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Impact of cold storage on semen quality in two divergent rabbit lines selected for resilience under heat stress condition

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ABSTRACT

Artificial insemination in rabbit farms is commonly performed using either fresh semen or doses stored at 14–18 °C for up to 3 days. However, exposure to elevated temperatures can impair sperm motility, concentration, and overall semen quality. This study aimed to evaluate the effect of cold storage on semen from two rabbit genetic lines divergently selected for resilience to thermal stress. A total of 254 ejaculates from 46 males belonging to the HE line (selected for reduced litter size variability) and the HO line (selected for increased litter size variability) were analyzed. Sperm motility and kinematic parameters, viability, acrosome reaction, reactive oxygen species (mROS), and mitochondrial membrane potential (MMP) were assessed. Bayesian methods were applied for statistical analysis. In fresh semen, both lines showed similar proportions of motile and progressive spermatozoa under both comfort and heat stress conditions. In contrast, after refrigeration, the HO line maintained higher motility than the HE line under comfort (75.3% vs. 66.0%; $P = 94\%$) and heat stress conditions (85.3% vs. 75.7%; $P = 97\%$). The HO line also exhibited a higher proportion of fast progressive spermatozoa in fresh semen under comfort (22.2% vs. 17.5%; $P = 96\%$) and heat stress (22.4% vs. 18.1%; $P = 93\%$) conditions. Conversely, mitochondrial activity was reduced in the HO line compared to the HE line under heat stress (56.9% vs. 67.1%; $P = 95\%$). These findings suggest that genetic selection for resilience in rabbits could improve the preservation and functional quality of refrigerated semen, particularly under thermal stress.

1. Introduction

Artificial insemination (AI) constitutes a highly efficient assisted reproductive technology that has become an established practice in commercial farming in rabbits (Viudes-de-Castro & Vicente, 2023). In most cases, fresh or refrigerated semen is employed, as frozen semen fails to achieve competitive fertility and prolificacy rates in rabbits (Bresciani et al., 2016). The assessment of potential fertility of spermatozoa is therefore essential for the evaluation of seminal quality. This requirement acquires particular relevance when semen is collected at different locations, given that recovery time, transportation, and refrigeration conditions may markedly increase sperm mortality. In rabbit farms artificial insemination is performed with fresh or semen doses refrigerated at 14–18 °C for 1 to 3 days rather than frozen because of the poor fertility resulting after thawing (Mocé & Vicente, 2009; Viudes-de-Castro & Vicente, 2023), thereby taking full advantage of the male rabbit's reproductive potential. Consequently, the profitability of AI rabbit centres relies fundamentally on the quality of fresh and refrigerated semen (Bielsa et al., 2022).

On the other hand, rising temperatures associated with global warming are primarily driven by increasing concentrations of greenhouse gases in the atmosphere. These emissions are projected to induce a global temperature increase of approximately 1–3.5 °C over the next century (Change, 1995). Climate change causes heat stress (HS) to become more frequent and severe (Dunshea, 2021). Detrimental effects on reproductive performance have been observed after HS, with semen quality being particularly compromised (Huang et al., 2023). Exposure to elevated temperatures has been shown to decrease sperm motility, concentration, and overall semen quality. Additionally, HS can lead to increased oxidative stress, resulting in DNA damage and apoptosis, further impairing fertility outcomes (Li et al., 2023; Sabés-Alsina et al., 2016; Sui et al., 2022). Furthermore, the deleterious effects of any stress may not be immediately detectable in fresh ejaculates, but they may be detected in their longevity or in their resistance to freezing or refrigeration processes (Johinke et al., 2014). Approaches such as nutritional supplementation (Ebeid et al., 2023), optimized management practices

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(Oladimeji et al., 2022), and selective breeding for thermotolerance (Boulbina et al., 2025) have been proposed to preserve semen quality under elevated temperature conditions. Among these strategies, genetic selection is the only one that is cumulative over time. Therefore, elevated environmental temperatures are expected to exert a significant impact on the reproductive capacity of living organisms, favouring the selection of species better adapted to changing climatic conditions and leading to declines in populations of less resilient species (Canale & Henry, 2010). In this sense, Blasco et al. (2017) proposed to improve resilience in maternal rabbit lines by directly selecting them for litter size residual variability. A divergent selection programme was applied. The HO line has been selected for reduced litter size variability, whereas the HE line has been selected for increased litter size variability. Since both lines are maintained under the same environmental conditions, the phenotypic differences observed between them can be attributed primarily to genetic factors. The HO line shows lower inflammatory response to infectious processes and greater resistance to diseases than the HE line. These results are consistent with the lower mortality at delivery, the better body condition, the lower percentage of litter mortality at birth and at weaning, and the higher homogeneity of litter weight at weaning found in the HO line (Argente et al., 2019); in short, HO line was more resilient than HE line (Argente et al., 2019; Bellouni et al., 2020; Serrano-Jara et al., 2025).

It is hypothesized that the reduced stress levels observed in males of the HO line, characterized by lower ocular surface temperature, cortisol, and TNF-α concentrations under both thermoneutral and heat stress conditions (Serrano-Jara et al., 2025), are associated with alterations in semen quality. Specifically, the attenuated physiological stress responses in HO males may influence key parameters of semen quality compared to HE males. Therefore, the aim of this study is to evaluate the effect of cold storage on semen quality in two genetic lines selected divergently for resilience in rabbits under thermal stress conditions.

2. Material and methods

2.1. Animals

Males belonged from the 17th generation of a divergent selection program at Miguel Hernández University. In the selection process for environmental variability of litter size, each line includes approximately 125 females and 25 males per generation. Selection is based on the phenotypic variance of litter size within each female, after correcting litter size for year-season effects and parity-lactation status. Once systematic effects are corrected, the intra-doe phenotypic variance reflects environmental variability. The HO line is selected for decreasing litter size variability and the HE line for increasing litter size variability The selection intensity for each generation is 30 % (see more details in Blasco et al., 2017).

This study involved 46 male rabbits, with 23 individuals representing the HE line selected by decreased litter size variability and 23 from the HO by increased litter size variability. Animals were housed in individual cages, fed with a commercial diet (16.1 % of crude protein; 3522 kcal/kg of digestible energy) and water was provided ad libitum. At 4.5 months of age, males were trained to ejaculate in an artificial vagina for 1 month.

Table 1
Distribution of the number of samples according to line and thermal conditions.

Thermal conditions	HO Line	HE Line	Total/line
Comfort	74	70	114
Stress	57	53	110
Total	131	123	254

Comfort: Temperature–humidity index, THI ≥ 27.8; Stress: THI < 27.8.

2.2. Semen collection, preparation, and dilution

Table 1 shows the number of semen samples for each line and stress conditions. Semen samples were collected using an artificial vagina maintained at 45 °C. The gel, if present, was immediately removed. Samples was diluted (diluted 1:20) in a extender composed of Tris (3.8 g), citric acid (2.2 g), and glucose (0.6 g) in 100 ml distilled water (pH 6.8, 300 mOsm/kg, Viudes-de-Castro et al., 1999). The stock solutions were sterilized by filtration (0.22 μm) and preserved at +4 to +7 °C for a maximum of two weeks before the day of semen collection.

Three aliquots of diluted semen were obtained. The first aliquot was immediately evaluated (fresh sample). The remaining two aliquots were stored at 14 °C (Viudes-de-Castro & Vicente, 2023) and analysed after 24 h (refrigerated samples): one was used for motility assessment by CASA system, while the other was evaluated for viability, mitochondrial membrane potential (MMP), and mitochondrial reactive oxygen species (mROS) production by flow cytometry.

2.3. Thermal conditions

All animals were housed in the same facilities between May and October 2024. The temperature-humidity index (THI) was used to classify thermal conditions. This index was previously described in rabbits by Marai et al. (2001):

$$THI = t - ((0.31 - 0.31 \times rh) \times (t - 14.4));$$

where t = average temperature of the farm and rh = relative humidity / 100.

The period in which data were collected were classified according to the THI into HS condition (THI ≥ 27.8) and comfort thermal condition (THI < 27.8). A sample was considered to come from a heat-stressed rabbit when the animal had completed its spermatogenesis during the heat stress period (Swierstra & Foote, 1965).

2.4. Motility assessment

A 5 μl aliquot of each diluted fresh and refrigerated sample was placed on a standard Goldcyto slide at a concentration of 20 million cell/ml (Goldcyto Biotech Corp., Guangzhou, People's Republic of China), and sperm parameters were evaluated using the computes-assisted sperm analysis (CASA system 6.6.59, Microptic, Barcelona, Spain). Videos were recorded at 50 frames per second using a 10x phase-contrast dry objective (NA 0.90). Each analysis consisted of 25 consecutive frames (0.5 s) with a time-out of 15 s per field. The particle area range was 20–150 μm^2 and the contrast threshold was set automatically (≈ 80). Only tracks detected for at least 15 frames were considered valid for motility assessment. At least five microscopic fields and a minimum of 1000 spermatozoa were capture for each sample, and then revised and corrected manually in order to avoid the possible problems due to sperm granules present in the rabbit semen plasma (Castellini et al., 2006). The percentages of total motility (TM; progressive and non-progressive), progressive motility (PM; straightness index >70 %), rapid (>50 μm/s), medium (10 – 25 μm/s), non-progressive (<10 μm/s), and immotile spermatozoa were recorded.

Kinematic parameters were also analysed: Curvilinear Velocity (VCL, μm/s), Straight Line Velocity (VSL, μm/s), Average Path Velocity (VAP, μm/s), Amplitude of Lateral Head Displacement (ALH, μm). Progression ratios were also calculated: Linearity (LIN, %): LIN=VSL/VCL × 100, Straightness (STR, %): STR=VSL/VAP × 100, Wobble (WOB, %): WOB=VAP/VCL × 100 and Beat Cross Frequency (BCF, Hz).

2.5. Flow cytometry analysis

Flow cytometry was used to assess the different quality parameters of the refrigerated samples. A BD LSRFortessa flow cytometer was used with fluorescent probes. This flow cytometer has five lasers with emission wavelengths of 355 nm (UV), 488 nm (blue), 561 nm (yellow-green), 405 nm (violet), and 640 nm (red). The instrument was operated using FACSDiva 8 software, with signals processed through logarithmic amplification and photomultiplier settings tailored to each specific staining protocol. A minimum of 10,000 cells per replicate were analysed, with a flow rate consistently maintained between 500 – 1500 cells per second.

Sperm parameters, including viability, MMP, and mROS production, were assessed using a triple fluorescent staining technique involving DAPI, Mitotracker Deep Red, and Mitosox™, as outlined by Gacem et al., (2023). DAPI (Merck, D9542) was employed to determine sperm viability, with DAPI-positive cells indicating dead spermatozoa and DAPI-negative cells representing live spermatozoa. DAPI was detected with peak excitation at 355 nm and emission at 450/40 nm BP. Mitochondrial membrane potential was evaluated using Mitotracker Deep Red (M22426; Invitrogen™; 100 nM), where MTDR-positive cells were classified as having high MMP and MTDR-negative cells as having low potential. MTDR was detected as a peak excitation of 640 nm and emission at 670/14 nm. Mitosox™ Red (M36008; Invitrogen™, 1 μM)) was used to quantify mROS, with MSOX-positive spermatozoa indicating high superoxide levels and MSOX-negative spermatozoa indicating low levels. M36008 was detected at peak excitation 561 nm and emission at 610/20 nm.

After 15 min of incubation at 37 °C in the dark, PBS was added to the samples with the fluorescent dye mixture and analyzed using flow cytometry. All flow cytometry analyses were performed at the Cell Culture and Flow Cytometry Core Facility within the Central Service for Experimental Research (SCSIE) at the University of Valencia.

2.6. Statistical analysis

For all traits studied under the two thermal conditions, i.e. comfort and HS, the model included the effects of line_conservation semen (4 levels: fresh semen of HO line, refrigerated semen of HO line, fresh semen of HE line, refrigerated semen of HE line), random effect of male and the error.

All analyses were performed using Bayesian methodology. Bounded uniform priors were used for all effects with the exception of the male permanent effect, considered normally distributed with mean 0 and variance σ^2_m. Residuals were a priori normally distributed with mean 0 and variance $\sigma^2{}_e$ and uncorrelated with the male effect. The priors for the variances were also bounded uniform. Features of the marginal posterior distributions for all unknowns were estimated using Gibbs sampling. The Rabbit program developed by the Institute for Animal Science and Technology (Valencia, Spain) was used for all procedures. We used a chain of 60,000 samples, with a burn-in period of 10,000. Only one out of every 10 samples were saved for inferences. Convergence was tested using the Z criterion of Geweke (Sorensen & Gianola, 2002), and Monte Carlo sampling errors were computed using time-series procedures, as described in Geyer (1992). In all Bayesian analyses, the Monte Carlo standard errors were small and lack of convergence was not detected by the Geweke test.

Means, standard errors and actual probability (P) were provided. P refers to the probability that the absolute value of the difference between two levels of a fixed effect will be greater than zero. P indicates the probability that the differences are above zero when HO – HE > 0, or below zero when HO – HE <0. Within a Bayesian framework, the notion of "statistical significance" in the classical sense does not apply; instead, the model directly estimates the probability of the differences being positive or negative. Notably, these probabilities may reach or even surpass 0.90, even in cases where the 95 % credible interval still encompasses zero. We consider that there are relevant differences when the P is greater than or equal to 90 %.

3. Results

Figs. 1 and 2 summarize the marginal posterior distributions of the differences between genetic lines and thermal conditions for sperm motility and kinetic parameters. In fresh semen (Fig. 1), both genetic lines exhibited comparable proportions of TM and PM, regardless of thermal condition. In contrast, when refrigerated semen was analyzed, the HO line showed a higher proportion of TM than the HE line, both under comfort conditions (75.3 % vs. 66.0 %; P = 94 %; Fig. 2) and under HS (85.3 % vs. 75.7 %; P = 97 %). Similarly, the rate of progressive spermatozoa was higher in the HO line compared with the HE line under comfort (D_{HO-HE} = 11.7 %; P = 95 %) and HS conditions (D_{HO-HE} = 12.7 %; P = 97 %). A 94 % - 97 % posterior probability (P) indicates strong evidence for a difference between the lines.

The HO line exhibited a higher percentage of fast progressive spermatozoa than the HE line in fresh semen under both comfort (22.2 % vs. 17.5 %; P = 96 %) and HS conditions (22.4 % vs. 18.1 %; P = 93 %; Table 2). No differences were detected between lines for medium progressive or non-progressive spermatozoa in fresh semen. In refrigerated semen, the HO line showed higher percentages of fast and medium progressive spermatozoa than the HE line under both comfort and HS conditions. Conversely, only under HS did the HO line presented a lower percentage of non-progressive spermatozoa compared with the HE line (18.1 % vs. 21.6 %; P = 95 %).

Concerning to kinetic parameters, the HO line exhibited higher VCL, VAP, VSL, ALH, and BCF values than the HE line in both fresh and refrigerated semen under comfort and HS conditions (P > 90 %; Figs. 1 and 2; Tables 3 and 4). However, STR values were similar between lines. For refrigerated seminal doses, LIN was higher in the HO line compared with the HE line under comfort (16.2 % vs. 14.3 %; P = 92 %) and HS (22.0 % vs. 19.5 %; P = 93 %) conditions. Under HS, refrigerated samples from the HO line also showed higher WOB than those from the HE line (D_{HO-HE} = 3.5 %; P = 97 %).

No differences in refrigerated samples were observed between lines for viability, mROS, or healthy spermatozoa under either comfort or HS conditions (Fig. 3). However, high MMP rate was lower in the HO line (56.9 %) than in the HE line (67.1 %) under HS (P = 95 %).

4. Discussion

AI is used in the rabbit industry, as in other livestock species, to improve breeding management. In rabbit farms, AI is performed with fresh or semen doses refrigerated at 15–18 °C rather than frozen because of the poor fertility resulting after thawing (Mocé & Vicente., 2009). In addition, it is well known that semen production and sperm quality can vary across different breeds. Several studies have been carried out to explore this variability between genetic lines (Lavara et al., 2017; Piles et al., 2013; El-Deghadi et al., 2025). The unique feature of this study is that it has been conducted using males from two rabbit lines that have undergone divergent genetic selection for resilience. This is the only known study in which resilience is treated as a trait observed through litter size variability. Specifically, the HO line was selected for reduced litter size variability, whereas the HE line was selected for increased variability (Blasco et al., 2017). As a result, the HO line exhibits lower response to stress and disease susceptibility, indicating greater resilience than the HE line either in females (Argente et al., 2019; Beloumi et al., 2020) and males (Serrano-Jara et al., 2025). Specifically, the consistently lower cortisol levels in the Low line, regardless of environmental conditions, suggest a more adaptive HPA axis response, which may prevent the detrimental effects of prolonged glucocorticoid release. In contrast, the High line appears to exhibit higher baseline activation of the HPA axis, which could indicate a reduced threshold for stress activation and a chronic stress profile (Serrano-Jara et al., 2025). Higher baseline activation of the HPA axis can negatively affect semen quality in rabbits, concretely may impair testicular function by suppressing the hypothalamic–pituitary–gonadal axis, reducing testosterone secretion,

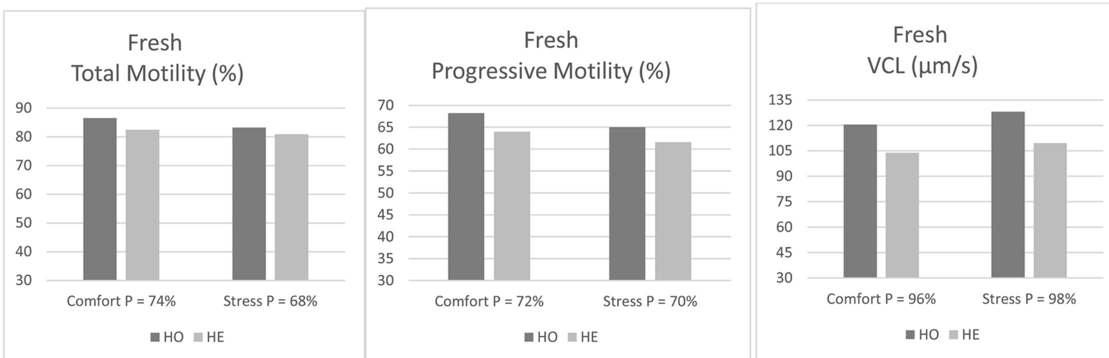

Fig. 1. Differences between lines for total motility, progressive motility spermatozoa and curvilinear velocity (VCL) in fresh semen and under comfort and heat stress conditions.
P: probability of the difference being >0 when HO—HE >0 or being <0 when HO—HE <0.

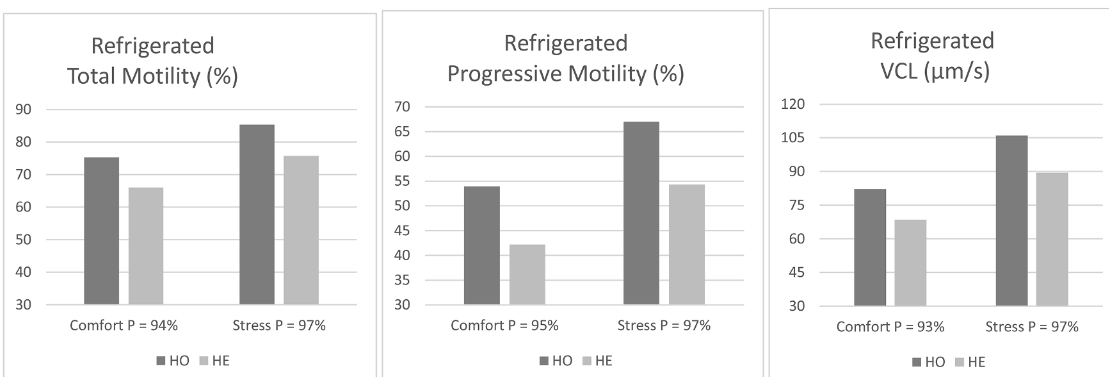

Fig. 2. Differences between lines for total motility, progressive motility spermatozoa and curvilinear velocity (VCL) in refrigerated semen and under comfort and heat stress conditions.
P: probability of the difference being >0 when HO—HE >0 or being <0 when HO—HE <0.

Table 2
Differences between lines for percentage of fast, medium and no progressive spermatozoa in fresh and refrigerated semen and under comfort and heat stress conditions.

Traits	Conservation	Thermal	HO n = 131	HE n = 123	$D_{HO—HE}$	$HPD_{95\ \%}$		P (%)
Fast Progressive (%)	Fresh	Comfort n = 114	22.2	17.5	4.7	- 0.3;	10.1	96
		Stress n = 110	22.4	18.1	4.3	- 1.8;	9.8	93
	Refrigerated	Comfort n = 114	12.3	7.9	4.4	- 0.8;	9.6	95
		Stress n = 110	19.5	14.5	5.0	- 1.0;	10.8	95
Medium Progressive (%)	Fresh	Comfort n = 114	47.3	47.7	0.4	- 10.3;	12.1	54
		Stress n = 110	42.6	43.8	- 1.2	11.8;	9.0	58
	Refrigerated	Comfort n = 114	41.4	34.4	7.0	−4.6;	17.3	90
		Stress n = 110	47.5	40.1	7.4	- 3.4;	17.5	92
No Progressive (%)	Fresh	Comfort n = 114	16.5	18.4	- 1.9	- 6.3;	2.4	80
		Stress n = 110	18.0	19.5	- 1.5	- 5.5;	2.8	78
	Refrigerated	Comfort n = 114	21.6	23.6	- 2.0	- 6.5;	2.2	82
		Stress n = 110	18.1	21.6	- 3.5	- 7.5;	0.6	95

HO: mean of the HO line. HE: mean of the HE line. $D_{HO—HE}$: differences between the HO and HE line. $HPD_{95\ \%}$: highest posterior density region at 95 %. P: probability of the difference being >0 when $D_{HO—HE}$ >0 or being <0 when $D_{HO—HE}$ <0.

and altering spermatogenesis (Gupta et al., 2023). This physiological imbalance can lead to decreased sperm concentration, motility, and viability, as well as increased oxidative stress within sperm cells (Huang et al., 2023). Conversely, rabbits that maintain more efficiently regulated HPA activity under stress conditions tend to preserve better semen quality, suggesting that resilience to stress at the neuroendocrine level contributes to superior reproductive performance. Therefore, differences in baseline HPA activation among genetic lines could partially explain variations in semen quality under challenging environmental conditions.

On the other hand, sperm motility is considered to be the most important parameter when assessing the potential reproductive performance of stored spermatozoa as it is believed to have a high correlation to the in vivo fertility of spermatozoa (Castellini, 2008). Additionally, Lavara et al. (2005) indicated that motility parameters, determined by CASA systems, in combination with sperm morphology analyses can provide some information about the fertilizing potential of rabbit sperm. In this study, in general, the HO line shows higher values for sperm motility and kinetics, mainly with refrigerated semen. Differences in sperm motility parameters between different genetic lines have already been observed in rabbits. Motility for fresh semen found is higher in the HO line than in another maternal rabbit line, but similar in the HE line

Table 3
Differences between lines for average path velocity (VAP) and straight-line velocity (VSL) in fresh and refrigerated semen and under comfort and heat stress conditions.

Traits	Conservation	Thermal	HO $n = 131$	HE $n = 131$	$D_{HO—HE}$	$HPD_{95\ \%}$		P (%)
VAP (µm/s)	Fresh	Comfort $n = 114$	54.5	44.5	10.0	0.5;	19.0	98
		Stress $n = 110$	62.1	50.5	11.6	1.7;	21.9	99
	Refrigerated	Comfort $n = 114$	32.8	26.0	6.8	- 2.8;	15.6	98
		Stress $n = 110$	47.8	37.3	10.5	0.2;	20.3	98
VSL (µm/s)	Fresh	Comfort $n = 114$	30.7	24.6	6.1	0.3;	11.8	98
		Stress $n = 110$	35.4	27.9	7.5	1.2;	14.2	99
	Refrigerated	Comfort $n = 114$	16.9	12.5	4.4	- 1.8;	9.7	95
		Stress $n = 110$	26.3	20.0	6.3	- 0.3;	12.6	97

HO: mean of the HO line. HE: mean of the HE line. $D_{HO—HE}$: differences between the HO and HE line. $HPD_{95\ \%}$: highest posterior density region at 95 %. P: probability of the difference being >0 when $D_{HO—HE}$ >0 or being <0 when $D_{HO—HE}$ <0.

Table 4
Differences between lines for straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) in fresh and refrigerated semen and under comfort and heat stress conditions.

Traits	Conservation	Thermal	HO $n = 131$	HE $n = 123$	$D_{HO—HE}$	$HPD_{95\ \%}$		P (%)
STR (%)	Fresh	Comfort $n = 114$	49.4	51.6	- 2.2	- 5.9;	2.0	86
		Stress $n = 110$	49.2	48.8	0.4	- 3.3;	3.7	58
	Refrigerated	Comfort $n = 114$	44.6	42.5	2.1	- 1.8;	6.2	85
		Stress $n = 110$	49.7	48.8	1.7	- 1.9;	5.2	85
LIN (%)	Fresh	Comfort $n = 114$	21.9	2.7	1.2	- 1.5;	3.3	81
		Stress $n = 110$	24.2	22.6	1.6	- 1.5;	5.0	83
	Refrigerated	Comfort $n = 114$	16.2	14.3	1.9	- 0.8;	4.4	92
		Stress $n = 110$	22.0	19.5	2.5	- 1.1;	5.7	93
WOB (%)	Fresh	Comfort $n = 114$	41.4	39.6	1.8	- 1.6;	5.5	83
		Stress $n = 110$	44.7	43.2	1.5	- 2.1;	5.0	79
	Refrigerated	Comfort $n = 114$	35.4	34.2	1.2	- 2.4;	4.8	73
		Stress $n = 110$	42.8	39.3	3.5	- 0.1;	7.2	97
ALH (µm)	Fresh	Comfort $n = 114$	3.0	2.6	0.4	- 0.1;	0.8	96
		Stress $n = 110$	3.1	2.7	0.4	- 0.1;	0.8	96
	Refrigerated	Comfort $n = 114$	2.2	1.9	0.3	- 0.2;	0.7	90
		Stress $n = 110$	2.8	2.4	0.4	−0.1;	0.8	97
BCF (Hz)	Fresh	Comfort $n = 114$	11.5	10.2	1.3	- 0.5;	3.3	92
		Stress $n = 110$	7.6	6.0	1.6	- 0.3;	3.6	95
	Refrigerated	Comfort $n = 114$	7.6	6.0	1.6	- 0.3;	3.6	95
		Stress $n = 110$	9.6	8.2	1.4	- 0.4;	3.2	93

HO: mean of the HO line. HE: mean of the HE line. $D_{HO—HE}$: differences between the HO and HE line. $HPD_{95\ \%}$: highest posterior density region at 95 %. P: probability of the difference being >0 when $D_{HO—HE}$ >0 or being <0 when $D_{HO—HE}$ <0.

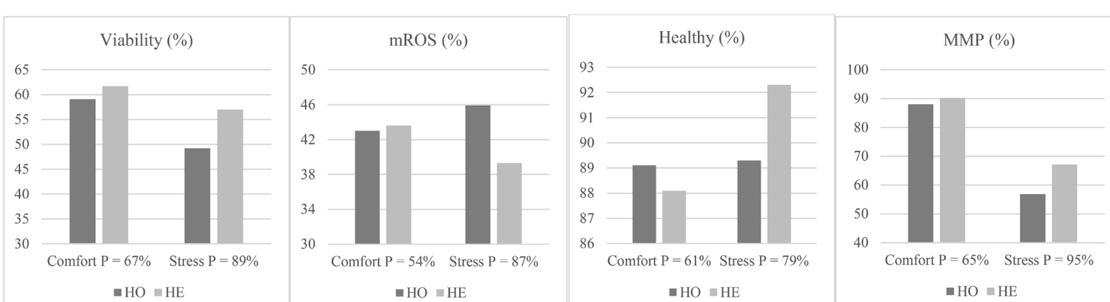

Fig. 3. Differences between lines for viability, mitochondrial reactive oxygen species (mROS), healthy, and high mitochondrial membrane potential (MMP) in refrigerated semen and under comfort and heat stress conditions.
P: probability of the difference being >0 when HO—HE >0 or being <0 when HO—HE <0.

(Lavara et al., 2005).

The cooling of spermatozoa for long-term preservation can disrupt the organization of membrane phospholipids (Paulenz et al., 2002), finally leading to cold shock. This phenomenon may cause the premature release of acrosomal contents and impair the capacity of the acrosome to undergo an appropriate reaction during fertilization (Esteves et al., 2007). Compared with other domestic species such as rams and bulls, rabbit spermatozoa exhibit greater resistance to cold shock, largely attributed to their elevated cholesterol-to-phospholipid ratio (Bailey et al., 2000; Mocé & Vicente, 2009), which enhances their ability to tolerate cooling to lower temperatures. This resistance is supported by our results, as both lines exhibited comparable integrity of membrane rate.

Damage to the sperm plasma membrane is primarily associated with in vitro semen handling, where spermatozoa are subjected to cooling and prolonged storage at low temperatures (Johinke et al., 2014). Under these conditions, oxidative stress constitutes a major factor contributing to the deterioration of sperm quality (Silvestre et al., 2021). Although low concentrations of reactive oxygen species (ROS) are essential for the regulation of key sperm functions, excessive ROS production during cooling and storage markedly compromises sperm viability and fertilizing capacity over time (Castellini et al., 2003; Laghouati et al., 2023).

Both lines exhibited similar levels of mitochondrial ROS, suggesting that oxidative stress is unlikely to be the primary factor explaining the observed differences in sperm motility. Our results agree with those observed by Bouldina et al. (2025), who, despite observing higher sperm motility in the adapted local breed, also found no significant differences in lipid peroxidation rates or reactive oxygen metabolites between two rabbit genetic lines. This observation aligns with previous studies indicating that variations in motility are not always directly linked to ROS production (Agarwal et al., 2014; Partyka et al., 2012). Instead, other physiological mechanisms may play a more prominent role. For instance, differences in mitochondrial function can affect ATP production, which is critical for progressive motility. Likewise, variations in sperm membrane integrity and acrosome stability may influence motility independently of oxidative stress. Additionally, thermotolerance adaptations, such as more efficient heat dissipation or endocrine regulation under thermal stress, could contribute to the superior motility observed in the HO line. These findings highlight the multifactorial nature of sperm quality under environmental stress and suggest that further studies should investigate mitochondrial performance, membrane integrity, and hormonal responses as potential determinants of line-specific resilience.

Several studies have highlighted the high sensitivity of rabbits to environmental fluctuations, particularly elevated temperatures, which have been associated with HS and consequent impairment of reproductive performance (Khalil et al., 2015). This negative impact appears to be further aggravated when high temperatures are combined with elevated humidity levels (Marai et al., 2002). The physiological basis for this susceptibility is linked to the limited thermoregulatory capacity of rabbits, as their sweat glands are poorly functional (Okab et al., 2008) and their dense fur hampers efficient heat dissipation (Khalil et al., 2015). As a result, HS has consistently been reported to compromise semen quality, leading to reductions in sperm concentration, progressive motility, and acrosome integrity (Fadl, 2020; Marai et al., 2002). In the present study, sperm motility was higher in the HO line than in the HE line under thermal stress conditions, particularly after semen refrigeration. The HO line exhibited a greater proportion of motile and progressively motile spermatozoa, as well as higher values for VCL, VAP, VSL, STR, LIN, WOB, ALH, and BCF. In contrast, a lower proportion of immotile and non-progressive spermatozoa was observed in this more resilient line. These findings may be attributed to the superior adaptation of the HO line to thermal stress, as evidenced by its lower eyeball temperatures and cortisol concentrations (Serrano-Jara et al., 2025). Recently, Boulbina et al. (2025) demonstrated that the local Algerian rabbit population, which is well adapted to thermal stress conditions, maintained better sperm quality during the early stages of storage and exhibited differences in antioxidant enzyme activity compared with the New Zealand White breed.

Several studies have reported a positive correlation between sperm motility and elevated mitochondrial activity (in humans: O'Connell et al., 2002; in bulls: Padrik et al., 2010; in rabbits: Johinke et al., 2014). In the present study, however, the HO line exhibited higher motility despite showing lower mitochondrial activity compared with the HE line in thermal stress conditions. Various studies have shown a correlation between ATP content and sperm motility (Amaral, 2022), including rabbit sperm (Gogol, 2013). Nevertheless, discrepancies between motility and high MMP rates have been observed previously in rabbit semen (Arkarsu et al., 2023; Gloria et al., 2021). Additionally, after refrigerated storage of rabbit semen for three days, a 50 % decrease in sperm motility was observed, but the decrease in the percentage of hMMP was only 8 % (Gogol et al., 2014). This may be because rabbit spermatozoa could use both the glycolytic pathway and OXPHOS in its strategy for obtaining ATP. The predominance of one metabolic pathway or the other seems to be influenced by the animal species, although it depends on the environmental conditions surrounding the sperm (Amaral, 2022). In this sense, the glycolytic pathway appears to be capable of maintaining sperm motility even with hMMP rate reduced by a mitochondrial inhibitor (Losano et al. 2017).

Another possible explanation for these conflicting results could be the different composition of seminal plasma among the different genetic lines may affect the direct relationship between MMP and sperm motility. Bouldina et al. (2025) observed that the local population had higher catalase activity and lower superoxide dismutase (SOD) activity in the seminal plasma, which could reduce hydrogen peroxide (H_2O_2) content. Although they did not observe significant differences in the parameters evaluating sperm oxidative stress. In the present study, we did not evaluate seminal plasma composition. The production of mitochondrial superoxide ($O_2\bullet^-$) is closely related to MMP (Cadenas et al., 2018), and it could be that higher MMP generates a greater amount of $O_2\bullet^-$. However, if there is greater SOD activity, it could generate more H_2O_2 that we are not detecting and could be affecting sperm total motility and kinetics. But, we did not evaluate H_2O_2 presence, but rather $O_2\bullet^-$ by Mitosox™ probe.

In summary, this study is among the first to investigate semen preservation in rabbit lines divergently selected for resilience under heat stress, representing a significant contribution to the fields of semen quality and genetic breeding. A key strength lies in the use of a Bayesian statistical framework, which enables a rigorous estimation of uncertainty and posterior probabilities, thereby enhancing the robustness of the findings. Additionally, the integration of multiple complementary semen quality indicators, such as motility, kinetic parameters, mitochondrial membrane potential (MMP), and mitochondrial reactive oxygen species (mROS), adds depth and reliability to the conclusions. By jointly assessing the effects of genetic selection and environmental stress, the study provides valuable insights for breeding programs seeking to improve reproductive performance under climate change conditions. Overall, these findings highlight the potential for developing and commercializing rabbit maternal lines better adapted to emerging climatic challenges. Nevertheless, further validation is needed to confirm that these adaptive traits translate into improved fertility and post-insemination prolificacy.

5. Conclusions

Genetic selection for resilience in rabbits represents a promising strategy to improve the preservation and quality of refrigerated semen, particularly under conditions of thermal stress. Like this, the resilience line maintained approximately 10–12 % higher motility after 24 h refrigeration under both comfort and heat stress conditions. Males from the more resilient line not only exhibit enhanced sperm motility and progressive movement, suggesting an adaptive advantage under challenging environmental conditions. These findings highlight the potential of resilience-focused breeding programs to support reproductive efficiency.

Future research should further investigate the underlying physiological mechanisms, such as sperm metabolic pathway, hormonal regulation, cellular stress responses, antioxidant enzyme activity, or sperm proteomics to optimize reproductive management strategies in rabbit populations exposed to environmental stressors.

Ethics approval

The experimental procedures with animals were approved by the General Directorate of Agriculture, Livestock and Fisheries of the Generalitat Valenciana with code 2022/VSC/PEA/0226.

Data availability statement

None of the data were deposited in an official repository. All data are available upon request.

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CRediT authorship contribution statement

D. Serrano-Jara: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **S. Gacem:** Writing – review & editing, Investigation, Formal analysis. **MJ. Argente:** Writing – review & editing, Methodology. **I. Agea:** Writing – review & editing, Methodology. **MA. Silvestre:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition. **ML. García:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis.

Declaration of competing interest

No potential conflict of interest relevant to this article was reported.

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