



# Influence of Non-conventional Sperm Quality Parameters on Field Fertility in Ovine

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### Specialty section:

This article was submitted to  
Animal Reproduction -  
Theriogenology,  
a section of the journal  
Frontiers in Veterinary Science

Received: 07 January 2021

Accepted: 06 April 2021

Published: 07 May 2021

### Citation:

Mendoza N, Casao A, Domingo J,  
Quintín F, Laviña A, Fantova E,  
Cebrián-Pérez JA, Muiño-Blanco T  
and Pérez-Pe R (2021) Influence of  
Non-conventional Sperm Quality  
Parameters on Field Fertility in Ovine.  
Front. Vet. Sci. 8:650572.  
doi: 10.3389/fvets.2021.650572

The prediction of the fertilizing ability of a seminal dose continues to be a primary aim in the field of artificial insemination (AI). To achieve this goal, in this study we have included the evaluation of some non-conventional sperm quality markers. A total of 3,906 ewes from 52 different farms were inseminated with 357 refrigerated seminal doses obtained from 45 mature *Rasa Aragonesa* rams. The same samples were used for sperm quality analysis including membrane integrity, capacitation status, oxygen consumption and apoptotic-like markers such as phosphatidylserine translocation (PS), plasmalemma disorganization/mitochondrial membrane potential, caspase activation and DNA damage. Seminal doses from the breeding (B) season presented higher percentages of intact membrane (IM), non permeant (NP) membrane with high mitochondrial membrane potential ( $\Delta\Psi_m$ ) and IM without PS translocation spermatozoa than those from the non-breeding (NB) season. Therefore, we can conclude that there were less spermatozoa showing apoptotic-like features in the seminal doses from the B than the NB season, although these differences did not affect field fertility. Only the percentage of intact membrane, non-capacitated (IM-NC) spermatozoa showed a significant correlation with *in vivo* fertility ( $P = 0.005$ ) and fecundity ( $P = 0.007$ ) values obtained after cervical AI when all data were evaluated. When the data were sorted by season and distance to the farms where AI was performed, the correlation between the percentage of IM-NC spermatozoa and reproductive parameters increased in the NB season and progressively with remoteness from the farms. Some other sperm parameters, like NP with high  $\Delta\Psi_m$ , IM sperm without active caspases and DNA-intact spermatozoa, also showed significant correlations with the reproductive parameters in the sorted data. Moreover, the increment in both the percentage of IM-NC and DNA-intact spermatozoa would increase the probability of obtaining a fertility higher than the mean (>52%), as revealed by a multiple logistic regression analysis. In conclusion, we have identified two seminal markers—the percentage of intact membrane, non-capacitated spermatozoa, and DNA intact spermatozoa—which could be used as a test to discard males in AI programs, which is highly important from an economic point of view and can contribute to achieving satisfactory fertility rates.

**Keywords:** ram, semen, quality, season, fertility, capacitation, apoptosis

## INTRODUCTION

The use of spermatozoa with high fertilizing potential is critical for obtaining good results in reproductive technologies with domestic animals, but this implies the need to know in advance the fertilizing ability of a seminal dose. It is generally assumed that ejaculated spermatozoa with good fertilizing capacity must have progressive motility, normal morphology and intact plasma and acrosomal membranes. However, the standard semen analyses based only on these parameters do not permit an accurate assessment of all sperm functional characteristics, due to over- or under-estimating the fertilizing capacity of a given sample (1–3). Therefore, the consideration of new aspects for the assessment of sperm functionality might help to establish the quality of a seminal dose (4, 5). Fertilization is a process that requires several sperm capacities, and thus the combination of different analysis techniques of semen quality and/or sperm functionality would allow improving the predictive power for the fertility rate (6–8).

Capacitation is a prerequisite for achieving fertilization, but this sperm state must be attained in the oocyte's surroundings. It is well-known that cryopreservation induces an increment in the percentage of capacitated spermatozoa but, in certain species, this increment happens even during cooling to temperatures above freezing (9–12). So, this premature capacitation could diminish the fertility achieved with refrigerated seminal doses when artificial insemination is carried out. Refrigeration has also accounted for an increase in spermatozoa showing certain features related with apoptosis in somatic cells, such as phosphatidylserine translocation, DNA fragmentation, activation of caspases or mitochondrial impairment (13–15). All these types of sub-lethal damage could also impair the reproductive success of refrigerated seminal samples. However, the detrimental effect of apoptotic spermatozoa on field fertility in ovine has not been reported to date. Oxygen is essential for the generation of cellular energy (ATP) via oxidative phosphorylation, and thus oxygen consumption is a key indicator of metabolic activity within cells (16). However, there are scarce reports that link oxygen consumption and fertility (17).

Fertility results in ovine are influenced by seasonality. Seasonal changes in testicular size, sperm production, mating activity and fertility have been described in rams (18, 19). Likewise, differences between breeding and non-breeding seasons in some sperm quality parameters and seminal plasma composition have been shown (20–23).

This work's main objective was to investigate a possible correlation between non-conventional sperm quality parameters, including apoptosis-related markers, capacitation status and oxygen consumption, and field fertility results after artificial insemination (AI) with refrigerated ram semen samples. Moreover, differences in the parameters between samples obtained in the breeding and non-breeding seasons were examined.

## MATERIALS AND METHODS

### Sperm Collection and Seminal Dose Preparation

All the experiments were performed using semen samples obtained during the course of 3 years from 45 mature *Rasa Aragonesa* rams maintained at the Centro de Transferencia Agroalimentaria (CTA) in Zaragoza, Spain. Samples were obtained throughout the year, but for comparing results we divided the data between breeding (B, September to March) and non-breeding (NB, April to August) seasons. All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD1201/05, which conforms to European Union Regulation 2010/63. Approval from the Ethics Committee of the University of Zaragoza was not a prerequisite for this study since we worked with the obtained semen samples. The semen samples were collected using an artificial vagina, within the regular collecting schedule at the AI station. A total of 357 different ejaculates were used in this study. Sperm concentration was calculated in duplicate using a Neubauer chamber (Marienfeld, Germany). Progressive individual motility evaluations were assessed subjectively by visual estimation under a phase-contrast microscope at  $\times 100$  magnification, maintained at 37°C. All the samples used ranged between 80 and 85% motility. Semen was diluted to  $4 \times 10^8$  cells/mL and seminal doses of 0.25 mL were prepared in straws and kept at 15°C until insemination time. Two straws of each sample were sent at the same temperature to our laboratory for analysis.

The artificial inseminations were carried out by the technical teams of ANGRA (National Association of *Rasa Aragonesa* Breeding) and UPRA-Grupo Pastores on different farms owned by these cooperative companies. A total of 3,906 ewes from 52 farms were inseminated. Data reported by the cooperatives were fertility (proportion of ewes that lambed per ewes inseminated), fecundity (number of lambs born per ewes inseminated) and prolificacy (number of lambs born per ewe lambed).

From now on these above-mentioned cooperatives will be named as Coop 1 and Coop 2. An essential difference in the reproductive management between both cooperatives was the use of melatonin implants in rams belonging to Coop 2 during the non-breeding season to avoid the reproductive effects of seasonality.

### Preparation of Sperm Samples for Laboratory Analyses

In order to remove the dilution medium, 200  $\mu$ L of each straw was diluted up to 1 mL with PBS and washed with 7 mL of sucrose buffer (10 mM NaCl, 222 mM sucrose, 2.5 mM KCl, 20 mM HEPES, 10 mM glucose, 1 mg/mL polyvinyl alcohol and polyvinyl pyrrolidone, pH 7.5) by centrifugation at  $500 \times g$  for 10 min. The supernatant was removed, leaving 1 mL to resuspend the pellet, and the sperm concentration was calculated using the Neubauer chamber (Marienfeld, Germany).

## Flow Cytometry Analysis

All the measurements were performed on a Beckman Coulter FC 500 (Beckman Coulter Inc., Brea, CA) with CXP software, equipped with two lasers of excitation (Argon ion laser 488 nm and solid-state laser 633 nm) and 5 filters of absorbance (FL1-525, FL2- 575, FL3-610, FL4-675 and FL5-755,  $\pm 5$  nm each bandpass filter). A minimum of 20,000 events was recorded in all the experiments. The sperm population was gated for further analysis on the basis of its specific forward (FS) and side scatter (SS) properties; other non-sperm events were excluded. A flow rate stabilized at 200–300 cells/s was used.

### Evaluation of Sperm Membrane Integrity

To determine plasma membrane integrity, 3  $\mu$ L of each stain, 1 mM carboxyfluorescein diacetate (CFDA) and 1.5 mM propidium iodide (PI) (both from Sigma-Aldrich, subsidiary of Merck KGaA, Darmstadt, Germany), and 3  $\mu$ L formaldehyde (0.005% final concentration) were added to 300  $\mu$ L of sperm samples (final concentration of  $5 \times 10^6$  cells/mL), based on a modification of the procedure described by Harrison and Vickers (24). Samples were incubated at 37°C in darkness for 15 min. The monitored parameters were FS log, SS log, FL1 (CFDA) and FL4 (PI). For the gated sperm cells, percentages of intact membrane (IM; CFDA+/PI-) spermatozoa were evaluated.

### Evaluation of the Plasmalemma Stability and Mitochondrial Membrane Potential

A double stain technique (25, 26) was performed with slight modifications, using YO-PRO-1 (1 mM in DMSO) and MitoTracker Deep Red (MitoT 10  $\mu$ M in DMSO), both from Thermo Fisher Scientific (Waltham, MA, USA). YO-PRO-1 is a DNA dye used as an apoptotic marker because it only permeates into cells that are beginning to undergo apoptosis. MitoTracker is a mitochondrion-selective stain that passively diffuses across the plasma membrane and accumulates in active mitochondria and is used to evaluate mitochondrial membrane potential ( $\Delta\Psi$ m). Two  $\mu$ L of each dye were added to 300  $\mu$ L sperm samples ( $4 \times 10^7$  spermatozoa) that were incubated at RT in darkness for 15 min. The monitored parameters were FS log, SS log, FL1 (YO-PRO-1) and FL5 (MitoT).

The MitoTracker data were analyzed as suggested by Hallap et al. (27) considering spermatozoa with high fluorescence as cells with high  $\Delta\Psi$ m. Four different sperm populations were classified according to staining (28): YO-PRO-1 -/MitoT+, non-permeant (NP) membrane cells with high  $\Delta\Psi$ m; YO-PRO-1 -/MitoT-, NP cells with low  $\Delta\Psi$ m; YO-PRO-1 +/-MitoT-, apoptotic cells that have lost the integrity of their plasmalemma and with low  $\Delta\Psi$ m; and YO-PRO-1 +/-MitoT+, cells that have lost the integrity of their plasmalemma although with high  $\Delta\Psi$ m.

### Detection of Membrane Phosphatidylserine (PS) Translocation

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). We used the simultaneous staining with FITC- Annexin V (Thermo Fisher Scientific, Waltham, MA, USA) to detect PS translocation and propidium iodide (PI, Sigma-Aldrich, subsidiary of Merck

KGaA, Darmstadt, Germany) to differentiate between live and dead cells, with or without PS translocation. Aliquots of 300  $\mu$ L ( $4 \times 10^6$  cells) were stained with FITC-Annexin V (1  $\mu$ L) combined with 7.5  $\mu$ M PI and incubated at 37°C in the dark for 15 min in binding buffer. The monitored parameters were FS log, SS log, FL1 (FITC-Annexin V) and FL4 (PI). Four sperm subpopulations were distinguished: intact membrane (IM) cells with (Annx+/PI-) or without PS translocation (Annx-/PI-); and membrane-damaged cells with (Annx+/PI+) or without PS translocation (Annx-/PI+).

### Detection of Activated Caspases

The caspase FITC-VAD-FMK *in situ* marker (Vybrant® FAM Caspase-3 and -7 Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) was used to detect active caspase-3 and -7. This cell-permeable caspase inhibitor peptide conjugated to FITC covalently binds to activated caspases, and functions as an *in situ* marker for apoptosis (29). Samples of 300  $\mu$ L ( $1 \times 10^6$  cells) containing FITC-VADFMK (5 nM) were incubated at 37°C and 5% CO<sub>2</sub> in the dark for 60 min. After washing twice with 100  $\mu$ L of washing buffer (supplied with the kit) by centrifuging at 600  $\times$  g 8 min at RT, the pellet obtained was resuspended with 300  $\mu$ L of washing buffer containing 7.3  $\mu$ M ethidium homodimer (Eth), and flow cytometry was performed within 10 min. The monitored parameters were FS log, SS log, FL1 (FITC-VAD-FMK) and FL4 (Eth). With this technique, four sperm subpopulations were distinguished: intact membrane cells (IM) with (FITC-VAD+/Eth-) or without active caspase-3 and -7 (FITC-VAD-/Eth-); and dead with (FITC-VAD+/Eth+) or without (FITC-VAD-/Eth+) active caspases.

### Evaluation of DNA Fragmentation

The presence of DNA strand breaks related to apoptosis in spermatozoa was evaluated by the TUNEL (terminal transferase-mediated dUDP nick end-labeling) assay using an *In Situ* Cell Death Detection Kit with fluorescein isothiocyanate (FITC)-labeled dUTP (Sigma-Aldrich, subsidiary of Merck KGaA, Darmstadt, Germany) (30). Sperm samples ( $4 \times 10^7$  cells/mL) were fixed with 4% paraformaldehyde in PBS at RT for 1 h. After two washes with 100  $\mu$ L of PBS, the samples were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The reaction was performed by incubating the pellet obtained with 50  $\mu$ L of labeling solution that contained the TdT enzyme and dUTP, for 1 h at 37°C in the dark. For each experimental set, a negative control was prepared by omitting TdT from the reaction mixture. Positive controls were simultaneously prepared by additional treatment with 10 IU DNase I for 10 min at 15–25°C before the labeling reaction. Two subsequent washes with PBS at 600  $\times$  g for 10 min at RT were performed to stop the reaction, and flow cytometry analysis was carried out. The monitored parameters were FS log, SS log and FL1 (TUNEL). TUNEL negative spermatozoa were considered DNA-intact.

### Evaluation of the Capacitation Status

The sperm capacitation state was evaluated using the chlortetracycline (CTC) assay (31) that we previously validated for the evaluation of capacitation and acrosome reaction-like

changes in ram spermatozoa (32). For staining, washed samples were diluted with PBS ( $4 \times 10^7$  cells/mL). CTC solution (750 mM, Sigma-Aldrich) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 mM cysteine, pH 7.8, and passed through a 0.22 mm filter (Merck KGaA, Darmstadt, Germany). To 18 mL of sperm sample, 2 mL of ethidium homodimer-1 (EthD-1, 23.3 mM) were added and then incubated at 37°C in the dark for 10 min. Thereafter, 20 mL of CTC solution and 5 mL of 12.2% (w/v) paraformaldehyde in 0.5 M Tris-HCl, pH 7.8, were added. An aliquot of 4 mL of stained sample was placed onto a glass slide at room temperature, and mixed with 2 mL of 0.22 M Triethylenediamine (an antifade-reagent, Merck KGaA, Darmstadt, Germany) in glycerol:phosphate buffered saline (PBS, 9:1). The samples were covered with  $24 \times 48$  mm coverslips, sealed with colorless enamel, and stored at 4°C in the dark.

For the evaluation of CTC patterns, the samples were observed under a Nikon Eclipse E-400 microscope with epifluorescence illumination using a V-2A filter at 1000 X magnification. At least 200 cells were counted in duplicate for each sample. Spermatozoa were classified into one of the following patterns (33): non-capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (showing no fluorescence on the head). The use of ethidium homodimer in this staining allows differentiating live and dead spermatozoa in these three types.

## Determination of Oxygen Consumption

Sperm oxygen consumption was measured with a Clark oxygen electrode linked to a recorder system software (Oxygraph, Hansatech Instruments Ltd., Norfolk UK), as previously described (13). The zero point was set by adding sodium dithionite to the chamber filled with distilled water at 37°C and maintained with constant stirring to ensure a homogeneous distribution of O<sub>2</sub>. Sperm samples were diluted with PBS up to  $3 \times 10^7$  cells/mL and 1 mL of the sperm solution was loaded into the pre-warmed chamber. The plunger was inserted to expel air, and O<sub>2</sub> consumption was monitored for 3 min at 37°C with constant stirring. Results were expressed as fmol O<sub>2</sub>·mL<sup>-1</sup>·min<sup>-1</sup> per million cells.

## Statistical Analysis

Statistical analyses were undertaken using the IBM SPSS Statistics software v. 21 (Armonk, NY, USA) and GraphPad Prism v.9.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

Normality of the data was analyzed with the Kolmogorov-Smirnov test. Correlations between tested sperm parameters and field fertility were calculated using the Spearman rank correlation method. The Mann-Whitney test was carried out to determine whether there were significant differences between groups in some of the above-mentioned parameters. Finally, multiple logistic regression analysis was used for determining the dichotomous field fertility rate (higher or lower than the mean fertility) with sperm parameters. Due to the unbalanced data registry, only the parameters that allowed us to have more than

100 observations in the logistic regression analysis were included in the model.

## RESULTS

### Sperm Quality and Reproductive Parameters of Refrigerated Samples

The mean values of seminal quality and the reproductive parameters obtained with refrigerated seminal doses are summarized in **Table 1**. When these refrigerated samples reached our laboratory, and after sucrose buffer washing, the mean percentage of intact membrane (IM) spermatozoa, estimated as PI- was around 50% ( $47.63 \pm 1.20\%$ ). However, percentages of IM spermatozoa that remained in a non-capacitated state (IM-NC), or without phosphatidylserine translocation or caspases activation, were relatively lower ( $19.12 \pm 0.61\%$ ,  $36.71 \pm 0.90\%$ , and  $32.82 \pm 1.05\%$ , respectively). When the mitochondrial membrane potential was evaluated together with membrane permeability, the percentage of non-permeant cells with high inner membrane potential ( $\Delta\Psi_m$ ) was also low ( $27.33 \pm 1.39\%$ ). Regarding other apoptotic parameters, the mean value of spermatozoa without DNA injury in all evaluated seminal doses was  $84.87 \pm 0.44\%$  (**Table 1A**). The oxygen consumption by these refrigerated samples was  $0.59 \pm 0.12$  fmol O<sub>2</sub>·mL<sup>-1</sup>·min<sup>-1</sup>/10<sup>6</sup> cells.

The reproductive results obtained after cervical inseminations were  $51.97 \pm 1.1\%$  fertility,  $0.83 \pm 0.2$  fecundity and  $1.56 \pm 0.2$  prolificacy (**Table 1B**).

### Influence of Season on the Analyzed Parameters

Comparing the results obtained in the breeding (B) and non-breeding (NB) season, we found significant differences in some of the analyzed sperm quality parameters (**Table 2**). The mean value of IN (PI-) spermatozoa was almost 10% higher in the B than in the NB season, this difference being highly significant ( $P = 0.001$ , **Table 2**). Regarding apoptotic-like markers, the percentage of NP spermatozoa with high  $\Delta\Psi_m$  and IM sperm without PS translocation was also higher in the B than in the NB season ( $P < 0.001$  and  $P = 0.05$ , respectively).

Moreover, spermatozoa in samples from the NB season showed significantly higher oxygen consumption than those in samples from the B season ( $P < 0.05$ ). Regarding capacitation, the percentage of IM-NC spermatozoa was slightly higher in B than in NB, with a tendency to significance ( $P = 0.087$ ).

Differences in the above-mentioned parameters did not lead to significant differences in the reproductive results between the two seasons. However, fertility, fecundity and prolificacy data were slightly higher in B than in NB (**Table 2**).

### Differences Between Sires and Reproductive Management

The rams used in this study belonged to two farming cooperatives, named here as Coop 1 and Coop 2, so we also compared sperm quality parameters between seminal doses from animals belonging to both companies (**Table 3**). Percentages of

**TABLE 1** | Mean, minimum and maximum values of (A) all analyzed parameters in refrigerated seminal doses, and (B) reproductive results obtained after artificial insemination with these seminal doses.

	<i>n</i>	Mean	S.E.M	Minimum	Maximum
<b>A) Sperm quality parameters</b>					
Intact membrane (IM) spermatozoa (PI-) (%)	286	47.63	1.20	3.00	95.00
<b>IM non-capacitated (IM-NC) spermatozoa (%)</b>	357	19.12	0.61	0.00	55.00
Non permeant (NP) sperm with high $\Delta\Psi_m$ (YO-PRO-1-/MitoT+) (%)	138	27.33	1.39	0.10	68.90
IM sperm without PS translocation (Annx-/PI-) (%)	346	36.71	0.90	0.2	85.20
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	277	32.82	1.05	0.00	78.50
DNA-intact spermatozoa (TUNEL-) (%)	357	84.87	0.44	50.00	99.00
Oxygen consumption (fmol O <sub>2</sub> ·mL <sup>-1</sup> ·min <sup>-1</sup> per million cells)	203	0.59	0.12	0.09	1.04
<b>B) Reproductive parameters</b>					
Fertility (ewes lambing per ewes inseminated, %)	357	51.97	1.10	0.00	100.00
Fecundity (number of lambs born per ewes inseminated)	357	0.83	0.20	0.00	2.14
Prolificacy (number of lambs born per ewe lambing)	357	1.56	0.20	0.00	3.00

*N* indicates the number of seminal doses evaluated and S.E.M. is the standard error of the mean.

**TABLE 2** | Differences in analyzed parameters between seminal doses from the breeding and the non-breeding season.

Parameter	Breeding season	Non-breeding season	Significance level ( <i>P</i> )
Intact membrane (IM) spermatozoa (PI-) (%)	51.97 ± 1.64	42.57 ± 1.66	<i>P</i> = 0.001
IM non-capacitated (IM-NC) spermatozoa (%)	19.92 ± 0.82	18.07 ± 0.90	<i>P</i> = 0.087
Non-permeant (NP) sperm with high $\Delta\Psi_m$ (YO-PRO-1-/MitoT+) (%)	36.68 ± 1.77	16.21 ± 1.12	<i>P</i> < 0.001
IM sperm without PS translocation (Annx-/PI-) (%)	39.06 ± 1.16	33.82 ± 1.39	<i>P</i> = 0.05
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	32.07 ± 1.18	33.63 ± 1.79	n.s.
DNA-intact spermatozoa (TUNEL-) (%)	85.17 ± 0.59	84.49 ± 0.66	n.s.
Oxygen consumption (fmol O <sub>2</sub> ·mL <sup>-1</sup> ·min <sup>-1</sup> per million cells)	0.55 ± 0.17	0.61 ± 0.17	<i>P</i> = 0.019
Fertility (ewes lambing per ewes inseminated, %)	53.01 ± 1.42	50.63 ± 1.72	n.s.
Fecundity (number of lambs born per ewes inseminated)	0.86 ± 0.02	0.79 ± 0.03	n.s.
Prolificacy (number of lambs born per ewe lambing)	1.59 ± 0.02	1.53 ± 0.03	n.s.

Results are expressed as a mean value ± standard error of the mean. Last column reflects the level of significance. n.s., non significant.

IM (PI-) and IM without PS translocation spermatozoa were significantly higher in samples from Coop 1 than Coop 2 (*P* < 0.001 and *P* < 0.05, respectively). In contrast, the Coop 2 seminal doses showed a higher value of DNA-intact spermatozoa (*P* < 0.001) than those from Coop 1. However, these differences did not lead to significant dissimilarities in the reproductive results of the two cooperatives (Table 3).

An important difference in the reproductive management between the cooperatives was the use of melatonin implants in rams belonging to Coop 2 during the NB season, in order to avoid the reproductive effects of seasonality. To investigate whether the observed differences between the two cooperatives were due to the melatonin treatment during the NB season, we compared the sperm quality values between cooperatives in the B and NB seasons. In NB, only the percentages of spermatozoa

with intact DNA were significantly higher in Coop 2 (implanted animals) than in Coop 1 (non-implanted) (88.72 ± 0.96 vs. 83.11 ± 0.79%, *P* < 0.001, respectively). However, differences in this parameter were also significant (*P* < 0.001) in the B season (Table 4).

## Relationship Between Reproductive and Sperm Quality Parameters

When the relationship between reproductive and sperm quality parameters was evaluated, only the percentage of IM-NC (intact membrane, non-capacitated) spermatozoa showed a significant correlation with field fertility (*P* = 0.005) and fecundity (*P* = 0.007), independently of the season, the cooperative and the distance (Table 5). When the effect of the season was evaluated, the percentage of IM-NC spermatozoa showed

**TABLE 3** | Differences in analyzed parameters between cooperatives.

Parameter	COOP 1	COOP 2	Significance level (P)
Intact membrane (IM) spermatozoa (PI-) (%)	50.68 ± 1.50	41.41 ± 1.84	$P < 0.001$
IM non-capacitated (IM-NC) spermatozoa (%)	19.36 ± 0.74	18.45 ± 1.01	n.s.
Non-permeant (NP) sperm with high $\Delta\Psi_m$ (YO-PRO-1-/MitoT+) (%)	26.08 ± 1.57	31.65 ± 2.95	n.s.
IM sperm without PS translocation (Annx-/PI-) (%)	37.92 ± 1.06	33.47 ± 1.70	$P = 0.024$
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	33.81 ± 1.29	30.97 ± 1.79	n.s.
DNA-intact spermatozoa (TUNEL-) (%)	83.63 ± 0.53	88.21 ± 0.66	$P < 0.001$
Oxygen consumption (fmol O <sub>2</sub> ·mL <sup>-1</sup> ·min <sup>-1</sup> per million cells)	0.59 ± 0.14	0.58 ± 0.22	n.s.
Fertility (ewes lambing per ewes inseminated, %)	51.37 ± 1.28	53.60 ± 2.12	n.s.
Fecundity (number of lambs born per ewes inseminated)	0.82 ± 0.02	0.84 ± 0.03	n.s.
Prolificacy (number of lambs born per ewe lambing)	1.56 ± 0.02	1.56 ± 0.03	n.s.

Results are expressed as a mean value ± standard error of the mean. Last column reflects the level of significance. n.s., non significant.

**TABLE 4** | Differences in analyzed parameters between seminal doses from the breeding and the non-breeding season for both cooperatives.

Parameter	Breeding season		Non-Breeding season	
	COOP 1	COOP 2	COOP 1	COOP 2
Intact membrane (IM) spermatozoa (PI-) (%)	56.71 ± 2.05	43.68 ± 2.40***	44.40 ± 2.02	38.06 ± 2.82
IM non-capacitated (IM-NC) spermatozoa (%)	20.23 ± 1.08	19.15 ± 1.01	18.23 ± 1.00	17.36 ± 2.07
Non-permeant (NP) sperm with high $\Delta\Psi_m$ (YO-PRO-1-/MitoT+) (%)	37.90 ± 2.34	34.61 ± 2.69	16.82 ± 1.11	n.d.
IM sperm without PS translocation (Annx-/PI-) (%)	40.72 ± 1.34	35.05 ± 2.19*	34.68 ± 1.62	31.16 ± 2.68
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	33.23 ± 1.55	30.39 ± 1.81	32.45 ± 1.19	33.79 ± 2.05
DNA-intact spermatozoa (TUNEL-) (%)	84.06 ± 0.73	87.87 ± 0.90***	83.11 ± 0.79	88.72 ± 0.96***
Oxygen consumption (fmol O <sub>2</sub> ·mL <sup>-1</sup> ·min <sup>-1</sup> per million cells)	0.56 ± 0.02	0.55 ± 0.02	0.61 ± 0.18	0.62 ± 0.42
Fertility (ewes lambing per ewes inseminated, %)	52.19 ± 1.72	54.99 ± 2.53	50.15 ± 1.96	51.46 ± 3.76
Fecundity (number of lambs born per ewes inseminated)	0.85 ± 0.03	0.88 ± 0.05	0.79 ± 0.03	0.79 ± 0.06
Prolificacy (number of lambs born per ewe lambing)	1.60 ± 0.03	1.57 ± 0.04	1.53 ± 0.03	1.55 ± 0.06

Results are expressed as a mean value ± standard error of the mean. \* $P < 0.05$  and \*\*\* $P < 0.001$  between cooperatives within season. n.d., not enough data to include in the comparative analysis.

significant correlation with all the *in vivo* reproductive results during the NB season ( $P = 0.025$ ,  $P = 0.004$ , and  $P = 0.007$  for fertility, fecundity and prolificacy, respectively), but only a tendency to significance with fertility ( $P = 0.082$ ) during the B season.

All seminal doses were prepared in the same AI centre and used in different farms distributed in a 250 km radius. The data were then divided in three categories according to the distance from the collection centre to the farm where the artificial insemination was carried out: short (<50 km), medium (50–100 km) and long (>100 km) distance. When the data were sorted by distance, the correlation between the percentage of IM-NC spermatozoa and reproductive parameters increased progressively with remoteness (Table 5).

Furthermore, three other sperm quality characteristics showed significant correlation with the reproductive parameters, but only in the B season: the oxygen consumption correlated

significantly with fertility ( $P < 0.05$ ), fecundity ( $P < 0.01$ ) and prolificacy ( $P < 0.05$ ); the percentage of spermatozoa with NP membrane and high  $\Delta\Psi_m$  (YO-PRO-1-/MitoT+) with fertility and fecundity ( $P < 0.05$ ); and the percentage of IM spermatozoa without active caspases with fecundity ( $P < 0.05$ ). When the data were divided into categories according to the farm distance, TUNEL negative cells showed a positive correlation with fertility ( $P < 0.1$ ) when the distance was > 100 km.

Multiple logistic regression analysis revealed that the increment in percentage of IM-NC ( $P = 0.02$ ) together with the rise in DNA-intact spermatozoa ( $P = 0.04$ ), would increase the probability of obtaining a fertility higher than the mean (>52%) (Table 6). ROC analysis (Figure 1) was used to identify the accuracy (area under the curve, which indicates discriminatory ability), sensitivity (true positive rate) and specificity (true negative rate) of the model. In our model, the area under the curve was 0.7328 ( $P < 0.001$ ) and sensitivity and specificity

**TABLE 5** | Spearman's rank correlation coefficient (Spearman's  $\rho$ ) between some analyzed sperm parameters and the reproductive results obtained after artificial insemination, considering overall data and data separated by season or farm distance to the semen collection point.

	Parameter	Fertility	Fecundity	Prolificacy
Overall data	IM non-capacitated (IM-NC) spermatozoa (%)	0.147**	0.143**	n.s
In B season	IM non-capacitated (IM-NC) spermatozoa (%)	0.122 <sup>†</sup>	n.s.	n.s.
	Oxygen consumption (fmol O <sub>2</sub> ·mL <sup>-1</sup> ·min <sup>-1</sup> per million cells)	0.245*	0.346**	0.212*
	NP sperm with high $\Delta\Psi_m$ (YO-PRO-1-/MitoT+) (%)	0.246*	0.291*	n.s.
	IM sperm without active caspases (FITC-VAD-/Eth-) (%)	n.s.	0.163*	n.s.
In NB season	IM non-capacitated (IM-NC) spermatozoa (%)	0.180*	0.232**	0.191*
Distance < 50 km	IM non-capacitated (IM-NC) spermatozoa (%)	n.s.	n.s.	n.s
Distance 50–100 km	IM non-capacitated (IM-NC) spermatozoa (%)	0.195*	0.176 <sup>†</sup>	n.s.
Distance > 100 km	IM non-capacitated (IM-NC) spermatozoa (%)	0.158*	0.188*	0.146 <sup>†</sup>
	DNA-intact spermatozoa (TUNEL-) (%)	0.144 <sup>†</sup>	n.s.	n.s.

n.s., non significant; <sup>†</sup> $P < 0.1$ ; \* $P < 0.05$ ; and \*\* $P < 0.01$ . B, breeding; NB, non-breeding; IM, Intact membrane; NP, non permeant membrane.

**TABLE 6** | Multiple logistic regression results of fertility >52% ( $n = 137$ ).

Variable	Beta	Odds ratios	95% CI for OR		P-value
			Lower limit	Upper limit	
Intercept	-9.478	$7.65 \times 10^{-5}$	$1.38 \times 10^{-7}$	0.02	0.002
Distance [<50 km]	-	1.000	-	-	-
Distance [50–100 km]	-0.917	2.503	0.865	7.516	0.09
Distance [> 100 km]	1.207	3.344	1.314	9.018	0.01
Cooperative [Coop 1]	-	1.000	-	-	-
Cooperative [Coop 2]	0.044	1.045	0.349	3.189	0.94
Season [breeding]	-	1.000	-	-	-
Season [non-breeding]	0.844	2.325	0.778	7.345	0.14
Intact membrane (IM) spermatozoa (PI-) (%)	0.041	1.042	0.998	1.091	0.07
IM sperm without PS translocation (Annx-/PI-) (%)	-0.027	0.973	0.928	1.018	0.24
IM non-capacitated (IM-NC) spermatozoa (%)	0.056	1.058	1.011	1.110	0.02
DNA-intact spermatozoa (TUNEL-) (%)	0.061	1.063	1.006	1.129	0.04
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	0.009	1.009	0.982	1.037	0.51
Non-permeant (NP) sperm with high $\Delta\Psi_m$ (YO-PRO-1-/ MitoT+) (%)	0.023	1.024	0.994	1.056	0.12

OR, Odds ratio; CI, confidence interval; (-) not applicable as the reference (most frequent value).

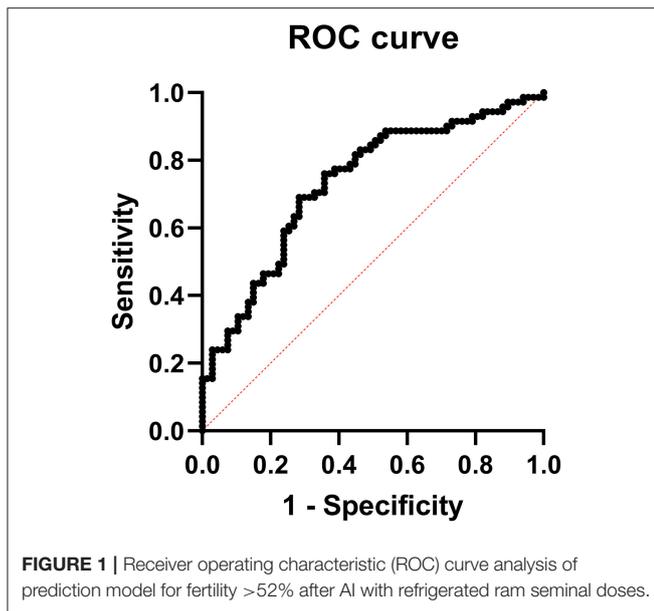
were 70.42 and 64.18%, respectively. The positive and negative predictive power was 67.57 and 67.19%, respectively.

## DISCUSSION

In this study, we determined the relationship between *in vivo* fertility and some sperm quality parameters, including membrane integrity, capacitation state, mitochondria functionality, metabolic activity and apoptotic-like markers, in rams. We found that, when all data was evaluated, only one marker significantly correlated with *in vivo* fertility and with fecundity. This parameter was the percentage of intact membrane non-capacitated (IM-NC) spermatozoa, evaluated using the chlortetracycline (CTC) assay in combination with ethidium homodimer. We had previously validated this procedure for the evaluation of capacitation and acrosome reaction-like changes in ram spermatozoa (32) following a

procedure reported earlier (34). This assay allows us to estimate simultaneously changes in the intracellular calcium distribution related to the capacitation status and plasma membrane integrity. Although some authors have also reported a correlation between capacitation status and *in vivo* fertility, their studies were conducted with frozen-thawed semen in other species (35, 36). However, to the best of our knowledge, this is the first study that correlates capacitation status with field fertility using refrigerated ram semen. Capacitation is a prerequisite for fertilization, but it has to occur in the female reproductive tract near the oocyte. If a high number of spermatozoa undergo capacitation during cryopreservation and transport, they will be less likely to survive until the time of insemination.

When the data from the seminal samples were divided into three categories according to the distance from the AI centre where the seminal doses were prepared to each farm, the relationship between the percentage of IM-NC spermatozoa



and the reproductive results was lost for the nearest farms and increased progressively with remoteness. Furthermore, the percentage of spermatozoa without DNA damage also showed a tendency to significance in its positive correlation with field fertility when inseminations were done on remote farms. These results suggest that these parameters, the percentage of IM-NC and DNA-intact spermatozoa could be useful indicators of the sperm resistance over time. The increment of both the percentage of IM-NC and the DNA-intact spermatozoa would increase the probability of obtaining a fertility higher than the mean, as revealed by a multiple logistic regression analysis.

Sheep are a seasonal, short-day breeder species. Thus, when we compared sperm quality parameters between the breeding (B) and non-breeding (NB) seasons, we found that seminal doses from the B season presented higher percentages of IM spermatozoa, NP with high  $\Delta\Psi_m$  spermatozoa, and IM without PS translocation sperm than in the NB season. Therefore, we can conclude that there are fewer spermatozoa showing apoptotic-like features in the seminal doses in the B than in the NB season. Although differences in sperm motility, morphology or viability between the two seasons have already been reported (20, 21, 37), this is the first time, to the best of our knowledge, that the effect of the season on apoptotic-like markers in ram ejaculates has been revealed. However, although fertility, fecundity and prolificacy results were slightly higher in the B than in the NB season, these differences in apoptotic-like parameters did not lead to significant differences in the reproductive results between the two seasons. Nonetheless, the correlation between the percentage of IM-NC spermatozoa and all the reproductive parameters, including prolificacy, increased when this was evaluated separately in the NB season data. This suggests that this marker would be more effective as a predictor of fertility when the sperm samples are “more stressed” or of less quality, as occurred when the time until insemination increased.

Plasma membrane integrity, evaluated as the non-permeability to certain dyes, is usually included in routine sperm analysis. However, this kind of evaluation does not detect subtle modifications in the sperm plasmalemma that reflect essential changes, such as the beginning of apoptosis, which lead the spermatozoa to certain death. The lack of correlation between membrane integrity and *in vivo* fertility has already been reported (2, 38–40) and is consistent with the results of the present study. Other authors found a significant, albeit limited, predictive capacity of this parameter on field fertility (41). However, it is worth pointing out that when PI was combined with an *in situ* activated caspase determination, or when we used more restrictive assays such as the double staining with YO-PRO-1 and Mitotracker Deep Red, which evaluates the beginning of plasmalemma destabilization and the loss of mitochondrial membrane potential, we found a significant correlation with the reproductive results in the B season.

The determination of sperm oxygen consumption using a Clark electrode has been used as a relatively simple method to evaluate the sperm mitochondrial function in several species such as stallion (42), human (43), boar (44), and ram (13). Oxygen consumption has often been considered a good indicator of the overall metabolic activity within cells, including spermatozoa (45, 46), and it has also been correlated with fertility in bulls (17). In this study, a positive correlation between this parameter and reproductive results was found, but only in the B season. Surprisingly, in samples from the NB season, the oxygen consumption was significantly higher than in those from the B season. This could be explained by the existence of a higher number of cells with mitochondrial membrane damage that could cause the membranes to become more “leaky” to protons, thereby increasing the oxygen consumption required for the maintenance of the proton gradient across the mitochondrial membrane and ATP synthesis (17).

We must highlight that the sires used in this study belonged to two farming cooperatives with different breeding schemes. Seminal doses from Coop 1, although presenting significantly higher membrane integrity values, also had higher DNA damage values than those from Coop 2. These results could be influenced by the use of melatonin implants in the NB season by Coop 2. We have already proved that melatonin implants during the NB season modify ram sperm motility characteristics and seem to improve fertilization results (47). However, in this study we found differences in sperm quality between the cooperatives in both seasons, not only during NB. Therefore, these differences could not be accurately attributed to the melatonin treatment. Furthermore, they did not lead to significant variations in the reproductive results achieved for both cooperatives.

In conclusion, this study reveals, for the first time, differences in apoptotic-like markers and oxygen consumption in ram seminal AI doses between the B and NB seasons. Moreover, we found two markers, the percentage of membrane intact non-capacitated spermatozoa, and the percentage of DNA-intact spermatozoa, that would allow discriminating between high and low fertility semen samples. Thus, these markers could be used to discard ejaculates with low fertilizing capacity and to test the value of particular males as semen donors after analyzing

an appropriate number of their ejaculates. Both procedures might improve fertility results obtained by artificial insemination in ovine.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the figshare repository (doi: 10.6084/m9.figshare.13521647).

## ETHICS STATEMENT

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD1201/05, which conforms to European Union Regulation 2010/63. Approval from the Ethics Committee of the University of Zaragoza was not a prerequisite for this study since we worked with the obtained semen samples. Written informed consent for participation was not obtained from the owners because ewes from farms associate to cooperatives were inseminated as always according to the breeding schedule by each cooperative, without any experimental procedure. Seminal doses for artificial insemination were collected at the Centro de Transferencia Agroalimentaria (CTA) in Zaragoza from rams belonging to cooperatives. We only analyzed in the lab an aliquot of the same seminal doses used for artificial inseminations, and veterinarians working for the cooperatives provide us results of fertility in order to study possible correlations.

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## AUTHOR CONTRIBUTIONS

RP-P and TM-B: conceptualization, experimental approach, and writing—original draft preparation. NM and JD: sperm quality analysis. FQ: semen samples preparation. AL and EF: responsible for artificial inseminations in farms. AC and NM: statistical analysis. AC and RP-P: data curation, writing—review, and editing. RP-P, JC-P, and TM-B: supervision. TM-B: project administration. TM-B and JC-P: funding acquisition. All authors contributed to the article and approved the submitted version.

## FUNDING

This research was supported by Grant DGA A26 (Gobierno de Aragón). NM was financed by a FPI fellowship (Ministerio de Ciencia e Innovación). Publishing fees were financed by IUCA.

## ACKNOWLEDGMENTS

The authors thank the Centro de Transferencia Agroalimentaria (Movera, Zaragoza) staff for their collaboration in the collection of semen samples and the preparation of doses for AI, veterinarians from the Technical Teams of ANGRA (Angel Macías and Elena Martín) and UPRA and Oviaragón for carrying out the artificial inseminations.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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