

Inter-male variability in sperm cryotolerance of Saanen bucks assessed using a purpose-built Sperm Quality Index

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ABSTRACT. Sperm cryopreservation is an essential tool for animal breeding and genetic conservation programs. However, the freeze–thaw process induces structural and functional alterations that compromise sperm survival and fertilizing ability. Although inter–individual variability in sperm cryotolerance has been widely reported in livestock species, integrative approaches for evaluating semen freezability in goats remain limited. Therefore, the present study evaluated sperm cryotolerance in Saanen bucks using composite Sperm Quality Indexes (SQI) derived from multiple post-thaw sperm functional parameters. A total of 124 ejaculates from seven adult Saanen bucks were cryopreserved and evaluated after thawing for progressive motility, viability, acrosome integrity, capacitation status, and plasma membrane fluidity. Two indices were calculated: SQI₁, which includes parameters associated with sperm functionality preservation, and SQI₂, which includes indicators of cryo-induced cellular damage. Significant inter–male differences were observed for both indices ($P < 0.05$). Male 1 exhibited lower SQI₁ values and higher SQI₂ values than males 6 and 7, indicating reduced sperm cryotolerance and increased susceptibility to cryo-induced membrane destabilization and capacitation-like changes. The remaining males showed intermediate responses without significant differences among them. The proposed SQIs successfully detected biologically meaningful inter-male differences in sperm cryotolerance and constitute a useful approach for identifying bucks with different freezing responses.

Keywords: *Capra hircus*; cryopreservation; freezability; spermatozoa; sperm quality index.

INTRODUCTION

Semen cryopreservation is a fundamental biotechnology in animal reproduction, allowing the long-term preservation and global distribution of valuable genetic material for artificial insemination, breeding programs, and biodiversity conservation (Watson, 2000). In goat (*Capra hircus*) production systems, the use of frozen semen is particularly important for accelerating genetic improvement and preserving desirable reproductive traits in dairy breeds, such as the Saanen goat. However, despite the practical advantages of sperm cryopreservation, the freeze–thaw process induces multiple structural and functional alterations in spermatozoa, frequently resulting in reduced post-thaw sperm quality and fertility potential (Watson, 2000; Purdy, 2006).

Cryopreservation-associated sperm damage is mainly related to osmotic stress, oxidative stress, intracellular ice crystal formation, and membrane phase transitions that occur during cooling and thawing (Purdy, 2006; Yáñez-Ortiz et al., 2022). These alterations compromise membrane integrity, mitochondrial activity, acrosomal stability, and sperm motility, thereby decreasing the fertilizing ability of thawed spermatozoa. Among these effects, changes in plasma membrane organization are considered one of the principal determinants of sperm cryosurvival, as sperm membranes are highly susceptible to lipid destabilization and oxidative injury during cryopreservation (Berean et al., 2024; Xue et al., 2025).

An important phenomenon widely recognized in sperm cryobiology is the existence of inter-individual variability in sperm cryotolerance. Within the same species, some males consistently maintain higher sperm quality after thawing and are commonly classified as “good freezers”, whereas others exhibit marked reductions in post-thaw sperm functionality and are considered “poor freezers” (Salamon & Maxwell, 2000; Perumal et al., 2016). This variability has been extensively documented in cattle, pigs, horses, and sheep (Yáñez-Ortiz et al., 2022) and is believed to be associated with intrinsic differences in sperm membrane composition, antioxidant capacity, seminal plasma proteins, and cellular resistance to stress (Cardenas-Padilla et al., 2024a).

In goats, sperm cryotolerance remains comparatively less studied, despite the increasing importance of caprine reproductive biotechnologies worldwide (Purdy, 2006; Sharma & Sood, 2020). Previous studies have suggested that differences in membrane lipid composition, seminal plasma components, and molecular signaling pathways may influence sperm freezability in bucks (Xu et al., 2022; Cardenas-Padilla et al., 2024b). Furthermore, cryopreservation may induce premature capacitation-like changes, commonly referred to as cryocapacitation, which are characterized by increased membrane fluidity, altered calcium dynamics, and destabilization of the sperm plasma membrane (Watson, 2000; Xue et al., 2025). These alterations negatively affect sperm longevity and fertilizing capacity after thawing.

Conventional semen evaluation generally relies on isolated parameters such as sperm motility, viability, or morphology. Although these indicators provide valuable information, they may not adequately reflect the multifactorial nature of cryo-induced sperm injury (Casas *et al.*, 2009; Morrell & Rodríguez-Martínez, 2011). Because cryopreservation simultaneously affects several structural and functional sperm attributes, integrative approaches combining multiple sperm quality indicators may provide a more biologically relevant assessment of sperm cryotolerance and semen freezability.

The Sperm Quality Index (SQI) represents one such integrative approach. By combining complementary sperm functional parameters into a composite metric, the SQI allows for a more comprehensive evaluation of the post-thaw sperm physiological status. Importantly, this approach can simultaneously incorporate parameters associated with sperm functionality preservation and indicators of cryo-induced cellular damage, providing a broader characterization of sperm cryosurvival capacity (Cardenas-Padilla & Medrano, 2025).

Therefore, the objective of the present study was to compare sperm cryotolerance among Saanen bucks using composite Sperm Quality Indexes derived from multiple post-thaw sperm functional parameters.

MATERIALS AND METHODS

Animals

The animals used in this study were provided by the Asociación Nacional de Criadores de Ganado Caprino de Registro A.C. and Caprinocultores Unidos de Guanajuato A.C. and maintained at the experimental facilities of the Education Centre for Agriculture and Animal Breeding, Faculty of Higher Studies Cuautitlan, National Autonomous University of Mexico (UNAM), Mexico.

Seven clinically healthy adult Saanen bucks aged between 2 and 3 years with a mean body condition score of 2.5 (5-point scale) were included in the study. The animals were maintained under uniform management conditions and were exposed to a natural photoperiod throughout the experimental period. The bucks were housed individually and fed a standardized diet consisting of alfalfa hay, commercial concentrate feed, and mineral blocks, with free access to water. The daily dry matter intake was adjusted to approximately 3% of the live body weight.

All experimental procedures involving animals were approved by the Institutional Subcommittee for the Care of Animals in Experimentation of the National Autonomous University of Mexico (SICUAE.DC-2020/3-3).

Semen Collection

Semen samples were collected weekly using an artificial vagina over one year, including both breeding and non-breeding seasons. A total of 124 ejaculates were ob-

tained from the seven bucks included in the study. Depending on semen availability and compliance with the inclusion criteria, each buck contributed between 15 and 19 ejaculates (Male 1, $n = 17$; Male 2, $n = 15$; Male 3, $n = 18$; Male 4, $n = 19$; Male 5, $n = 18$; Male 6, $n = 18$; and Male 7, $n = 19$). Only ejaculates meeting the minimum quality requirements were included for cryopreservation: semen volume ≥ 0.5 mL, progressive motility $\geq 70\%$, sperm concentration $\geq 2 \times 10^9$ spermatozoa/mL, and $\geq 80\%$ morphologically normal spermatozoa.

Initial Sperm Evaluation

After collection, the ejaculates were macroscopically evaluated for volume, color, appearance, and the presence of contaminants. Subsequently, a diluted aliquot of each sample 1:100 (v/v) in 0.9% NaCl was used for microscopic assessment of sperm quality, following the procedure reported by Cardenas-Padilla *et al.* (2024b). Sperm analyses were conducted using a Leica DM1000 LED microscope equipped with differential interference contrast optics and a Leica DMLS epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

The parameters evaluated included progressive motility, viability, acrosome integrity, capacitation status, plasma membrane fluidity, and sperm concentration. Briefly, progressive motility (percentage of spermatozoa showing forward movement) was subjectively assessed at $20 \times$ magnification. Sperm viability (plasma membrane integrity) and morphology were evaluated using eosin-nigrosine staining at $100 \times$ magnification. Sperm concentration was determined using a hemocytometer after dilution (1:200, v/v) in a formaldehyde-saline solution (0.3%). Acrosome integrity (percentage of spermatozoa with a normal apical ridge) was assessed at $100 \times$ magnification.

Sperm capacitation status was evaluated using a chlorotetracycline (CTC) fluorescence assay, and spermatozoa were classified into F (non-capacitated, acrosome-intact), B (capacitated, acrosome-intact), and AR (acrosome-reacted) patterns. Plasma membrane fluidity was assessed using the merocyanine 540 assay to distinguish between low- and high-fluidity sperm populations. All microscopic evaluations were performed according to established procedures at $100 \times$ magnification, and a minimum of 200 sperm cells were analyzed per sample.

Semen Cryopreservation

Semen cryopreservation was performed using a conventional Tris-based extender, following the protocol described by Cardenas-Padilla *et al.* (2024b). Fresh semen was diluted to a final concentration of 200×10^6 spermatozoa/mL in a freezing extender composed of 250 mM Tris, 104 mM citric acid, 28 mM glucose, 0.05% streptomycin, 500 IU/mL penicillin, 12% (v/v) fresh hen egg yolk, and 4% (v/v) glycerol (Cabrera *et al.*, 2005).

Following dilution, the semen was packaged into 0.25-mL French straws, sealed with polyvinyl alcohol powder, and equilibrated at 5 °C for 3.5 h. Subsequently, the straws were placed horizontally 4 cm above the surface of liquid nitrogen for 10 min to allow freezing in nitrogen vapors before being plunged into liquid nitrogen (–196 °C) for long-term storage.

Post-Thaw Sperm Evaluation and Sperm Quality Index Calculation

Post-thaw sperm evaluation was performed immediately after thawing (37 °C in a water bath for 30 s) using the same parameters and methodologies described for fresh semen assessment. Subsequently, two composite Sperm Quality Indexes (SQI) were calculated according to the integrative approach previously proposed by Cardenas-Padilla and Medrano (2025).

SQI₁ included sperm parameters associated with the preservation of sperm functionality after cryopreservation:

$$SQI_1 = \frac{\text{Progressive motility \%} + \text{Viability \%} + \text{F pattern \%} + \text{Acrosome integrity \%}}{4}$$

Higher SQI₁ values were interpreted as indicative of greater sperm cryotolerance and improved preservation of sperm functional integrity after thawing.

SQI₂ included parameters associated with cryo-induced cellular destabilization:

$$SQI_2 = \frac{\text{AR pattern \%} + \text{Hyper-fluid membranes \%}}{2}$$

Higher SQI₂ values were interpreted as indicative of increased cryo-induced membrane destabilization and capacitation-like changes.

Table 1. Season-adjusted estimated marginal means (EMMeans) for Sperm Quality Indexes (SQI₁ and SQI₂) in frozen-thawed semen from seven Saanen bucks.

Male	Ejaculates (n)	SQI ₁ EMMean ± SE	SQI ₂ EMMean ± SE
1	17	28.3 ± 1.92 ^a	73.5 ± 2.12 ^a
2	15	32.7 ± 2.06 ^{ab}	67.2 ± 2.26 ^{ab}
3	18	34.7 ± 1.87 ^{ab}	66.9 ± 2.06 ^{ab}
4	19	34.8 ± 1.82 ^{ab}	65.8 ± 2.00 ^{ab}
5	18	33.5 ± 1.87 ^{ab}	66.6 ± 2.06 ^{ab}
6	18	40.1 ± 1.87 ^b	61.5 ± 2.06 ^b
7	19	38.4 ± 1.82 ^b	64.5 ± 2.00 ^b

Values are season-adjusted EMMeans ± SE from linear mixed-effects models. Different superscript letters within the same column indicate significant Tukey-adjusted pairwise differences (*P* < 0.05).

Statistical Analysis

Data are presented as season-adjusted estimated marginal means (EMMeans) ± standard error (SE). SQI₁ and SQI₂ were analyzed separately using linear mixed-effects models. Buck was included as a fixed effect because the primary objective was to compare the seven bucks included in the study, and season (spring, summer, autumn, and winter; coded as collection periods 1–4 in the dataset) was included as a fixed adjustment factor. The collection occasion, defined as the combination of season and chronological ejaculate sequence, was included as a random intercept to account for observations obtained during the same collection occasion. Season-adjusted EMMeans were calculated by equally weighting all four seasons. Pairwise comparisons among bucks were evaluated using Tukey-adjusted tests. As a secondary sensitivity analysis, models including the buck × season interaction were compared with the additive models using likelihood-ratio tests because seasonal variation was not the primary biological hypothesis, and inference concerning inter-male variability was based on the season-adjusted marginal comparisons. Model assumptions were assessed using residual-versus-fitted and normal probability plots. Differences were considered statistically significant at *P* < 0.05.

RESULTS

Regarding inter-male variability in SQI₁, the mixed-effects model detected a significant effect of buck (Wald χ^2 = 25.13, *df* = 6, *P* = 0.0003). Season also affected SQI₁ in the additive model (*P* = 0.045). The season-adjusted EMMeans are presented in Table 1. Pairwise comparisons revealed that male 1 had significantly lower SQI₁ values than males 6 (*P* = 0.0004) and 7 (*P* = 0.0040), whereas no other pairwise comparisons reached statistical significance (Figure 1).

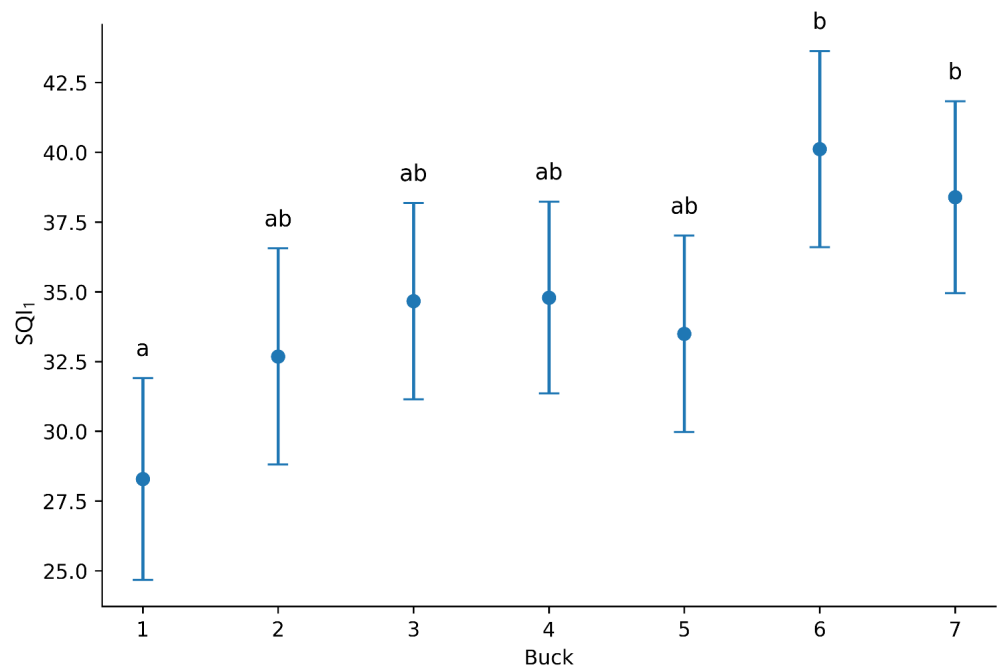


Figure 1. Season-adjusted estimated marginal means for SQL_1 in frozen-thawed semen from seven Saanen bucks. Points represent EMMeans and error bars represent 95% confidence intervals. Different letters indicate significant Tukey-adjusted pair-wise differences ($P < 0.05$).

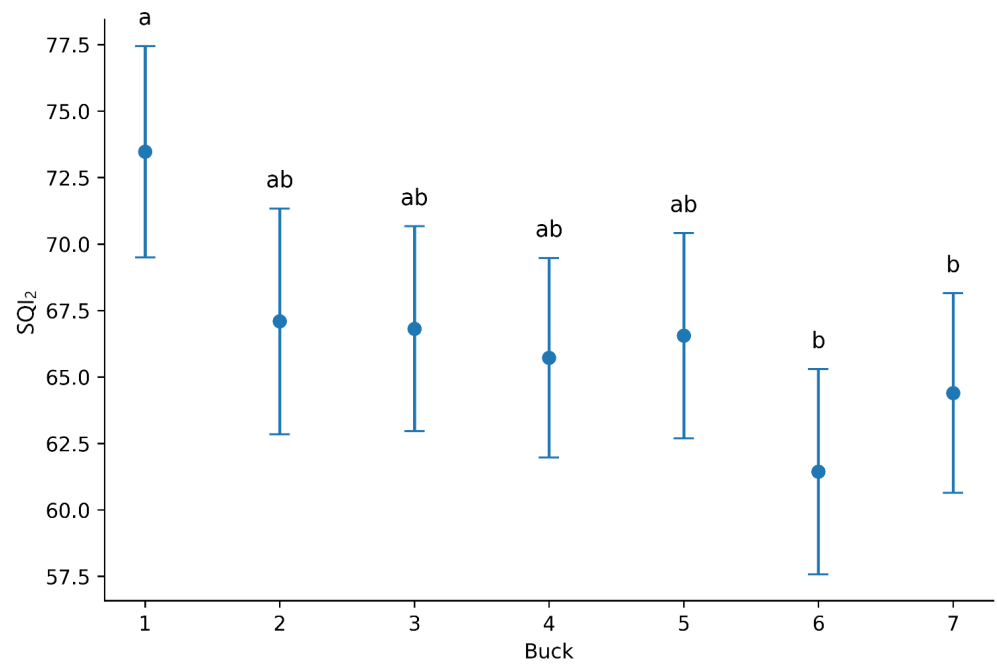


Figure 2. Season-adjusted estimated marginal means for SQL_2 in frozen-thawed semen from seven Saanen bucks. Points represent EMMeans and error bars represent 95% confidence intervals. Different letters indicate significant Tukey-adjusted pair-wise differences ($P < 0.05$).

In terms of inter-male variability in SQL_2 , the mixed-effects model also detected a significant effect of buck (Wald $\chi^2 = 19.35$, $df = 6$, $P = 0.0036$). The effect of season was not statistically significant ($P = 0.052$). The Season-adjusted EMmeans are presented in Table 1. Male 1 showed significantly higher SQL_2 values than males 6 ($P = 0.0010$) and 7 ($P = 0.0297$), whereas no other pairwise comparisons reached statistical significance (Figure 2).

When evaluating concordance between indices and performing sensitivity analysis, the adjusted male profiles were concordant: males with higher SQL_1 generally had lower SQL_2 scores. Male 1 showed the least favorable combined profile, whereas males 6 and 7 showed the most favorable profiles. Secondary models indicated that the magnitude of male differences varied among seasons (buck $\times$ season likelihood ratio tests: SQL_1 , $P = 0.004$; SQL_2 , $P = 0.015$). Nevertheless, the season-adjusted marginal analysis consistently identified male 1 as being less cryotolerant than males 6 and 7.

DISCUSSION

Sperm cryotolerance is considered a multifactorial characteristic influenced by the structural and functional properties of spermatozoa, particularly membrane stability during freezing and thawing processes. The season-adjusted mixed-model analysis demonstrated significant inter-male variability in both the SQL_1 and SQL_2 scores. Importantly, the same biological contrast was detected by both indices: male 1 showed poorer preservation of sperm functionality and greater cryo-induced destabilization than males 6 and 7. This agreement strengthens the interpretation that SQL s capture complementary biological dimensions of semen freezability rather than isolated assay variations.

Inter-individual variability in sperm cryosurvival has been documented in several domestic species, including cattle, pigs, horses, and sheep (Yáñez-Ortiz *et al.*, 2022; Perumal *et al.*, 2016). Males capable of maintaining superior post-thaw sperm quality are commonly referred to as “good freezers,” whereas those exhibiting greater sperm damage are considered “poor freezers” (Salamon & Maxwell, 2000). In the present study, male 1 exhibited lower SQL_1 values and elevated SQL_2 values, suggesting increased susceptibility to cryo-induced damage. Conversely, males 6 and 7 showed greater preservation of post-thaw sperm functionality, indicating a superior cryotolerance. These findings reinforce the concept that sperm freezability is an intrinsic characteristic of each individual male.

The biological basis underlying the differences in sperm cryotolerance remains incompletely understood; however, membrane composition appears to play a central role in determining sperm resistance to cryopreservation (Parks & Graham, 1992; Xue *et al.*, 2025). Sperm plasma membranes are highly enriched in polyunsaturated fatty acids, which confer the membrane fluidity required for

fertilization but simultaneously increase susceptibility to lipid peroxidation and oxidative injury during freezing and thawing (Watson, 2000). Evidence from studies on small ruminants has demonstrated that oxidative damage is closely associated with a decline in post-thaw sperm quality and that improving antioxidant protection during cryopreservation can significantly preserve sperm functionality (Bucak *et al.*, 2009; Berean *et al.*, 2024). Recent evidence further suggests that sperm freezability is influenced not only by membrane lipid organization but also by endogenous antioxidant defense mechanisms, seminal plasma composition, and melatonin-mediated regulation, all of which may modulate the sperm response to cryopreservation-induced stress (Cardenas-Padilla *et al.*, 2024b). Accordingly, variations in membrane lipid organization, cholesterol:phospholipid ratio, antioxidant capacity, and seminal plasma composition may therefore contribute to differences in post-thaw sperm survival among bucks (Cabrera *et al.*, 2005). Beyond membrane destabilization, metabolomic studies have demonstrated that cryopreservation induces profound alterations in energy metabolism, amino acid turnover, and lipid metabolism in goat spermatozoa, suggesting that metabolic resilience may also contribute to inter-male differences in cryotolerance (Li *et al.*, 2024).

Recent studies conducted in Saanen bucks provide additional evidence supporting the biological basis for the variability observed in the present study. Cardenas-Padilla *et al.* (2024b) demonstrated that semen collected during the breeding season exhibited improved post-thaw sperm quality, accompanied by seasonal changes in the expression of sperm melatonin receptors MT1 and MT2. Subsequently, Cardenas-Padilla and Medrano (2025) reported that higher expression of both receptors was associated with greater semen freezability and a lower proportion of acrosome-reacted spermatozoa after cryopreservation. Because SQL_2 integrates the proportion of acrosome-reacted spermatozoa and sperm exhibiting high plasma membrane fluidity, the association between melatonin receptor expression and post-thaw sperm quality reported previously suggests a potential biological mechanism underlying part of the variability observed among bucks in this index.

Cryopreservation also induces capacitation-like changes, known as cryocapacitation, which are characterized by increased membrane fluidity, calcium influx, redistribution of membrane phospholipids, and destabilization of the sperm plasma membrane (Watson, 2000; Bailey *et al.*, 2000). These alterations accelerate premature sperm activation and reduce sperm longevity after thawing. In agreement with this concept, bucks with elevated SQL_2 values in the present study exhibited increased proportions of spermatozoa with high membrane fluidity and acrosome-reacted CTC patterns, indicating greater membrane destabilization and enhanced susceptibility to cryo-induced capacitation-like events. In addition to membrane

remodeling, recent evidence indicates that cryopreservation can activate regulated cell death pathways, including apoptosis and ferroptosis, which may further compromise post-thaw sperm function (Hai et al., 2024).

Conventional semen evaluation frequently relies on isolated parameters, such as sperm motility, viability, and morphology. Although these indicators provide useful information, they may not fully reflect the multifactorial nature of cryo-induced sperm injury (Casas et al., 2009; Morrell & Rodríguez-Martínez, 2011). Cryopreservation simultaneously affects several structural and functional sperm attributes, including plasma membrane integrity, mitochondrial activity, capacitation status, acrosomal stability, and membrane fluidity. Therefore, integrative approaches that combine multiple functional parameters may provide a more biologically relevant assessment of sperm cryosurvival capacity.

In the present study, the SQI approach allowed the simultaneous integration of parameters associated with sperm functionality preservation (SQI_1) and markers of cryo-induced cellular destabilization (SQI_2). The concordant discrimination of male 1 as the least cryotolerant and males 6 and 7 as the most cryotolerant demonstrates the value of combining complementary functional indicators, as sperm freezability cannot be adequately characterized by a single sperm parameter. Moreover, the seasonal variation observed in the magnitude of these differences suggests that sperm cryotolerance is a dynamic phenotype resulting from the interaction between intrinsic male characteristics and seasonal influences. Therefore, repeated evaluations throughout the year may provide a more reliable classification of semen donors for cryopreservation and artificial insemination programs than assessments based on a single ejaculate.

The practical implications of these findings may be particularly relevant for caprine breeding programs and semen banks. The identification of males with superior semen freezability could improve the efficiency of cryopreservation protocols, optimize semen selection for artificial insemination programs, and contribute to the preservation of valuable genetic resources. In addition, integrative sperm quality indexes may be useful tools for the routine evaluation of frozen semen quality under commercial and research conditions.

The limitations of the present study should be acknowledged. Fertility outcomes were not evaluated, and molecular mechanisms associated with cryotolerance, including oxidative stress biomarkers, membrane lipidomics, and mitochondrial function, were not measured in each ejaculate. In addition, the study included only seven bucks, and the detected buck $\times$ season interaction indicated that larger balanced studies are needed to characterize individual seasonal trajectories. Future studies integrating functional, molecular, and fertility-related parameters may contribute to a more comprehensive understanding of sperm freezability in goats. Experimental studies in Saanen bucks have

already demonstrated that improving antioxidant capacity significantly enhances post-thaw sperm quality (Yi et al., 2024), whereas recent evidence indicates that mitochondrial energy metabolism is closely associated with sperm cryosurvival (Zang et al., 2025). Therefore, incorporating oxidative stress biomarkers, mitochondrial function, and metabolic profiling into future investigations may improve the biological interpretation of individual cryotolerance differences.

Overall, composite sperm quality indices discriminated Saanen bucks with different average cryotolerance capacities after adjustment for season. The complementary SQI_1 and SQI_2 results support the usefulness of an integrative evaluation, whereas the seasonal interaction highlights the importance of repeated sampling across the year before classifying semen donors.

CONCLUSIONS

The mixed-effects analysis demonstrated significant inter-male variability in sperm cryotolerance among Saanen bucks following semen cryopreservation. Bucks exhibiting higher SQI_1 values simultaneously showed lower SQI_2 values, indicating greater preservation of sperm functionality and reduced susceptibility to cryo-induced membrane destabilization after thawing.

The integrative Sperm Quality Index approach allowed effective discrimination between males with different semen freezability capacities by simultaneously evaluating the parameters associated with sperm functional preservation and cryo-induced cellular damage. Therefore, the integrative SQI approach can distinguish bucks with contrasting average semen freezability more effectively than isolated parameters can.

The application of integrative sperm quality indices may contribute to improved selection of semen donors for cryopreservation programs, optimization of artificial insemination strategies, and preservation of valuable genetic resources in caprine production systems. However, validation against fertility outcomes and molecular biomarkers is required before routine predictive application.

Competing Interests Statement

The authors declare no conflict of interest.

Ethics Statement

The animal study protocol was approved by the Institutional Subcommittee for the Care of Animals in Experimentation, UNAM (Subcomité Institucional para el Cuidado de Animales en Experimentación, Universidad Nacional Autónoma de México; Approval No. SICUAE. DC-2020/3-3).

Author Contributions

Conceptualization, A.J.C.-P. and A.M.; methodology, A.J.C.-P. and A.M.; validation, A.J.C.-P. and A.M.; formal analysis, A.J.C.-P. and A.M.; investigation, A.J.C.-P. and A.M.; writing—original draft preparation, A.J.C.-P.; writing—review and editing, A.J.C.-P. and A.M.; visualization, A.J.C.-P. and A.M.; supervision, A.M.; project administration, A.M.; funding acquisition, A.J.C.-P. and A.M. All authors have read and agreed to the published version of the manuscript.

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