R. Bras. Zootec., 55:e20250151, 2026
https://doi.org/10.37496/rbz5520250151

Reproduction
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

The effect of male age, extender, cooling time and freezing method on post-thaw Brahman cattle sperm motility

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Received: August 4, 2025
Accepted: January 21, 2026

How to cite: Murillo, L.; Sevilla, F.; Araya-Zúñiga, I.; Rodríguez, J.; Molina-Montero, R.; Silvestre, M. A. and Valverde, A. 2026. The effect of male age, extender, cooling time and freezing method on post-thaw Brahman cattle sperm motility. Revista Brasileira de Zootecnia 55:e20250151. https://doi.org/10.37496/rbz5520250151

Editors:
José Nélio de Sousa Sales
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ABSTRACT - The objective of this study was to evaluate how male age, semen extender, cooling (equilibration) time and freezing method influence the quality of Brahman bulls' sperm after cryopreservation, as measured by Computer-Assisted Sperm Analysis (CASA) parameters. Six Brahman bulls, with an average age of 48.6±11.5 months, were used. Two ejaculates were collected for each animal on different days, with an interval of approximately one week between collections. Semen was diluted with three extenders: Andromed®, BioXcell®, and OptiXcell®. The samples were equilibrated for either four or six hours and frozen using either static/manual or controlled programmable methods, resulting in 12 treatments for bovine semen cryopreservation. Results showed significant differences (P<0.05) in sperm motility and kinematics based on the extender used. Bull age had a significant effect (P<0.05) on the percentage of fast and medium sperm. Cooling time did not affect motility variables (P>0.05). No differences were found in total motility rate between freezing methods, but significant differences (P<0.05) were noted in sperm kinematic variables. Freezing of Brahman semen was more effective when samples were extended with OptiXcell®, cooled for 4 h, and frozen using an automatic method.

Keywords: *Bos indicus*, livestock, reproduction, semen, spermatozoon, Zebu cattle

1. Introduction

Semen dilution and cooling influence sperm motility (De Ambrogi et al., 2006), the maintenance of plasma membrane integrity (Wysokińska et al., 2015), and resistance to hypothermic stress associated with the instability of phase transitions in sperm membrane lipids (Parks and Lynch, 1992; Drobnis et al., 1993). Plasma membrane permeability has been associated with cation outgassing, reduced enzyme activity, and altered membrane proteins, all of which impact the ability of sperm to adapt to their environment (De Leeuw et al., 1990; Davis et al., 1995; Waterhouse et al., 2006; Schmid et al., 2013).

Animal breeding plays a pivotal role in enhancing livestock production and adaptability to various challenges, including climate change and disease (Godde et al., 2021; Wanjala et al., 2024). Genetics, alongside health and nutrition, constitutes a cornerstone for the development of the livestock sector (Brito et al., 2020; Prache et al., 2022). In Latin America, genetic improvement of the Brahman breed is critical for increasing meat production and optimizing farming practices, thereby contributing to sustainable and efficient livestock development (Márquez-Godoy et al., 2024; Scholtz et al., 2011). In addition to genetic improvement programs, assisted reproduction techniques can improve the reproductive parameters of this breed in tropical areas, where beef cattle production management systems are generally extensive or semi-intensive (Baruselli et al., 2023; Mikkola et al., 2024; Rubio Lozano et al., 2021; van Arendonk, 2011).

Semen analysis is fundamental for livestock reproductive management (Valverde et al., 2020; Zuidema et al., 2021). This process evaluates sperm motility, morphology, DNA fragmentation, and swimming patterns (Valverde and Madrigal-Valverde, 2018). Advanced semen analysis systems are indispensable for predicting male fertility, facilitating the application of natural mating (Araya-Zúñiga et al., 2024) and assisted reproductive techniques, such as artificial insemination (AI) (Sundararaman et al., 2012; Zuidema et al., 2021). Livestock production has been significantly enhanced by the more efficient use of genetically superior bulls for AI without the need for natural mating (Mueller and Van Eenennaam, 2022; Singh et al., 2022). When combined with cryopreservation techniques, AI becomes an effective method for preserving and disseminating high-quality genetic material over extended periods (Rosete Fernández et al., 2021; Bosch et al., 2020). The primary goal of semen freezing is to maintain sperm viability and functionality at temperatures below 0 °C (Ozimic et al., 2023) over extended periods. Despite its effectiveness, cryopreservation can damage cellular structures through ice crystal formation and cellular dehydration (Grötter et al., 2019; Tamburrino et al., 2023; Woods et al., 2004). These cryopreservation-induced damages can compromise sperm viability, reducing fertility and productivity (Peris-Frau et al., 2020; Yeste, 2016).

The study of cryopreservation in Brahman is particularly justified due to the breed's importance in tropical regions, where it serves as a cornerstone of beef production systems (Cooke et al., 2020). Despite its widespread use, the current state of research reveals significant gaps in understanding the breed's specific responses to semen freezing protocols. The variability in results from previous studies can be attributed to genetic differences among individuals, environmental factors, and the use of varying cryopreservation techniques (Jaiswal and Vagga, 2022). While some studies suggest that Brahman sperm exhibits enhanced resistance to cryogenic damage, others indicate heightened susceptibility, highlighting the need for standardized and optimized protocols (Rubio-Guillén et al., 2007; Shannon and Vishwanath, 1995). Addressing these inconsistencies is essential to improving the post-thaw quality of Brahman semen, ensuring better fertility outcomes, and enhancing the efficiency of assisted reproductive technologies in this economically significant breed. Considering the factors involved in the freezing process, this study evaluates how male age, semen extender, cooling (equilibration) time, and freezing method influence the quality of Brahman bulls' sperm after cryopreservation, as measured by Computer-Assisted sperm Analysis (CASA) parameters.

2. Material and methods

2.1. Ethical approval

The study followed the laws and regulations for conducting experiments with live animals in Costa Rica. Throughout the study, animals were handled with care to avoid unnecessary stress. This study was further carried out following ethical principles and with the approval of the Committee of the Research and Development Center for Sustainable Agriculture in the Humid Tropics of the Costa Rican Institute of Technology (CIDASTH-ITCR), under protocol numbers Section 08/2023, article 5.0, DAGSC-075-2023, and CIE-206-2023, and was conducted in accordance with the Three Rs principle.

2.2. Experiment description and location

The study was conducted in collaboration with the Agricultural Production Program of the Agronomy School at the Costa Rica Institute of Technology, San Carlos Campus. The Beef Cattle Unit operates the La Vega Farm in La Vega de Florencia, San Carlos (CRTM05 X: 442863.442 Y: 1152494.364), located at an altitude of 70 MASL. The study period spanned from December 2023 to March 2024.

2.3. Animals

Brahman bulls used in this study were previously selected based on the following criteria: total sperm motility greater than 50%, morphological sperm abnormalities below 30%, and a minimum scrotal circumference of 32 cm at two years of age. Six bulls out of 10 were deemed suitable, with an average age of 48.6±11.5 months, and were divided into two age groups: three bulls aged 24–48 months and three bulls older than 48 months. At least two ejaculates were collected for each animal on different days, with an interval of approximately one week between collections.

2.4. Ejaculate collection

Males were handled using a hydraulic chute (Priefert®, model S04). Before ejaculate collection by electrostimulation procedure, rectal feces were evacuated, and a 1–2-minute transrectal massage to stimulate the accessory glands (vesicular glands and ampullae of the deferent ducts) and prostate was performed, enhancing sexual response and relaxing the anal sphincter. A 75 mm diameter transrectal probe (Pulsator V, Lane Manufacturing, Denver, CO, USA) was used with a preprogrammed cycle, applying increasingly intense stimuli until ejaculation. Each bull underwent up to two cycles of 36 electrical stimuli, starting with six at 1–2 V, nine at 3–5 V, twelve at 6 V, and nine at 7 V, each lasting 3.5 seconds, concurrent with rectal massage. Semen was collected directly from the penis using a sterile collection bag. Three types of commercially available semen extenders (Andromed®, Bioxcell®, and Optixcell®) with distinct compositions and manufacturers were used in this study. After collection, three aliquots of semen:extender were prepared in a 1:1 ratio (vol:vol) in Eppendorf® tubes which were prewarmed at 37 °C to avoid temperature shock.

2.5. Semen dilution

In the laboratory, semen samples were further diluted at a 1:20 ratio, achieving an average concentration of 17.5–27.5 ($\times 10^6$/mL) in 15 mL Eppendorf® tubes. Both the semen and diluent were maintained at 37 °C. Semen samples were analyzed both fresh and after refrigeration for 4 and 6 hours (Table S1 and Table S2). The samples were cooled at room temperature (21 °C) for 10 minutes before being stored in a refrigerator at 5 °C for 4–6 hours to complete the cooling process. For this stage, all diluents were used, and each ejaculate was divided into three fractions. Each fraction, corresponding to a specific extender, was divided into two additional fractions according to equilibration time (Figure 1). For each treatment fraction, seven straws were used in the freezing process. A total of 1008 straws were prepared.

2.6. Freezing and thawing process

Following each cooling time, semen samples were packaged into 0.5 mL straws (Minitube), using a suction pump (IMV, L'Aigle, France), and sealed with sealing powder (IMV, L'Aigle, France). Immediately after packaging, 50% of the straws (n = 504) were subjected to cryopreservation using a controlled programmable freezer (IMV Digitcool, IMV, L'Aigle, France), following the protocol: cooling at −5 °C/min from +4 °C to −10 °C; then at −100 °C/min from −10 °C to −100 °C; and finally at −20 °C/min from −100 °C to −140 °C. The remaining straws (n = 504) were frozen using a static method. They were placed horizontally on racks positioned 4 cm above the liquid nitrogen surface in a polystyrene container for 15 min, after which they were directly immersed in liquid nitrogen and stored in a 20 L liquid nitrogen tank for one month prior to thawing.

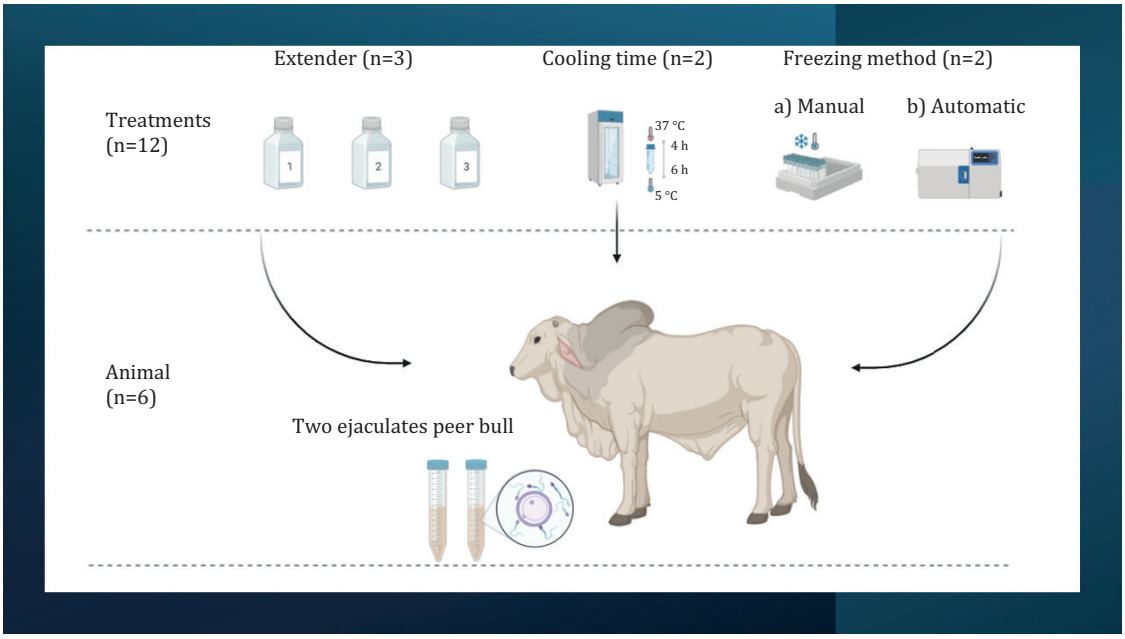

Figure 1 - Description of total treatments used. Created by BioRender.com.

Three semen cryopreserved straws for each treatment were randomly chosen. A total of 432 frozen semen straws were thawed. Straws were thawed in a water bath at 37 °C for 30 seconds (Solís et al., 2024). The contents of three straws of the same bull, collection, extender type, cooling time, and freezing method were dispensed in a 1.5 mL tube and kept at 37 °C while analyses were performed. Following thawing, sperm kinematic and motility parameters were evaluated on a heating plate maintained at 37 °C ± 0.5 °C.

2.7. Sperm kinematics and motility assessment

Each sample was run in duplicate by placing 3 µL of homogenized semen in a 20 µm Spermtrack® counting chamber. Semen analysis was performed using the CASA-Mot ISAS®v1 system (Proiser R + D, Valencia, Spain), which included a Proiser 782 M video camera operating at 40 frames per second with a resolution of 768 × 576 pixels. The camera was attached to a UB203 microscope equipped with a 1× ocular and a 10× phase contrast objective (AN 0.25), and an integrated heated stage maintained at 37.0 ± 0.5 °C. The analysis employed the default bovine settings provided by the manufacturer. For each counting chamber, seven fields from CASA systems were captured, totaling at least 600 cells. The analyzed motility and kinematic variables included total motility (TM, %), progressive motility (PM, %), spermatozoa with movement categorized as fast (>45 µm·s^{-1}), medium (25 ≤ x < 45 µm·s^{-1}), slow (10 < y < 25 µm·s^{-1}), linearity (LIN, %), straightness (STR, %), wobble (wave-like movement; WOB, %), straight line velocity (VSL, µm·s^{-1}), curvilinear velocity (VCL, µm·s^{-1}), average path velocity (VAP, µm·s^{-1}), amplitude of lateral head displacement (ALH, µm), and beat cross frequency (BCF, Hz).

2.8. Statistical analysis

The assumptions of normality and homoscedasticity were tested using the Shapiro–Wilk and Levene tests, respectively, and normal probability plots were used to evaluate the distribution of all analyzed sperm variables. Analysis of variance (ANOVA) and generalized linear models were used to determine the effects of age, extender, cooling/equilibration time and freezing method, as well as their interaction effects on sperm quality variables.

The statistical model was:

$$Y_{ijk...u} = A_i + Ej(A)_j + Ag_k + P(AgEj)_l + Ex_m + C_n + F_o + (Ag*Ex)_p + (Ag*C)_q + (Ag*F)_r + (Ex*C)_s + (Ex*F)_t + (C*F)_u + e_{ijk...v}$$

in which Y is the variable; A_i = i-th random effect of animal; $Ej(A)_j$ = j-th random effect of ejaculate nested within animal; Ag_k = k-th age effect; $P(AgEj)_l$ = l-th straw effect nested within ejaculate nested within animal; Ex_m = m-th extender effect; C_n = n-th cooling-time effect; F_o = o-th freezing-method effect; $Ag*Ex_p$ = n-th age × extender interaction effect; $Ag*C_q$ = q-th age × cooling-time interaction effect; $Ag*F_r$ = r-th age × freezing-method interaction effect; $Ex*C_s$ = s-th extender × cooling-time interaction effect; $Ex*F_t$ = t-th extender × freezing-method interaction effect; $C*F_u$ = u-th cooling-time × freezing-method interaction effect; e = experimental error. Residual (co)variances were modeled to account for the repeated-measures structure, and the model with the lowest BIC and AIC values was selected as the best fit.

Differences between means for the effects of age, extender, cooling/equilibration time and freezing method were analyzed using the Bonferroni test ($P<0.05$). Results were expressed as mean ± standard error of the mean. Data were analyzed using the IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, US).

3. Results

No significant differences on total or progressive motility between the two age groups were found (Table 1). However, post-thaw velocities parameters exhibited significant age-dependent differences (Table 1; $P<0.05$). Specifically, semen from bulls older than 48 months demonstrated significantly greater values for rapid and medium motility, alongside lower values for slow motility, when compared with semen from bulls 24–48 months old. Regarding the cooling process, no differences ($P>0.05$) were observed in total and progressive motility between the two cooling time conditions (Table 1).

The effect of age on spermatozoa kinetic variables are reported in Table 2. Bulls aged 24–48 months exhibited significantly greater values for VCL, ALH, and BCF compared to bulls older than 48 months. Conversely, bulls older than 48 months had greater values for LIN, STR, and WOB ($P<0.05$). Regarding the impact of cooling time on sperm kinetic parameters, an increase in LIN ($P<0.05$) was observed at 4 hours of cooling compared to 6 h cooling.

There was an effect of extender on fresh semen prior to freezing on the motility variables ($P<0.05$). OptiXcell® presented the highest values of TM in the initial analysis (83.19±0.89%), followed by BioXcell® (63.01±0.93%) and finally Andromed® presented the lowest values for TM (25.97±1.25%). There was an impact of extender type on TM, PM, and fast-, medium-, and slow-speed spermatozoa post-thaw ($P<0.05$; Table 3). OptiXcell® extender had the greatest value for TM, PM, and slow speed spermatozoa ($P<0.05$), whereas BioXcell® extender had the greatest value for medium speed spermatozoa ($P<0.05$). Additionally, Andromed® had the lowest values for PM, and medium speed spermatozoa ($P<0.05$).

Table 1 - Sperm quality variables (percentage means ± standard error of the mean) post-thaw according to cooling time and age in Brahman bull semen

Variable (%)	Cooling time (h)		Age (months)	
	4	6	24-48	>48
Total motility	24.39±1.14	26.58±1.19	25.22±1.27	25.68±1.02
Progressive motility	13.35±0.94	15.66±0.99	14.39±1.05	14.52±0.85
Fast spermatozoa*	5.60±1.33	8.17±1.39	2.85±1.48y	11.19±1.19x
Medium speed spermatozoa*	5.98±0.45	5.99±0.47	5.16±0.50y	6.88±0.40x
Slow speed spermatozoa*	11.94±1.25	11.80±1.30	16.26±1.12x	7.05±1.35y

* Spermatozoa with movement categorized as fast (>45 µm·s^{-1}), medium (25 ≤ x < 45 µm·s^{-1}), slow (10 < y < 25 µm·s^{-1}).
Different letters (x–y) within the same row indicate statistical differences among bull age groups ($P<0.05$).

Table 2 - Kinematic variables (means ± standard error of the mean) post-thaw according to cooling time and age in Brahman bull semen

Variable	Cooling time (h)		Age (months)	
	4	6	24-48	>48
VCL (μm·s^{-1})	76.22±1.16	78.10±1.05	80.15±0.43x	73.98±1.56y
VSL (μm·s^{-1})	32.55±0.70	32.50±0.63	32.22±0.26	32.85±0.94
VAP (μm·s^{-1})	39.33±0.67	40.49±0.60	40.53±0.24	39.29±0.90
LIN (%)	42.35±0.55a	40.73±0.50b	39.71±0.20y	43.46±0.74x
STR (%)	74.34±0.66	72.82±0.59	72.33±0.24y	74.87±0.88x
WOB (%)	52.74±0.41	52.59±0.37	51.50±0.15y	53.94±0.55x
ALH (μm)	3.14±0.04	3.17±0.04	3.29±0.02x	3.02±0.06y
BCF (Hz)	7.32±0.11	7.35±0.10	7.56±0.04x	7.09±0.15y

VCL - curvilinear velocity; VSL - straight line velocity; VAP - average path velocity; LIN - linearity of forward progression; STR - straightness; WOB - wobble; ALH - amplitude of lateral head displacement; BCF - beat-cross frequency.
Different letters (a–b) within the same row indicate statistical differences among cooling times (P<0.05), whereas different letters (x–y) within the same row indicate statistical differences among bull age groups (P<0.05).

Table 3 - Sperm quality variables (percentage means ± standard error of the mean) post-thaw according to the extender used for cryopreservation of Brahman bull semen

Variable	Extender		
	Andromed®	BioXcell®	OptiXcell®
Total motility	18.92±1.98b	23.97±1.66b	31.61±0.62a
Progressive motility	6.86±1.64c	15.42±1.37b	19.30±0.52a
Fast spermatozoa*	3.89±2.30c	7.67±1.90b	8.28±0.72a
Medium speed spermatozoa*	4.51±0.77b	6.99±0.65a	6.20±0.24ab
Slow speed spermatozoa*	10.04±2.16b	8.33±1.81b	16.36±0.67a

* Spermatozoa with movement categorized as fast (>45 μm·s^{-1}), medium (25 ≤ x < 45 μm·s^{-1}), slow (10 < y < 25 μm·s^{-1}).
Different letters (a–c) within the same row indicate statistical differences among extenders (P<0.05).

The effect of extender type on sperm kinematic variables was analyzed and reported in Table 4. The OptiXcell® extender exhibited significantly greater values for VCL, VSL, VAP, ALH, and BCF. In contrast, the BioXcell® extender had greater values for LIN, STR, and WOB. However, the Andromed® extender produced the lowest values for VSL, VAP, LIN, STR, WOB, and BCF.

Table 4 - Kinematic sperm variables (means ± standard error of the mean) post-thaw according to the extender used for cryopreservation of Brahman bull semen

Variable	Extender		
	Andromed®	BioXcell®	OptiXcell®
VCL (μm·s^{-1})	83.03±1.04b	79.53±0.26c	118.60±0.18a
VSL (μm·s^{-1})	30.35±0.60c	44.91±0.15b	50.90±0.10a
VAP (μm·s^{-1})	41.11±0.58c	48.70±0.15b	64.52±0.10a
LIN (%)	37.28±0.40c	55.72±0.10a	43.57±0.07b
STR (%)	69.31±0.46c	87.45±0.12a	75.63±0.08b
WOB (%)	51.19±0.28c	61.61±0.07a	55.84±0.09b
ALH (μm)	3.48±0.04b	2.87±0.01c	4.15±0.01a
BCF (Hz)	7.70±0.09c	9.77±0.02b	10.18±0.02a

VCL - curvilinear velocity; VSL - straight line velocity; VAP - average path velocity; LIN - linearity of forward progression; STR - straightness; WOB - wobble; ALH - amplitude of lateral head displacement; BCF - beat-cross frequency.
Different letters (a–c) within the same row indicate statistical differences among extenders (P<0.05).

No differences (P>0.05) were observed in total motility (26.07±0.12%; 24.58±1.21%) and progressive motility (14.26±0.93%; 14.70±1.01%) across the evaluated freezing procedures (manual and automatic, respectively). However, as illustrated in Table 5, the effect of freezing methods on sperm kinematic variables revealed that the automatic method yielded greater values for VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF (P<0.05) when compared with the manual method.

There was no interaction effect between age, extender type, freezing method, and cooling time on total and progressive motility (P>0.05). However, there was an interaction (P<0.05) between extender type, cooling time, and freezing method for VCL (Figure 2), VSL (Figure 3), VAP (Figure 4), LIN (Figure 5),

Table 5 - Sperm kinematic variables (means ± standard error of the mean) post-thaw for different freezing methods of Brahman bull semen

Variable	Freezing method	
	Automatic	Manual
VCL (μm·s^{-1})	83.71±0.88a	71.23±1.26b
VSL (μm·s^{-1})	36.58±0.53a	28.81±0.76b
VAP (μm·s^{-1})	44.47±0.50a	35.78±0.72b
LIN (%)	42.66±0.42a	40.45±0.60b
STR (%)	74.66±0.50a	72.52±0.71b
WOB (%)	53.66±0.31a	51.76±0.45b
ALH (μm)	3.29±0.03a	3.04±0.05b
BCF (Hz)	7.82±0.08a	6.89±0.12b

VCL - curvilinear velocity; VSL - straight line velocity; VAP - average path velocity; LIN - linearity of forward progression; STR - straightness; WOB - wobble; ALH - amplitude of lateral head displacement; BCF - beat-cross frequency.
Different letters (a–b) within the same row indicate statistical differences among freezing methods (P<0.05).

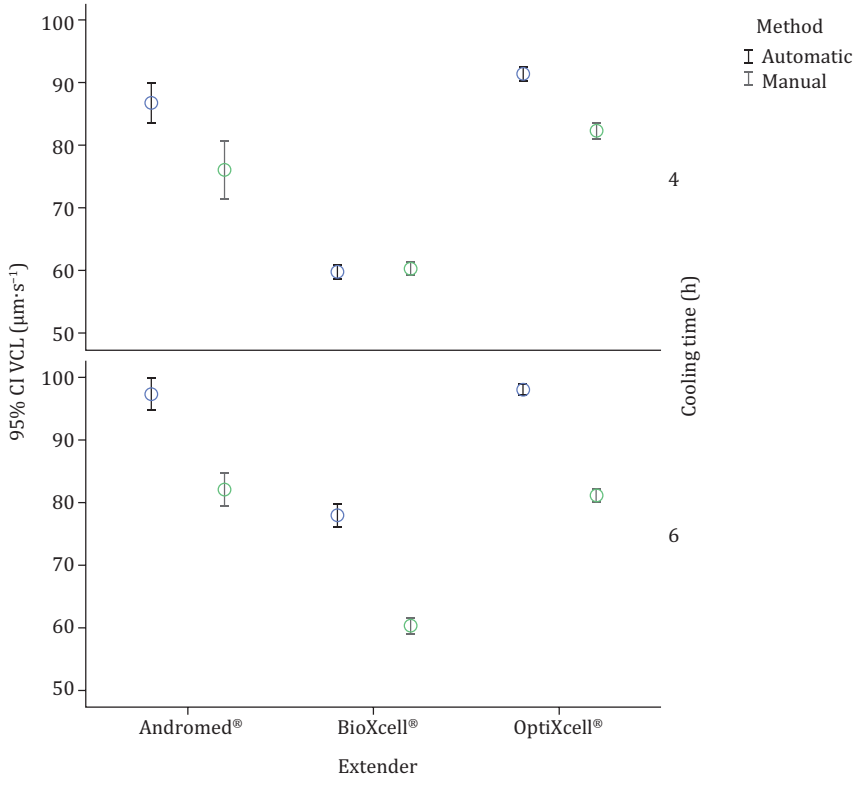

Figure 2 - Interaction effect between cryopreservation extender, freezing methods, and cooling time on curvilinear velocity (VCL, μm·s^{-1}) in thawed Brahman bull semen, using a confidence interval (CI) of 95%.

and BCF (Figure 6). The greatest VCL value was observed for the Andromed® extender at 6 h of cooling when frozen using the automatic technique (P<0.05) while the lowest VCL value was observed for the BioXcell® extender at 6 h of cooling when using the manual technique (P<0.05); additionally, at 4 h of cooling, both automatic and manual techniques showed similar values (P>0.05). Greater kinematic values were observed with the Andromed® extender, the automatic freezing method, and a 6-hour cooling time (P<0.05). The lowest values were recorded with OptiXcell®, a 4-hour cooling time, and the manual method (Figure 2).

The interaction effect between extender type, freezing method, and equilibration time on the VSL variable was assessed (P<0.05). The greatest values for VSL were observed for the OptiXcell® extender at 6 h of cooling when using the automatic freezing method (P<0.05). The lowest VSL values were observed with the Andromed® extender at 4 h of cooling when using the manual freezing method (P<0.05; Figure 3).

Significantly greater VAP values (P<0.05) were reported when the OptiXcell® extender was used in conjunction with the automatic freezing method for both cooling times. By contrast, BioXcell® and Andromed® extenders produced significantly lower (P<0.05) VAP, especially when associated with the manual freezing method at both cooling times (Figure 4).

Among the extenders, BioXcell® consistently resulted in the greatest LIN values, while Andromed® exhibited the lowest (P<0.05). The observed variability in LIN values depended on the extender and the cooling time, and it was found that the Andromed® diluent exhibited greater variability. The interaction of BioXcell®, 4 h of cooling, and the automatic method yielded the highest LIN values (P<0.05; Figure 5).

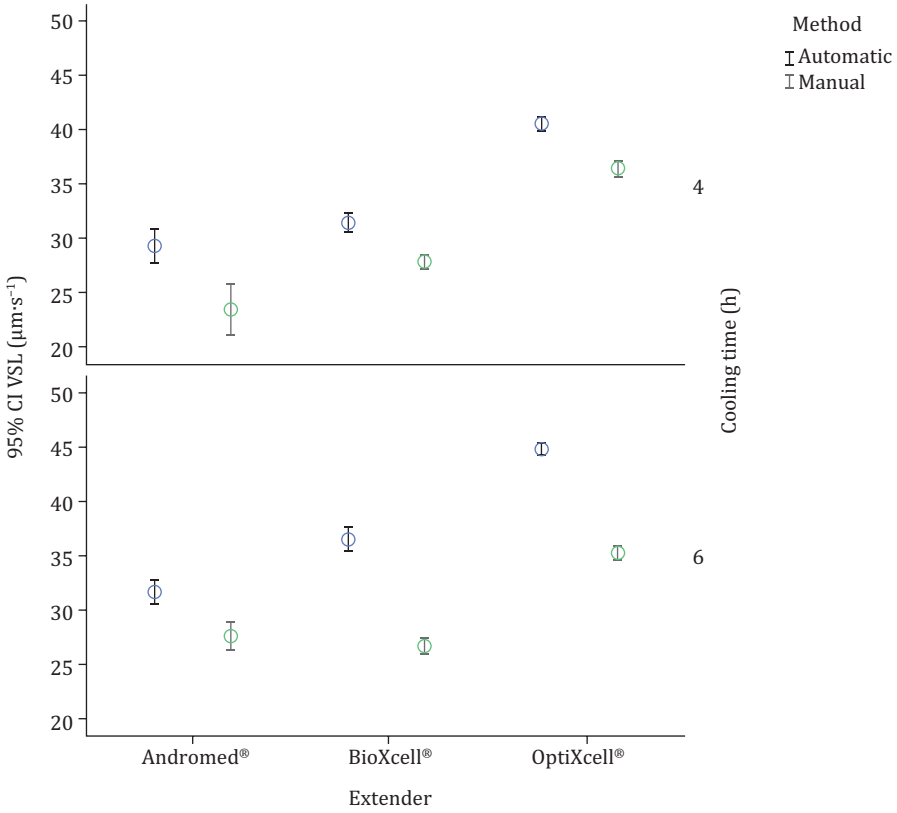

Figure 3 - Interaction effect between cryopreservation extender, freezing methods, and equilibration time on straight line velocity (VSL, µm·s^{-1}) in thawed Brahman bull semen using a confidence interval (CI) of 95%.

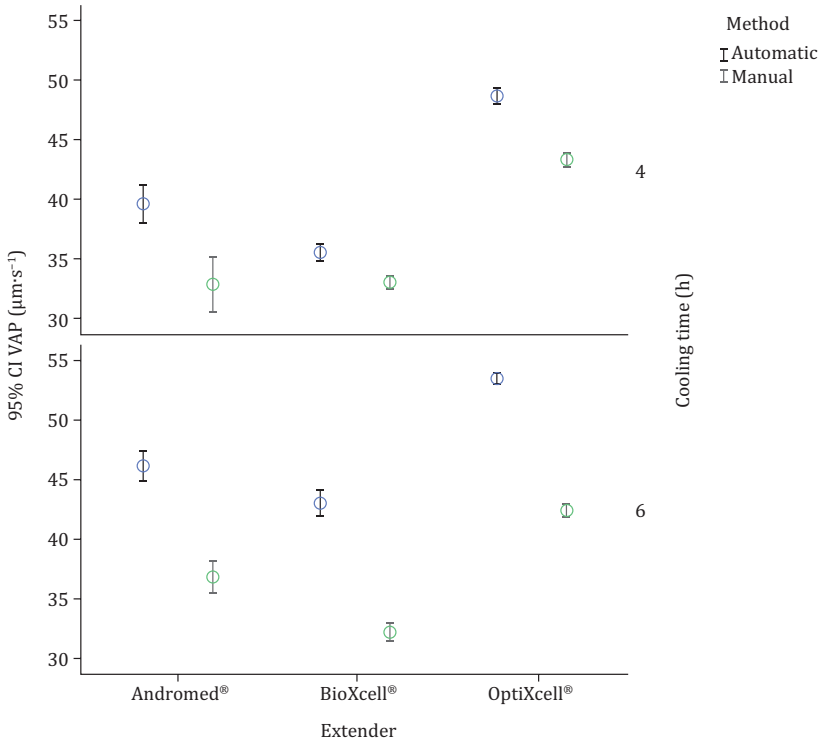

Figure 4 - Interaction effect between cryopreservation extender, freezing methods, and equilibration time on average path velocity (VAP, μm·s^{-1}) in thawed Brahman bull semen using a confidence interval (CI) of 95%.

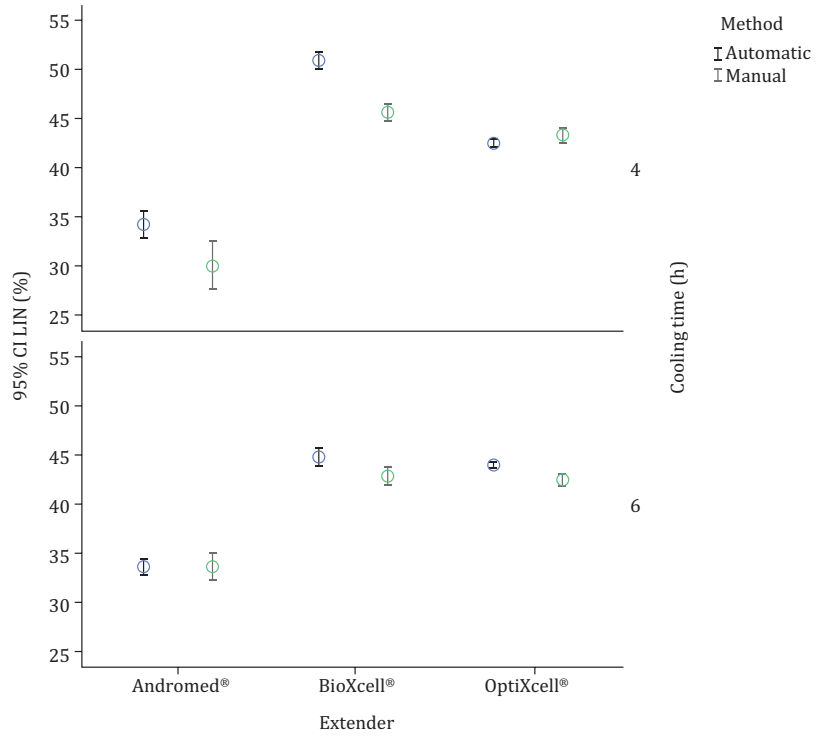

Figure 5 - Interaction effect between cryopreservation extender, freezing methods, and equilibration time on linearity index (LIN, %) in thawed Brahman bull semen using a confidence interval (CI) of 95%.

Among the extenders evaluated, using the automatic processing method, similar values were observed at 4 h of cooling time. However, at 6 h, the BCF values with the BioXcell® extender were lower (P<0.05). When the manual method was analyzed, at 4 h of cooling, the highest BCF values were observed with the OptiXcell® extender, and the lowest values were observed with Andromed® (P<0.05). At 6 h of cooling times, using the manual method, the highest values were observed with Andromed®, whereas the lowest were observed with BioXcell®. When cooling time was evaluated at 4 h, the BCF values for the Andromed®, BioXcell®, and OptiXcell® diluents were similar. At 6 h, no differences in BCF values were found between the automatic and manual methods for the Andromed®, and these values were similar to those observed using the automatic method with OptiXcell® (Figure 6).

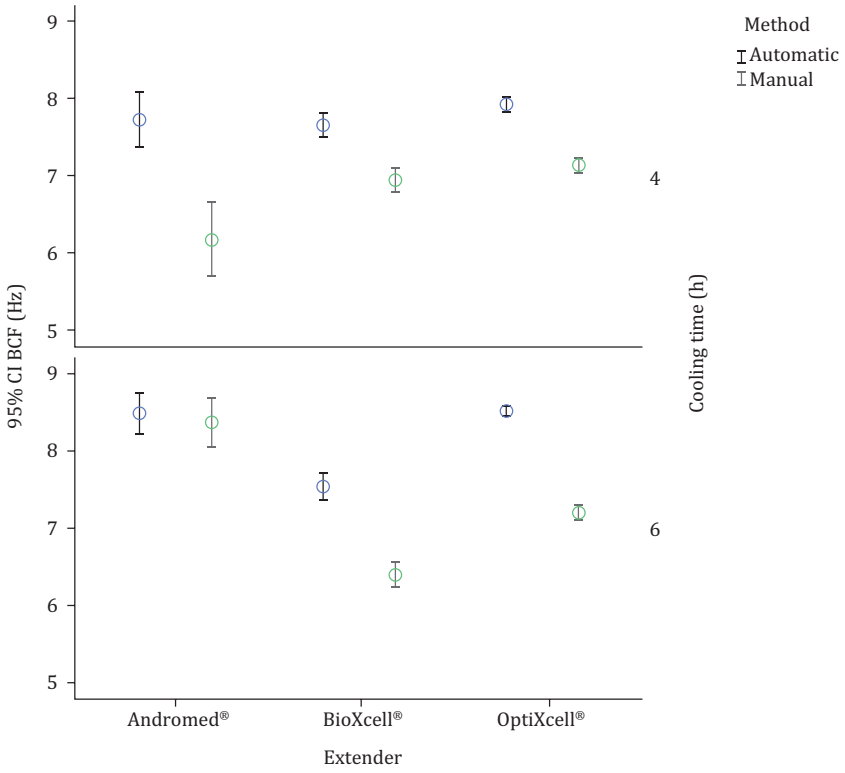

Figure 6 - Interaction effect between cryopreservation extender, freezing methods, and equilibration time on beat cross frequency (BCF, Hz) in thawed Brahman bull semen using a confidence interval (CI) of 95%.

4. Discussion

Many factors could influence the efficiency of the freezing process of spermatozoa. Most freezing protocols implement an equilibration period of 4 h or 6 h, but increasing the equilibration time did not improve motility patterns (Murphy et al., 2018a). Some authors have observed that increasing the equilibration period up to 72 h can improve sperm motility but does not affect field fertility (Fleisch et al., 2017). The results of the present study demonstrated that semen cooling time did not significantly affect total and progressive motility. Consistent with these findings, previous studies have also reported no significant differences in post-thaw motility between cooling times ranging from 4–9 hours (Herold et al., 2004; Murphy et al., 2018a). In other works in bulls, rams, and dogs, it has been observed that a 6-h cooling period resulted in greater sperm motility and progressive movement after thawing compared to shorter cooling times (1 h, 2 h, 3 h, 4 h) and 8 h of cooling, regardless of the freezing technique used (Belala et al., 2016; Dias et al., 2018; Murphy et al., 2018a; Vozaf et al., 2021).

Other studies in bulls and Buffalo have suggested that the improvement in post-thaw motility with extended equilibration periods may be linked to alterations in the sperm membrane, which facilitate adaptation to low temperatures and increase cryotolerance (Arunkumar et al., 2022; Inyawilert et al., 2024). With respect to the effect of equilibration period on kinematic variables, greater linearity was observed at 4 h. There was an interaction between extender, cooling time, and freezing method for LIN. The increase in LIN at 4 h is likely due to a reduction in values for BioXcell® at 6 h, whereas the other treatments appeared similar. During cooling, sperm cells undergo membrane and axonemal alterations, which impair their ability to move in a straight line, reducing key kinematic parameters, such as linearity and straightness, and ultimately leading to sperm death during freezing and thawing (Rasul et al., 2001). No significant interaction effect of extender × cooling time × freezing method was observed for STR, WOB, and ALH kinematic variables between the two cooling times, which agrees with several previous studies (Mehdipour et al., 2016; Rasul et al., 2001).

The effect of age on motility revealed that bulls older than 48 months exhibited a higher percentage of spermatozoa with fast and medium movement compared to younger bulls, which showed a greater proportion of slow spermatozoa. This finding is consistent with previous studies that reported improved motility after thawing in adult bulls (Brito et al., 2002; Murphy et al., 2018b). Some researchers suggest that these age-related differences may be linked to physiological changes associated with sexual maturity, such as the development of the testes and accessory glands (Bhasin et al., 2022; Majić Balić et al., 2012; Vince et al., 2018). Progressive motility typically indicates proper spermatogenesis and sperm maturation during epididymal transit (Tanga et al., 2021). In males, as individuals age, the testicular parenchyma undergoes changes associated with the aging process, affecting both the interstitial space and the germinal compartment, and leading to dysfunctions in testicular spermiogenesis and sperm maturation (Teves and Roldan, 2022). These factors may help explain the observed effects on sperm motility and morphology (Elbashir et al., 2021; Kühnert and Nieschlag, 2004). In addition, as bulls age, the percentage of sperm morphological anomalies increases, a trend that has also been reported in humans (Kleshchev et al., 2023; Ulubay et al., 2022). Variations in cryopreserved semen may arise due to an individual bull's ability to tolerate freezing and thawing, which can impact both internal and external cellular membranes, primarily through membrane lipoperoxidation (Khan et al., 2021; Kowalczyk et al., 2020; Sharafi et al., 2022; Upreti et al., 1998). The integrity of the plasma membrane is likely a key determinant of sperm motility in stallions (Akbarinejad et al., 2020) and bulls (Reis et al., 2016). Bulls older than 48 months demonstrated greater values for LIN, STR, and WOB in frozen semen, compared to younger bulls, which exhibited greater wave-like movement and more circular motion. These findings are consistent with those reported in fresh Brahman semen (Araya-Zúñiga et al., 2023). Additionally, the observed reduction in linearity and oscillation suggests sperm hyperactivation (Kathiravan et al., 2011) may also indicate premature capacitation/hyperactivation. This phenomenon may reflect the immaturity of sperm from younger bulls (Carreira et al., 2017).

Several commercial diluents are used to freeze bovine spermatozoa to avoid cryodamage with varying results (Ugur et al., 2019). The analysis of post-thaw sperm motility revealed significantly greater total motility, progressive motility, and the percentage of fast-moving sperm in semen diluted with OptiXcell® compared to Andromed® and BioXcell®. The difference in semen diluted with OptiXcell prior to freezing was substantial; on the order of 50–200% higher; which could be attributable to the pre-freeze effects of this extender**.** Consistent with these findings, previous studies have reported increased total and progressive motility when using OptiXcell® (Fleisch et al., 2017; Kumar et al., 2015). Furthermore, superior post-thaw sperm motility with OptiXcell® in comparison to both Andromed® and BioXcell® has been observed (Singh et al., 2018). The BioXcell® extender, made with soybean lecithin, produces low sperm quality in 80% of cryopreserved semen batches (Crespilho et al., 2012). This could be explained by the limited efficacy of soy lecithin as a primary source of lipoproteins, which are critical for sperm protection during cryopreservation, further underscores its limitations. In contrast, other studies have reported that the potential fertility of ejaculates may be reduced when stored short-term with AndroMed® (Fernandez-Novo et al., 2021).

The post-thaw kinematic analysis showed superior velocity values in semen cryopreserved with OptiXcell®, aligning with other studies that also reported better results compared to egg yolk and

soybean lecithin extenders (Kang et al., 2019; Robayo et al., 2008). The higher value of LIN for BioXcell is largely due to increased LIN at 4 h, under automated freezing conditions, as well as higher straightness index and oscillation values, indicating improved progressive motility (Araya-Zúñiga et al., 2023). This suggests that a greater STR reflects a more uniform sperm trajectory with reduced amplitude, while greater linearity indicates a smaller amplitude of curvilinear movement aligned with a straight path (Leite et al., 2010). Other works have suggested that the values of curvilinear velocity and lateral head displacement amplitude observed with Bioxcell® could be due to the extender's chemical composition, which may increase viscosity and induce curvilinear movement, like that in cervical mucus and suggests that improved preservation of flagellar integrity or enhanced ATP production, which supports increased crossing frequency (Celeghini et al., 2008). Moreover, BioXcell® also demonstrated the highest WOB values compared to Andromed® and OptiXcell®. The greater STR values in cattle correlated with lower lateral head displacement amplitude, indicating linear sperm trajectories (Pichardo-Matamoros et al., 2023; Víquez et al., 2020). Such movement, constrained by the oviduct walls, favors progressive and rectilinear motion, as the oviduct's striated structure limits circular movement (Hyakutake et al., 2019). The Andromed® extender exhibited the lowest values for velocity and progressive motility, a finding consistent with other studies that reported poor semen quality post-thaw in buffalo when using Andromed®, compared to egg yolk extenders (Herold et al., 2004). Similarly, the lower values were observed for average velocity, straight line velocity, crossing frequency, linearity, and oscillation in bulls when Andromed® was used (Fernandez-Novo et al., 2021). Comparable results were also reported in ram semen (Khalifa et al., 2013), in buffalo (Singh et al., 2018), and bulls (Fleisch et al., 2017). None of these studies, however, provided a definitive explanation for the reduced semen quality associated with Andromed®. Fernandez-Novo et al. (2021) suggested that the observed effects might be due to differences in the extender's composition. Since the precise composition of the extender is not disclosed by manufacturers, the interpretation of these results remains challenging.

Bovine sperm freezing is usually carried out with either static or programmable freezers (Vishwanath and Shannon, 2000). In our study, although total and progressive motility were not different between freezing methods, there was an increase in all post-thaw sperm kinematic parameters when semen was frozen with an automated programmable system compared to the static method. These findings are consistent with previous research reporting similar semen quality outcomes when comparing manual/static freezing and programmable automatic freezing techniques (Kabir et al., 2022). Furthermore, previous studies have demonstrated that static semen freezing, achieved by positioning straws 4–5 cm above liquid nitrogen for 10–15 min, produces favorable results in cattle and rams (Jha et al., 2019). Static freezing, achieved by reducing temperature in a manual cooling chamber using nitrogen vapor, is a more cost-effective and efficient method for buffalo semen cryopreservation (Kabir et al., 2022), but precise control is essential for high-quality post-thaw semen (Abud et al., 2014). The same study highlights that variation in factors such as polystyrene box size, liquid nitrogen levels, and lab conditions can negatively affect post-thawing semen quality. Similarly, in sheep, manual freezing yielded results comparable to those obtained with automatic programmable freezing, underlining its applicability in field settings where lower costs are a priority (Vozaf et al., 2021). Conversely, other works have reported no significant differences in motility between conventional and automated freezing techniques for bovine semen (Ribeiro-Peres et al., 2014). Automated programmable systems, however, offer strict control over cooling and freezing rates, reducing the impact of ambient temperature fluctuations (Jaiswal and Vagga, 2022; Khan et al., 2021; Liu and Li, 2020). Programmable cryopreservation units are ideal for large-scale semen freezing, though their high cost may limit accessibility (Purdy, 2006; Zhang et al., 2024). In our study, results showed that the automatic freezing method produced superior post-thaw sperm velocity variables, linearity index, and wobble (WOB) compared to the static method. Similarly, post-thaw sperm kinematics in semen frozen using a programmable unit were superior to those of semen frozen in a polystyrene box (Silva and Guerra, 2011). Also, a significant increase in swimming patterns such as average velocity, straight-line velocity, and curvilinear velocity was observed when using the automatic freezing protocol (Shah et al., 2016).

5. Conclusions

In our study, semen cryopreservation was significantly influenced by male age. The extender effect was found on post-thaw sperm motility and kinetics variables. A 4-hour cooling period was positively correlated with improved sperm linearity. The automatic freezing method positively influenced kinematic values but did not significantly affect total or progressive motility compared to the manual method. Freezing Brahman semen was more effective when semen was extended with OptiXcell®, cooled for 4 h, and frozen with an automatic method. Given the commercial importance of the Brahman breed, these findings should be used in future studies to highlight their relevance and applicability in research with Zebu cattle.

Supplementary material

The supplementary material of this article can be found online at: https://www.rbz.org.br/wp-content/uploads/articles_xml/1806-9290-rbz-55-e20250151/1806-9290-rbz-55-e20250151-suppl01.pdf

Data availability

The raw data supporting the conclusions of this article are available in the TECdatos Repository, Costa Rica Institute of Technology. Data identification number: https://doi.org/10.18845/RDA/CI5VOG

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the Costa Rica Institute of Technology (ITCR) for financing this study. This work was supported by the Costa Rica Institute of Technology [Vice-Chancellor's office of Research and Extension; VIE (Vicerrectoría de Investigación y Extensión; Project VIE-2151083)] and Postgraduate Office of ITCR. The funders had no role in the study's design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support

This research was funded by the Costa Rica Institute of Technology (Vice-Chancellor's office of Research and Extension; VIE (Vicerrectoría de Investigación y Extensión). This work was part of the research project VIE-2151083, "Optimización de la conservación y búsqueda de parámetros de la fertilidad en espermatozoides de animales de interés productivo"). The APC was funded by Costa Rica Institute of Technology (Vice-Chancellor's office of Research and Extension; VIE (Vicerrectoría de Investigación y Extensión).

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