, C18:2n6t regarding equine sperm freezability deserves to be further tested in other independent studies involving a larger number of horses. Moreover, to confirm and explore the specific role of these fatty acids, it should also be necessary to analyze them before and after sperm freezing, as well as modify the lipid composition of the membrane of spermatozoa, both by *in vitro* approaches and/or by nutritional intervention. For example, it is very likely that the levels of C18:2n6t in the total lipids of PF spermatozoa could be due to specific diets, taken by these stallions, and containing foods rich in this non-standard fatty acid, that derives from industrial processes of hydrogenation of vegetal oils [33,34]. Future studies should be dedicated to measure the same fatty acids, possibly together with their probable peroxidation products, in post-thaw samples (Fig. 2). This would allow confirming the hypothesis that a high level of unsaturated fatty acids, as well as of certain *trans* fatty acids, represents a detrimental factor for the freezability and cryopreservation of spermatozoa, through the formation of a higher level of organic hydroperoxides [31].

Although more samples will be needed to fully validate these findings, this is the first time that the presence of linolelaidic acid (C18:2n6t) is documented in the membranes of fresh equine spermatozoa and that its content is strongly associated with the reduced vitality/motility in fresh and post-thaw cells, as well as with the lower sperm freezability. Finally, we found that a high ratio of unsaturated to saturated fatty acids is associated with reduced sperm functionality and resistance to freezing.

CRediT authorship contribution statement

Sergio Oddi: Conceptualization, Methodology, Formal analysis, Multivariate analysis, Writing – original draft, preparation and writing-review and editing. **Augusto Carluccio:** Conceptualization, Methodology, Formal analysis, Writing – original draft, preparation and writing-review and editing. **Francesca Ciaramellano:** Investigation. **Marcello Mascini:** Investigation, Gas chromatographic analysis. **Roberta Bucci:** Investigation, Methodology, Formal analysis. **Mauro Maccarrone:** Writing – original draft, preparation and writing-review and editing. **Domenico Robbe:** Methodology, Formal analysis, Writing – original draft, preparation and writing-review and editing. **Enrico Dainese:** Conceptualization, Writing – original draft, preparation and writing-review and editing.

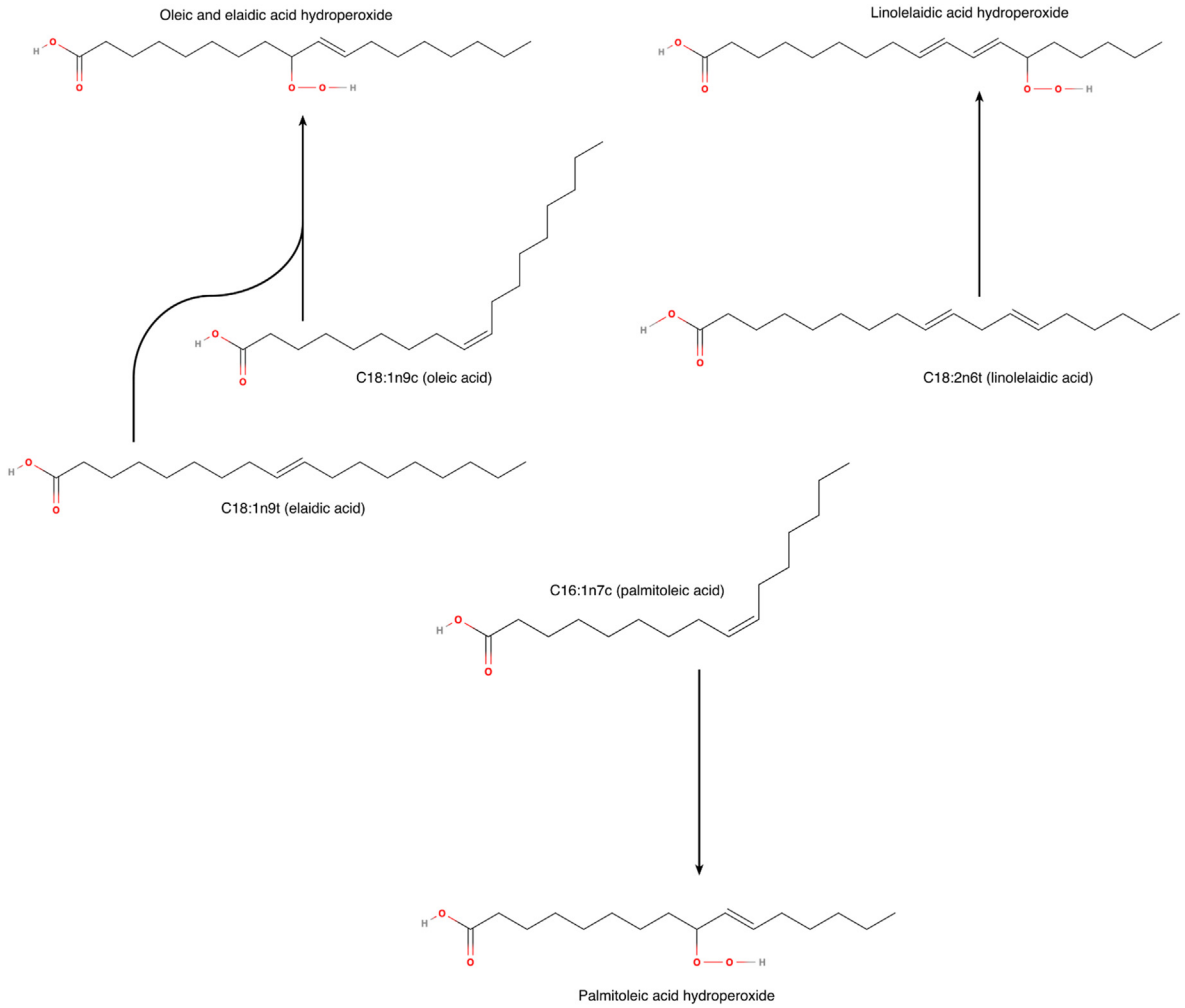

Fig. 2. Representation of the chemical structures of the main unsaturated fatty acids found in sperm membranes of GF and PF stallions, along with their possible hydroperoxide derivatives.

Declaration of competing interest

The authors declared no conflict of interest.

Acknowledgements

The present study has been carried out in the framework of the Project "Demetra" (Dipartimenti di Eccellenza 2018–2022, CUP_C46C18000530001), funded by the Italian Ministry for Education, University and Research.

References

[1] Ezzati M, Shanehbandi D, Hamdi K, Rahbar S, Pashaiasl M. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. Cell Tissue Bank 2020;21. https://doi.org/10.1007/s10561-019-09797-0.
[2] Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. Reprod Biomed Online 2018;37:327–39. https://doi.org/10.1016/j.rbmo.2018.05.012.
[3] Barbas JP, Mascarenhas RD. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 2009;10:49–62. https://doi.org/10.1007/s10561-008-9081-4.
[4] Tischner M. Evaluation of deep-frozen semen in stallions. J Reprod Fertil Suppl 1979;53–9.
[5] Vidament M, Dupere AM, Julienne P, Evain A, Noue P, Palmer E. Equine frozen semen: freezability and fertility field results. Theriogenology 1997;48:907–17. https://doi.org/10.1016/S0093-691X(97)00319-1.
[6] Al-Essawe EM, Johannisson A, Wulf M, Aurich C, Morrell JM. Addition of seminal plasma to thawed stallion spermatozoa did not repair cryoinjuries. Anim Reprod Sci 2018;196:48–58. https://doi.org/10.1016/j.anireprosci.2018.06.018.
[7] Brinsko S, Van G, Jg-J of reproduction. 2000 U. Motility, morphology and triple stain analysis of fresh, cooled and frozen-thawed stallion spermatozoa. J Reprod Fertil 2000;56:111–20.
[8] Amann RP, Pickett BW. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7:145–73. https://doi.org/10.1016/S0737-0806(87)80025-4.
[9] Mandal R, Badyakar D, Chakrabarty J. Role of membrane lipid fatty acids in sperm cryopreservation. Adv Androl 2014;2014:1–9. https://doi.org/10.1155/2014/190542.
[10] Macías García B, González Fernández L, Ortega Ferrusola C, Morillo Rodríguez A, Gallardo Bolaños JM, Rodríguez Martinez H, et al. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. Theriogenology 2011;75:811–8. https://doi.org/10.1016/j.theriogenology.2010.10.021.
[11] Esmaeili V, Shahverdi AH, Moghadasian MH, Alizadeh AR. Dietary fatty acids affect semen quality: a review. Andrology 2015;3:450–61. https://doi.org/10.1111/andr.12024.
[12] Macías García B, González Fernández L, Ortega Ferrusola C, Salazar-Sandoval C, Morillo Rodríguez A, Rodríguez Martinez H, et al. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. Reprod Domest Anim 2011;46:141–8. https://doi.org/10.1111/j.1439-0531.2010.01609.x.
[13] Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology 2005;63:1519–27. https://doi.org/10.1016/j.theriogenology.2004.07.010.
[14] Ortega-Ferrusola C, Sotillo-Galán Y, Varela-Fernández E, Gallardo-Bolaños JM, Muriel A, González-Fernández L, et al. Detection of 'apoptosis-like' changes during the cryopreservation process in equine sperm. J Androl 2008;29:

213–21. https://doi.org/10.2164/jandrol.107.003640.
[15] Lenzi A, Picardo M, Gandini L, Dondero F. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum Reprod Update 1996;2:246–56. https://doi.org/10.1093/humupd/2.3.246.
[16] Aitken RJ, Wingate JK, De Iuliis GN, Koppers AJ, McLaughlin EA. *Cis* -unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. J Clin Endocrinol Metab 2006;91:4154–63. https://doi.org/10.1210/jc.2006-1309.
[17] Ortega Ferrusola C, González Fernández L, Morrell JM, Salazar Sandoval C, Macías García B, Rodríguez-Martinez H, et al. Lipid peroxidation, assessed with BODIPY-C 11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction 2009;138:55–63. https://doi.org/10.1530/REP-08-0484.
[18] Hotelling H. Analysis of a complex of statistical variables into principal components. J Educ Psychol 1933;24:417–41. https://doi.org/10.1037/h0071325.
[19] Wold S, Esbensen K, Geladi P. Principal component analysis. Chemometr Intell Lab Syst 1987;2:37–52. https://doi.org/10.1016/0169-7439(87)80084-9.
[20] Cabrera T, Ramires-Neto C, Belaz KRA, Freitas-Dell'aqua CP, Zampieri D, Tata A, et al. Influence of spermatozoal lipidomic profile on the cryoresistance of frozen spermatozoa from stallions. Theriogenology 2018;108:161–6. https://doi.org/10.1016/j.theriogenology.2017.11.025.
[21] Al-Essawe EM, Wallgren M, Wulf M, Aurich C, Macías-García B, Sjunnesson Y, et al. Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. Theriogenology 2018;115:99–107. https://doi.org/10.1016/j.theriogenology.2018.04.021.
[22] Loomis PR. Advanced methods for handling and preparation of stallion semen. Vet Clin N Am Equine Pract 2006;22:663–76. https://doi.org/10.1016/j.cveq.2006.07.002.
[23] Folch J, Lees M, Stanley Sloane GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.
[24] Bitman J, Wood L, Hamosh M, Hamosh P, Mehta N. Comparison of the lipid composition of breast milk from mothers of term and preterm infants. Am J Clin Nutr 1983;38:300–12.
[25] Helrich K. Official Methods Of Analysis Of The Association Of Official Analytical Chemists. Fifteenth. Arlington; 1990.
[26] R Core Team. R. A Language And Anvironment For Statistical Computing. 2019.
[27] Harrell F. Hmisc: Harrell Miscellaneous. 2020.
[28] Le S, Josse J, Husson F. FactoMineR: an R package for multivariate analysis. J Stat Software 2008;25:1–18.
[29] Wickham H. ggplot2: Elegant Graphics For Data Analysis. New York: Springer-Verlag: Springer-V; 2016.
[30] Dziuban CD, Shirkey EC. When is a correlation matrix appropriate for factor analysis? Some decision rules. Psychol Bull 1974;81:358–61. https://doi.org/10.1037/h0036316.
[31] Alvarez JG, Storey B. Evidence of increased lipid peroxidative damage and loss of superoxide activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992;13:232–41.
[32] Khosrowbeygi A, Zarghami N. Fatty acid composition of human spermatozoa and seminal plasma levels of oxidative stress biomarkers in subfertile males. Prostagl Leukot Essent Fat Acids 2007;77:117–21. https://doi.org/10.1016/j.plefa.2007.08.003.
[33] Bhattacharya A, Banu J, Rahman M, Causey J, Fernandes G. Biological effects of conjugated linoleic acids in health and disease. J Nutr Biochem 2006;17:789–810. https://doi.org/10.1016/j.jnutbio.2006.02.009.
[34] Gonçalves DC, Lira FS, Carnevali LC, Rosa JC, Pimentel GD, Seelaender M. Conjugated linoleic acid: good or bad nutrient. Diabetol Metab Syndrome 2010;2. https://doi.org/10.1186/1758-5996-2-62.