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ORIGINAL ARTICLE OPEN ACCESS

Predictive Indicators of Cryotolerance and Fertility in Bovine Sperm: Evaluating Fresh Semen Quality to Improve AI Outcomes With Frozen–Thawed Sperm

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Received: 21 June 2024 | **Revised:** 30 September 2024 | **Accepted:** 27 October 2024

Funding: This work was supported by Ministry of Science and Innovation, Spain (AGL2017-88329-R and PID2020-113320RB-I00).

Keywords: bull ejaculates | cryotolerancia | fertility | sperm quality

ABSTRACT

The success of artificial insemination (AI) with frozen–thawed semen in cattle is influenced by both female factors and sperm quality. In terms of sperm quality, prior studies indicate that the ability of frozen–thawed bovine sperm to fertilise an oocyte is dependent on their quality and resilience to cryopreservation. Cryopreservation induces oxidative stress, leading to ultrastructural damage in the sperm. This study aimed to determine whether the quality of fresh semen can identify bulls with good and poor sperm freezability. This difference between fresh and frozen semen from the same bull allows us to predict fertility. Motility and kinetic parameters were assessed using computer-assisted sperm analysis (CASA), while six functional variables were evaluated through flow cytometry, both before and after the freeze–thaw process on the sperm from 13 bulls. In vivo fertility was measured using 90-day non-return rates. The principal component analysis (PCA) of eight sperm variables post-thaw identified one principal component explaining 81.19% of the total variance and classified the bulls into two groups: Poor freezability bulls (progressive motility: 48.12% ± 8.41%; viability: 77.51% ± 7.61%) and good freezability bulls (progressive motility: 58.64% ± 6.64%; viability: 88.12% ± 2.52%). Bulls with higher freezability showed better sperm viability and motility, as well as lower levels of ROS, superoxides and intracellular calcium before cryopreservation that were significantly correlated with higher non-return rates (NRR). The results underscore the importance of assessing the quality and functionality of fresh semen to predict the fertility potential of cryopreserved sperm. This approach can aid in selecting ejaculates with the best potential for successful artificial insemination, ultimately improving reproductive performance in dairy cattle.

1 | Introduction

Artificial insemination (AI) with frozen–thawed semen is a common, widely employed breeding technique in the cattle industry. One of the reasons why cryopreserved sperm is frequently used for artificial insemination is that the freeze-thawing protocol is relatively standardised (REF). In fact, bovine spermatozoa are known for their relatively high cryotolerance compared to those from horses, pigs and dogs (Peris-Frau et al. 2020).

In dairy cattle, low reproductive performance is usually attributed to the female because of an inadequate energy

balance that is often associated with low body condition score or excessive body condition loss. Several studies indicate that cows with lower body condition scores and loss of weight have lower conception rates, embryonic resorption and decreased efficiency of heat detection in comparison to cows with regular condition scores (Nazhat et al. 2021). In spite of this—and in addition to female factors—the semen also plays a significant role in the reproductive success, as non-return rates (NNRs) are widely reported to vary among bovine sires (Stålhammar, Janson, and Philipsson 1994). While some of the male factors underlying differences in individual fertility include proteins from sperm, including histones (Kutchy et al. 2017), and from seminal plasma (Kasimanickam et al. 2019), why NNRs differ between bulls remains in most cases unknown.

Despite many researchers having realised that the semen is also important for cattle fertility, it has often been ignored that one of the reasons why fertility could differ between bulls would not be the intrinsic fertility of sperm but rather their ability to withstand freeze-thawing. In this context, worthy of notice is that cryopreservation induces oxidative and osmotic stress, which results in ultrastructural sperm damage—including reduction of sperm motility and survival, DNA fragmentation and impaired mitochondrial function—that ultimately leads to decreased fertility (Khan et al. 2021; Akhtar et al. 2022; Yánez-Ortiz et al. 2022). Among other proteins, aquaporins (AQPs), which have been identified in bovine spermatozoa, play a significant role in the transport of water and permeable cryoprotectants across the plasma membrane; particularly, AQP3, AQP7 and AQP11 have been found to be related to sperm cryotolerance (Prieto-Martínez et al. 2017; Morató et al. 2018). Noticeably, the relative content of AQP11 in fresh bovine sperm was even seen to predict the in vitro and in vivo fertilising ability of frozen–thawed sperm (Morató et al. 2018). These findings suggest that not only does the capability of frozen–thawed bovine sperm to fertilise an oocyte rely upon their intrinsic fertility but also upon their resilience to cryopreservation. Because of the aforementioned low reproductive performance of dairy cattle, it is imperative, particularly when AI is conducted using cryopreserved sperm, to ensure that the best sperm in terms of both cryotolerance and fertility is selected, and thus a strategy to determine which ejaculates are worthy to be cryopreserved is needed. For this reason, addressing whether or not the evaluation of sperm quality and functionality in fresh semen can predict the fertility of cryopreserved sperm is of substantial relevance for dairy cattle breeding.

The aim of this study was, therefore, to determine if the quality of fresh semen can allow for the identification of good and poor freezability bulls, which does not only include function and survival of frozen–thawed sperm but also their fertilising ability. In order to address this aim, sperm motility was evaluated by means of a computer-assisted sperm analysis (CASA), and viability and other functional variables were determined with flow cytometry before and after freeze-thawing. This provided, for each sperm quality/functionality variable, the ratio between fresh and frozen–thawed sperm, as a measure of cryotolerance. Also, frozen–thawed sperm was used for AI, and conception rates were recorded.

2 | Materials and Methods

2.1 | Semen Samples

The study involved 13 bulls from Asturiana de los Valles (*Bos taurus*, $n=6$) and Holstein ($n=7$) breeds, with ages ranging from 14 to 24 months. Bulls were housed in a regular artificial insemination (AI) centre (Centro de Inseminación Artificial de Cenero; Gijón, Asturias, Spain). Animals were regularly collected using an artificial vagina (inside temperature: 45°C), and semen was evaluated based on volume, sperm concentration, sperm morphology and motility by subjective visual assessment using a phase contrast microscope at 200× magnification.

2.2 | Sperm Cryopreservation

Semen was extended with Bioxcell (IMV, L'Aigle, France) at 20°C to a final concentration of 92×10^6 sperm/mL using a photometer (SDM 4; Minitüb, Tiefenbach, Germany). The extended semen was cooled from 22°C to 5°C for 1.5 h (at a cooling rate of approximately 0.2°C/min) and then left at 5°C for further 2.5 h. At the end of the cooling period (4 h), each semen sample was split into two aliquots. One was transported to the laboratory for analysis (chilled semen), and the other was cryopreserved. For this purpose, the sperm were packaged into 0.25 mL straws (23×10^6 sperm/straw); these straws were then loaded into a programmable freezer (Digitcool; IMV Technologies, L'Aigle, France) and cryopreserved using the standard curve for bovine sperm (5°C/min from 4°C to −10°C; 40°C/min from −10°C to −100°C and 20°C/min from −100°C to −140°C). For thawing, straws were thawed at 37°C for 40 s; for each ejaculate, three straws were simultaneously thawed, and their contents were pooled in a 5 mL tube. The thawed sperm were incubated at 37°C in the dark for 2 h.

2.3 | Evaluation of Sperm Motility

Total and progressive motility and sperm kinetic parameters were determined with a CASA system (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). Five microlitre per sample (fresh or frozen–thawed) was placed onto a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and observed under a negative phase contrast field (Olympus 10×0.30 PLAN objective; Olympus, Tokyo, Japan). The CASA system used was based on the analysis of 16 consecutive, digitalised photographic images which were taken on a time lapse of 0.64 s, which implied a velocity of image-capturing of one photograph every 40 ms. For each sample, 1000 sperm were counted, and three different replicates were examined. The following sperm motility parameters were recorded: Total motility (TMOT, %); progressive motility (PMOT, %); curvilinear velocity (VCL, mm/s); straight-line velocity (VSL, mm/s); average pathway velocity (VAP, mm/s); amplitude of lateral head displacement (ALH, mm); beat-cross frequency (BCF, Hz); linearity (LIN, %) that resulted from LIN = VSL/VCL × 100; straightness (STR, %), which was calculated as VSL/VAP × 100 and oscillation index (WOB, %), obtained from VAP/VCL × 100. A motile sperm was defined as having VAP ≥ 10 mm/s, and a sperm cell

was considered as progressively motile when its STR was at least 70%. In addition, the percentage of progressively motile spermatozoa among the population of motile sperm was calculated. For each parameter, the corresponding mean and standard error of the mean (SEM) were calculated.

2.4 | Flow Cytometry

Six different parameters were assessed through flow cytometry in ejaculated bulls and in sperm doses: Sperm plasma membrane integrity, acrosome membrane integrity, membrane lipid disorder, intracellular levels of superoxide (O_2^-) radicals, intracellular levels of hydrogen peroxide (H_2O_2) and intracellular levels of calcium. All fluorochromes were obtained from ThermoFisher Scientific (Waltham, MA, United States) unless otherwise stated. Sperm samples were diluted in prewarmed PBS to a final concentration of 2×10^6 cells/mL in a final volume of 200 μL before they were stained with a combination of fluorochromes with the corresponding protocol. After staining, samples were incubated at 38°C in the dark. A total of 10,000 sperm cells for each of the two technical replicates were evaluated for every sample and sperm parameter.

These assessments were conducted using a Cell Lab Quanta SC cytometer (Beckman Coulter; Fullerton; CA, USA). Samples were excited with an argon ion laser (488 nm) at a power of 22 mW. Cell diameter/volume was assessed using the Coulter principle, which measures electrical resistance changes caused by suspended, non-conductive particles in an electrolyte solution. In this system, forward scatter (FS) was replaced by electronic volume (EV), and for EV channel calibration, 10 μm Flow-Check fluorospheres (Beckman Coulter) were positioned at channel 200 on the EV scale.

Three optical filters were used for fluorescence detection: FL1 (Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width 505–545 nm); FL2 (DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm); and FL3 (LP filter: 670/730 nm, detection width: 655–685 nm). FL1 was used to detect green fluorescence from SYBR-14, YO-PRO-1, fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA; PNA-FITC), JC-1 monomers, Fluo-3, 2′,7′-dichlorofluorescein (DCF^+), and FL3 was used to detect red fluorescence from propidium iodide (PI), merocyanine 540 (M540) and ethidium (E^+). The signal was logarithmically amplified, and photomultiplier settings were adjusted according to particular staining methods.

2.4.1 | Evaluation of Plasma Membrane Integrity

Sperm viability was evaluated by assessing their membrane integrity using the LIVE/DEAD sperm viability kit (Molecular Probes; Eugene, OR, USA), which follows a modified protocol from Garner and Johnson (1995). Sperm were incubated in the presence of SYBR-14 (final concentration: 100 nmol/L) for 10 min, and PI (final concentration: 12 μmol/L) was subsequently added prior to an additional incubation of 5 min.

After staining and evaluation with the flow cytometer, three sperm populations could be discriminated in dot-plots: (1) viable green-stained sperm ($SYBR\text{-}14^+/PI^-$); (2) non-viable red-stained spermatozoa ($SYBR\text{-}14^-/PI^+$) and (3) moribund spermatozoa stained both green and red ($SYBR\text{-}14^+/PI^+$); unstained, non-sperm particles ($SYBR\text{-}14^-/PI^-$) were not included for the calculation of the three previously mentioned populations.

2.4.2 | Evaluation of Acrosome Integrity

Acrosome membrane integrity was evaluated following the protocol from Nagy et al. (2003). Sperm were co-incubated in the presence of PNA-FITC (final concentration: 2.5 μg/mL) and PI (final concentration: 12 μmol/L) for 10 min.

Samples were co-stained with peanut agglutinin (PNA) conjugated to fluorescein isothiocyanate (FITC; PNA-FITC) and PI. As sperm were not permeabilised, four sperm populations were identified: (1) viable sperm with an intact acrosome ($PNA\text{-}FITC^-/PI^-$); (2) non-viable sperm with an outer acrosome membrane that could not be fully intact ($PNA\text{-}FITC^+/PI^+$); (3) non-viable sperm with an exocytosed acrosome ($PNA\text{-}FITC^-/PI^+$); and (4) sperm with damaged plasma membrane ($PNA\text{-}FITC^+/PI^-$). The percentage of $SYBR\text{-}14^-/PI^-$ particles corresponding to non-sperm debris was used to correct the percentage of viable sperm with an intact acrosome ($PNA\text{-}FITC^-/PI^-$), and the percentages of the other sperm populations were recalculated.

2.4.3 | Evaluation of Sperm Membrane Lipid Disorder

Membrane lipid disorder of sperm was evaluated with M-540 and YO-PRO-1 co-staining, following the procedure of Rathi et al. (2001) with minor modifications (Yeste et al. 2014). In brief, samples were co-stained with M540 (final concentration: 2.6 μmol/L) and YO-PRO-1 (final concentration: 25 nmol/L) and then incubated for 10 min. When packaging of phospholipids decreases, the M-540 fluorochrome intercalates in the outer monolayer of the sperm plasma membrane. The combination of the two fluorochromes allowed for the discrimination of four populations: (1) viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$); (2) viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$); (3) non-viable spermatozoa with low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$) and (4) non-viable spermatozoa with high membrane lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$). The percentage of $SYBR\text{-}14^-/PI^-$ particles corresponding to non-sperm debris was used to correct the percentage of viable spermatozoa with a low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$), and the percentages of the other sperm populations were recalculated. Data were not compensated. Membrane lipid disorder was assessed through the calculation of the percentage of viable sperm with a low membrane lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$ sperm) from the population of viable sperm ($YO\text{-}PRO\text{-}1^-$).

2.4.4 | Evaluation of Intracellular Levels of ROS

Total levels of ROS were detected through staining with H_2DCFDA and PI following the protocol from Guthrie and Welch (2006). In the presence of ROS, the non-fluorescent probe H_2DCFDA is intracellularly de-esterified and oxidised

into highly fluorescent DCF^+. Samples were incubated with H_2DCFDA (final concentration: 200 μmol/L) and PI (final concentration: 12 μmol/L) at 38°C for 20 min. Four subpopulations were identified in dot-plots: (1) viable sperm with low levels of ROS (DCF^-/PI^-); (2) non-viable sperm with low levels of ROS (DCF^-/PI^+); (3) viable sperm with high levels of ROS (DCF^+/PI^-) and (4) non-viable sperm with high levels of ROS (DCF^+/PI^+). The percentage of SYBR-$14^-/PI^-$ particles corresponding to non-sperm debris was used to correct the percentage of viable sperm with low ROS levels (DCF^-/PI^-), and the percentages of the other sperm populations were recalculated.

2.4.5 | Evaluation of Intracellular Levels of Superoxide (O_2^-) Radicals

Superoxide levels (O_2^-) were detected following a modification of the procedure described by Guthrie and Welch (2006). In brief, samples were co-stained with hydroethidine (HE) and YO-PRO-1. In the presence of O_2^- radicals, HE is oxidised into ethidium (E^+). Samples were incubated with HE (final concentration: 4 μmol/L) and YO-PRO-1 (final concentration: 25 nmol/L) at 38°C for 20 min. Four subpopulations were identified: (1) viable sperm with low levels of superoxides (E^-/YO-PRO-1^-); (2) non-viable sperm with low levels of superoxides (E^-/YO-PRO-1^+); (3) viable sperm with high levels of superoxides (E^+/YO-PRO-1^-) and (4) non-viable sperm with high levels of superoxides (E^+/YO-PRO-1^+). The percentage of SYBR-$14^-/PI^-$ particles corresponding to non-sperm debris was used to correct the percentage of viable sperm with low intracellular levels of O_2^- (E^-/YO-PRO-1^-), and the percentages of the other sperm populations were recalculated.

2.4.6 | Evaluation of Intracellular Calcium Levels

Intracellular calcium levels in sperm were determined using the protocol described by Harrison, Mairet and Miller (1993), with minor modifications. Samples were incubated at 38°C for 10 min with Fluo3-AM (final concentration 1 μM) and PI (final concentration: 12 μM). Four sperm populations were discriminated in dot-plots: (1) viable sperm with low levels of intracellular calcium (Fluo3-AM^-/PI^-); (2) viable sperm with high levels of intracellular calcium (Fluo3-AM^+/PI^-); (3) non-viable sperm with low levels of intracellular calcium (Fluo3-AM^-/PI^+) and (4) non-viable sperm with high levels of intracellular calcium (Fluo3-AM^+/PI^+). The percentage of SYBR-$14^-/PI^-$ particles corresponding to non-sperm debris was used to correct the percentage of viable sperm with low intracellular levels of calcium (Fluo3-AM^-/PI^-), and the percentages of the other sperm populations were recalculated.

2.4.7 | Evaluation of Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined following a modified protocol from Ortega-Ferrusola et al. (2008). Three sperm populations could be distinguished in flow cytometry dot-plots: (1) sperm with low mitochondrial membrane potential (green-stained); (2) spermatozoa with high mitochondrial membrane potential (orange-stained) and (3) spermatozoa with heterogeneous mitochondria (green- and orange-stained in the same cell).

2.5 | In Vivo Fertility

In vivo fertility was assessed through 90-day non-return rates (NRR). These rates corresponded to the proportion of cows that did not return to estrus after 90 days of AI and were obtained by dividing the number of pregnant cows by the total number of inseminations. The average number of total inseminated cows per bull was 557 (with a minimum of 300 calves per bull).

2.6 | Statistical Analyses

Data were analysed with a statistical package (IBM SPSS for Windows, Ver. 27.0; IBM Corp., Armonk, NY, USA). First, data were checked for normal distribution and homogeneity of variances with Shapiro–Wilk and Levene tests. To set the two freezability groups, eight CASA and flow cytometry variables evaluated at post-thaw (total motility, progressive motility, %SYBR$14^+/PI^-$ sperm, %PNA^-/PI^- sperm, %$M540^-$/YO-PRO-1^- sperm, %E^-/YO-PRO-1^- sperm, %DCF^-/YO-PRO-1^- sperm and %Fluo$3^-/PI^-$ sperm) were subjected to principal component analysis (PCA; settings: Varimax procedure and Kaiser normalisation). As a result, a regression score for the principal component was returned for the statistical case (i.e., each sample). These scores were then used as continuous variables to run a two-step cluster analysis (settings: Distance likelihood distance and Bayesian information criterion), which allowed for the classification of individuals as with good or poor freezability. Thereafter, and for each sperm variable, a resistance percentage (%R), resulting from the quotient between the value after freeze-thawing and the value before freeze-thawing and multiplied by 100, was calculated. %R is a variable that determines the percentage of spermatozoa that survive freezing. It is an intrinsic property unique to each bull. Consequently, the resistance can vary from the ejaculate to the thawed semen, depending on the individual. Following this, the resistance percentages of sperm variables were compared between the two groups of freezability (i.e., good and poor) with a *t* test for independent samples. Also, the values of each sperm variable before and after freeze-thawing were compared through a linear mixed model (intrasubject factor: Before and after freeze-thawing; intersubject factor: Good or poor freezability) with post hoc Sidak's test for pairwise comparisons. Correlation analysis was also conducted between all sperm variables (including resistance percentages [%R]) and NRR after 90 days of servicing. The level of significance was set at $p \leq 0.05$.

3 | Results

3.1 | Principal Component Mainly Summarises the Variance of Post-Thaw Bovine Sperm Quality

The PCA ran with eight sperm variables evaluated at post-thaw (% total motility, % progressive motility, % SYBR$14^+/PI^-$ sperm, % PNA-$FITC^-/PI^-$ sperm, % $M540^-$/YO-PRO-1^- sperm, % E^-/

YO-PRO-1$^-$, % DCF$^-$/PI$^-$ and % Fluo3$^-$/PI$^-$ sperm) retrieved one principal component that explained 81.19% of the total variance (Tables 1 and 2).

3.2 | Cluster Analysis Revealed Two Groups of Bull in Terms of Sperm Cryotolerance

Based on the regression scores of principal component one, a cluster analysis was conducted with data from the 13 bulls. Animals were classified as with optimal ($n=7$) and non-optimal freezability ($n=6$). The two groups were further compared in terms of sperm quality before (Figure 1) and after freeze-thawing (Figure 2), as well as on the resistance percentage to cryopreservation (Figure 3).

3.3 | Ejaculates With Greater Sperm Viability and Motility Before Cryopreservation Exhibit a Greater Resilience to Freeze-Thawing

Ejaculates from bulls with higher freezing %R exhibited greater percentages of progressively motile (58.64% ± 6.66%) and viable (88.12% ± 2.52%) sperm compared to ejaculates that froze less effectively (48.12% ± 8.41% and 77.50% ± 9.61%, respectively) ($p<0.05$). Remarkably, bulls with optimal freezability demonstrated lower levels of total ROS, superoxides (O_2^-) and intracellular calcium (Ca^{2+}) before cryopreservation. Remarkably, this significant difference ($p<0.05$) was observed in fresh semen prior to freezing (Figure 1).

3.4 | Evaluation of the Resistance Parameter Provides Further Information on Ejaculate Freezability

In this study, the results obtained for the thawed seminal doses and the calculation of seminal resistance percentage indicated that bulls' semen exhibiting higher viability, less acrosomal damage, reduced lipid disorder and lower levels of ROS, O_2^- and intracellular calcium during freezing, demonstrating better resistance to it (Figure 2).

The evaluation of the resistance to freezing of the separate variables (Figure 3) was similar to the results observed at post-thawing. Indeed, as regards to fresh samples, bulls with greater resistance to freezing were those with the least sperm cell damage; when it comes to post-thawing samples, the bulls with the highest amount of total ROS and with viable sperm but damaged acrosome also resist freezing the best.

3.5 | Resistance Percentage of Total Motility, Percentages of DCF$^+$/PI$^-$ Sperm and of Fluo3$^+$/PI$^-$ to Correlation Is Correlated to NRR

Among all the parameters, three were found to predict the freezability of fresh semen: total motility. Therefore, if the frozen parameters' resistance relative to fresh ones is calculated, we can predict the fertility of frozen semen, calcium, and ROS exhibited variations before and after freezing (sperm %R).

As Table 3 shows, %R of three sperm variables to cryopreservation was significantly correlated to NRR. Specifically, higher resistance percentage of total motility and of the percentage of Fluo3$^+$/PI$^-$ to cryopreservation were associated with greater fertility of frozen–thawed sperm ($p<0.05$). On the other hand, the correlation between ROS and fertility was negative, indicating that the resistance of the percentage of viable sperm with high ROS levels (DCF$^+$/PI$^-$) was negatively correlated to NRR.

In contrast to the aforementioned, the two groups of bulls did not show significant differences in their NRR.

Interesting correlations between the eight variables studied were found ($p<0.05$). Specifically, lower lipid membrane disorder was associated with higher sperm viability, higher intracellular calcium levels, less acrosomal damage and a lower number of reactive oxygen species (ROS) in the sperm cells.

TABLE 1 | Components obtained after principal component analysis (PCA), which included % total motility, % progressive motility, % SYBR14$^+$/PI$^-$ sperm; % PNA-FITC$^-$/PI$^-$ sperm, % M540$^-$/YO-PRO-1$^-$ sperm, % E$^-$/YO-PRO-1$^-$ sperm, % DCF$^-$/PI$^-$ sperm and % Fluo3$^-$/PI$^-$ sperm as input variables.

	Initial eigenvalues			Extraction sums of squared loadings		
Component	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	6.49	**81.19**	81.19	6.49	81.19	81.19
2	0.91	11.43	92.62			
3	0.30	3.84	96.47			
4	0.15	1.97	98.44			
5	0.07	0.89	99.33			
6	0.02	0.34	99.68			
7	0.02	0.26	99.94			
8	0.00	0.05	100.00			

Note: Bold value explained the percentage of total variance.

TABLE 2 | Rotated component matrix for principal component 1 (PC1), which retrieved that all original eight variables evaluated in frozen–thawed sperm had a loading score >0.75, representing a variance of 81.19%.

Sperm variables	Loading factors
% Total motility	0.759
% SYBR14$^+$/PI$^-$ sperm	0.967
% Progressive motility	0.753
% PNA-FITC$^-$/PI$^-$ sperm	0.899
% M540$^-$/YO-PRO-1$^-$ sperm	0.957
% E$^-$/YO-PRO-1$^-$ sperm	0.951
% DCF$^-$/PI$^-$ sperm	0.929
% Fluo3$^-$/PI$^-$ sperm	0.963

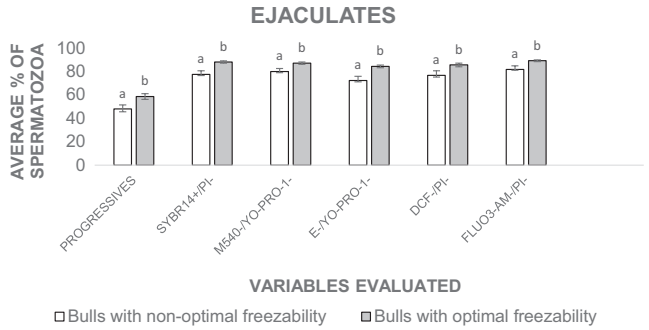

FIGURE 1 | Mean values and standard deviations for six of the eight variables included in the principal component analysis (Table 2), which were determined in the fresh semen of optimal and non-optimal freezability bulls. Different letters (a, b) above columns indicate significant differences ($p < 0.05$) between the two groups.

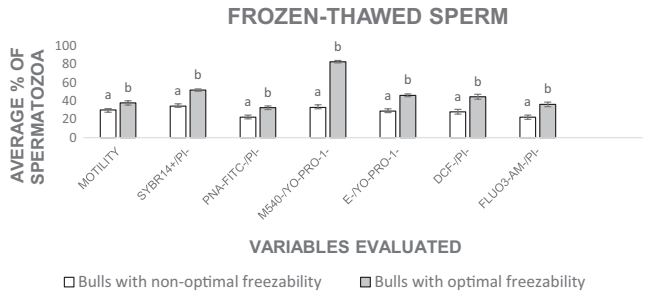

FIGURE 2 | Mean values and standard deviations for seven of the eight variables included in the principal component analysis (Table 2), which were determined in the frozen–thawed sperm of optimal and non-optimal freezability bulls. Different letters (a, b) above columns indicate significant differences ($p < 0.05$) between groups.

4 | Discussion

Our findings found that a greater resistance of total motility and the percentages of DCF$^+$/PI$^-$ and Fluo3$^+$/PI$^-$ is correlated with the fertility rates of frozen–thawed sperm. This indicates that a greater resilience to freeze-thawing is related to a higher reproductive performance, as bulls exhibiting better resistance experienced less damage, as evidenced by enhanced viability, diminished acrosomal damage and lower lipid disorder. These results underscore the significance of assessing semen quality not only post-thaw but also in comprehending the factors influencing resistance during the freezing process and its subsequent correlation with fertility. In addition, the correlation between ROS and fertility was negative, indicating that as ROS levels increased, fertility tended to decrease.

The identification of a potential biomarker for bull semen capable of predicting its freezability and fertility has been a longstanding goal of the cattle artificial insemination industry (Ryu et al. 2019). Consequently, numerous proteomic studies across different species have explored the relationship between specific proteins in bull ejaculates and their capacity for freezing (Wang et al. 2014; Yánez-Ortiz et al. 2022; Dietrich and Ciereszko 2018). Few studies have, however, focused on evaluating seminal quality variables of ejaculated semen to determine biomarkers capable of predicting both freezability and fertility. This gap in research is noteworthy, considering the observed variations in freezing between ejaculates from different bulls and even within ejaculates from the same bull (Alm-Kristiansen 2023).

In cattle, cryopreservation typically results in a gradual reduction of up to 50% in sperm motility and viability post-thawing (Khalil et al. 2018), which is consistent with the results of our study. Our results also demonstrated that ejaculates from bulls with high resistance to freezing exhibit a higher proportion of sperm with progressive motility (58.64%) and a greater percentage of viable sperm (88.12%) compared to ejaculates that freeze less effectively (48.12% and 77.5%, respectively). Similar results were reported by Alm-Kristiansen (2023), who identified a positive correlation between the population of rapidly moving spermatozoa in ejaculates and the quality of frozen semen from the same ejaculate. Yet, Ibanescu, Siuda, and Bollwein (2020) reported that ejaculates with greater sperm viability post-thawing predominantly comprised sperm exhibiting rapid but non-linear movements. These variables, namely motility and viability, are typically considered when assessing semen quality, particularly after cryopreservation. According to Casas et al. (2009), evaluating these parameters enables not only the prediction of sperm fertility potential but also an understanding of their ability to withstand the freezing process across different individuals.

It is widely acknowledged that mammalian spermatozoa are susceptible to cryo-injury resulting from cryopreservation processes, including the formation of ice crystals, the generation of reactive oxygen species (ROS), lipid peroxidation and other factors (Ugur et al. 2019), leading to cellular damage, membrane rupture, acrosomal damage and cell death. Importantly, not all semen freeze uniformly, and certain males with high-quality semen subjected to freezing may experience severe cellular damage, significantly compromising sperm quality (Evans et al. 2021). Related to the above, the sperm quality resulting from freezing in our study has allowed us to classify the animals into optimal and non-optimal freezers.

The variables assessed in this study, specifically membrane lipid disorder, acrosome integrity and ROS were found to be interconnected, as alterations in one of these variables could

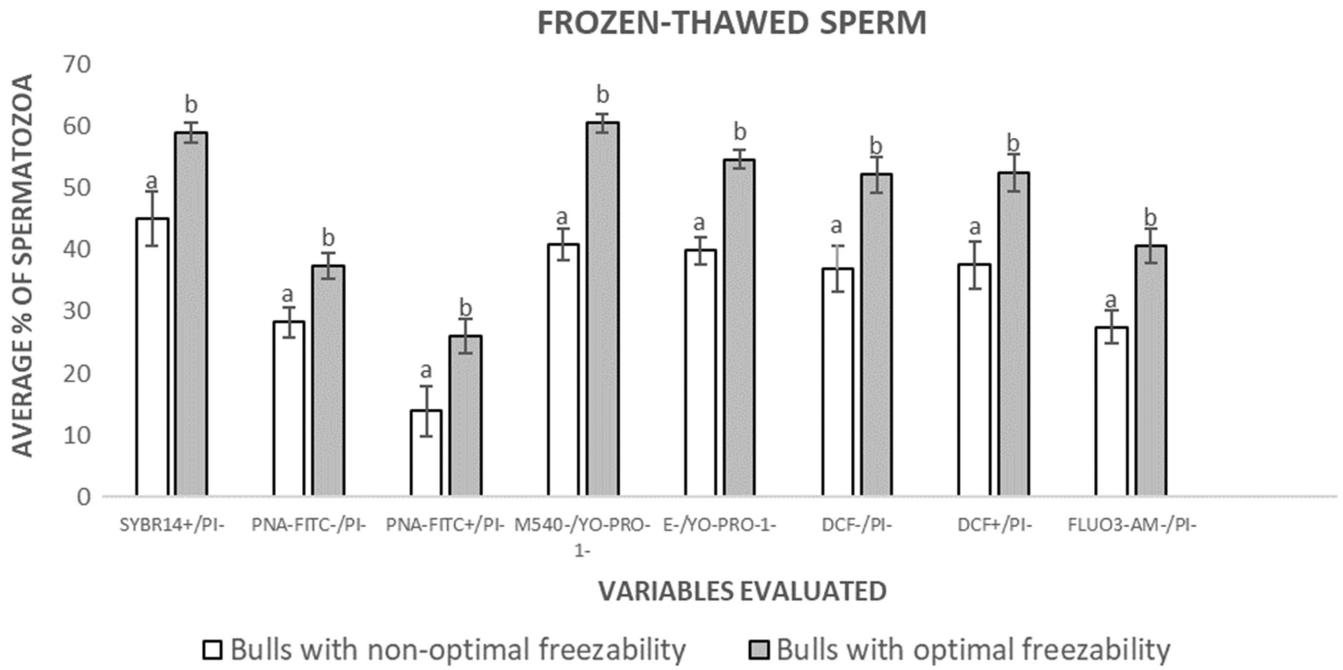

FIGURE 3 | Mean values and standard deviations for eight variables calculated through dividing the value of each variable after freeze-thawing (frozen–thawed sperm) by the value of the same variable before freeze-thawing (fresh semen). Different letters (a, b) above columns indicate significant differences ($p < 0.05$) between groups.

TABLE 3 | Correlations between 90-day non-return rates (NRR_{90}) and the resistance variables (i.e., value of the variable in frozen–thawed sperm divided by the value of the same variable in fresh semen) corresponding to total motility, percentages of viable sperm with high ROS content (DCF^+/PI^-) and percentages of viable sperm with high calcium levels ($Fluo3^+/PI^-$).

	Total motility	DCF^+/PI^-	$Fluo3^+/PI^-$
NRR_{90}	0.621*	−0.648*	0.560

*Indicates that the correlation is statistically significant $p < 0.05$.

have consequences for others. The lipid composition of sperm membranes, including polyunsaturated fatty acids (PUFA), plays a crucial role in sperm physiology and integrity, rendering them susceptible to ROS-induced damage, which affects acrosomal integrity and sperm motility (Evans et al. 2021). Furthermore, changes in acrosome integrity before freezing are associated with post-thawed sperm viability, indicating a close relationship between acrosomal damage and freezing resistance (Khan et al. 2021). Consistent with our findings, reduced damage in these sperm variables prior to freezing suggests improved resistance to freezing and higher post-thaw sperm quality.

Regarding intracellular calcium levels, our results suggested that ejaculates from bulls with good freezing characteristics exhibit lower intracellular calcium levels, resulting in better cryotolerance and post-thaw sperm quality. Our findings align with those of Sushadi et al. (2023), who demonstrated that elevated calcium levels may impede the penetration of cryoprotectants into sperm cells, diminishing their effectiveness in protecting sperm during freezing and thawing, thereby disrupting ion balance across the membrane and leading to membrane damage and reduced sperm viability post-thawing. Additionally, excessive calcium levels can induce oxidative stress and damage the acrosomal membrane, thereby affecting sperm quality (Lahimer et al. 2023).

Several studies have indicated that the use of extracellular and intracellular calcium chelators in semen extenders can have distinct effects on prolonging sperm fertility, underscoring the importance of regulating calcium levels for maintaining fertility. Reducing intracellular calcium has been shown to enhance sperm fertility preservation in certain mammals (Sushadi et al. 2023). Nevertheless, a definitive relationship between intracellular calcium levels and male fertility could not be established based on our findings. In summary, of the spermatozoa from ejaculates that better resist freezing, when thawed, a higher percentage survive and correlate with a higher rate of non-return at 90 days.

5 | Conclusions

A strong correlation is observed between freezing resistance and sperm quality and fertility in bulls. Bulls with better resistance to freezing exhibited less damage, including higher viability, reduced acrosomal damage and lower lipid disorder. The difference between fresh and frozen semen allowed us to predict the fertility of the various bulls in the study.

Production of reactive oxygen species during cryopreservation is related to the fertilising capacity of frozen–thawed bovine sperm. Lower intracellular calcium levels in ejaculates from bulls with good freezing resistance suggested better cryotolerance and post-thaw sperm quality. The findings highlight the

significance of evaluating the quality and functionality of unfrozen semen to anticipate the fertility prospects of frozen sperm. This method can assist in identifying ejaculates with the highest likelihood of successful artificial insemination, leading to enhanced reproductive outcomes in dairy cattle.

Author Contributions

Carolina Tamargo: writing – review and editing, writing – original draft, validation, investigation, formal analysis. **Ferran Garriga:** writing – review and editing, validation, investigation, formal analysis. **Marc Yeste:** writing – review and editing, resources, formal analysis. **Elisabeth Pinart:** resources, supervision, investigation. **Rodrigo Muiño:** writing – review and editing, conceptualization, supervision, validation. **María Teresa Carbajo:** validation, supervision. **Carlos Olegario Hidalgo:** validation, supervision, project administration, investigation, funding acquisition, conceptualization.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in DUGi at http://dugi.udg.edu/.

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