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VIABILITY OF VITRIFIED EMBRYOS FROM ROMOSINUANO AND TROPICAL MILKING CATTLE OVER TWO SEASONS OF THE YEAR IN VERACRUZ, MEXICO

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ABSTRACT

The seasons of the year in tropical climates affect the behavior of female cattle of different breeds. The aim of this study was to determine the effect of the weather season on the viability of vitrified embryos on the Tropical Milking (TM) and Romosinuano (RM) breeds. Ultrasound-guided follicular aspiration sessions, *in vitro* fertilization, and embryo vitrification were carried out during two seasons: hot-dry (HD) and hot-humid (HH). A total of 31 RM and 27 TM donors were used, and data were collected on the viability of oocytes and embryos. The response variables observed in the devitrified embryos were the capacity of eclosion (CE), blastocele reconstitution (BL), trophoblast (TR), zona pellucida (ZP), and cell mass (CM). For cell viability, the number of live cells (LIV) and dead cells (DEC) were considered. The data were analyzed using a generalized linear model with the GENMOD procedure of SAS®. Multiple comparison tests and adjusted mean differences were performed with the Tukey test. An effect on the season × breed interaction ($p \leq 0.0001$) was observed in the characteristics studied. The TM breed displayed higher values in five characteristics ($p \leq 0.0001$), and the HH season ($p \leq 0.001$) in four characteristics. No breed effect was found on cell viability. However, in the HH season, the number of dead cells was lower ($p \leq 0.04$). Seasonal climatic variation in Southeastern Mexico had effects on the vitrified embryos.

Keywords: *Bos taurus* L., criollo breeds, genetic resources, hot climates, intertropical region, reproductive biotechnologies.

INTRODUCTION

In 2021, the global production of *in vivo* bovine embryos was 386 374, while *in vitro* was 1 521 018 (Stringfellow and Seidel, 2000; Viana, 2022), of which 65 % were transferred fresh and 35 % cryopreserved. One of the most important advances in bovine reproduction technology has been the vitrification of embryos. The percentages of gestation using devitrified embryos range from 65 to 27 % (Youngs, 2011; Naranjo-Chacón *et al.*, 2016). Therefore, the reproductive biotechnology of vitrified embryos is improving to obtain greater percentages of gestation.

The total percentage of eclosion observed 72 h after cultivation following devitrification is variable, ranging from 29 to 51.8 % (Cho *et al.*, 2002; Silva *et al.*, 2013). These differences may be influenced by factors external to the *in vitro* production process in the laboratory, including the climatic seasons of the year. These variations affect the reproduction capability of the bovine female, particularly the ovarian follicular dynamics and the embryo viability (Tomita *et al.*, 2023). Crossbred cows (*Bos taurus x Bos indicus*) showed more follicles during the rainy season (15 ± 1.3) than during the dry season (7.6 ± 0.9) (Cansino-Arroyo *et al.*, 2014). There is also evidence that the quality of collected embryos varies according to the season of the year. In Saudi Arabian cows, embryos were of excellent quality in 59 % of cases in the winter and 27 % in the summer (Rayn *et al.*, 1992).

The rise in the ambient temperature and the different tolerance to it in females affect their reproductive behavior by reducing the concentrations of hormones such as estrogen and the luteinizing hormone (LH), which negatively impacts the follicular dynamic (Rodrigues *et al.*, 2016). Heat stress activates the hypothalamus-pituitary-adrenal (HPA) axis, increasing cortisol levels, inhibiting the hypothalamus-pituitary-gonadal (HPG) axis, and reducing the release of the gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and follicle stimulating hormone (FSH). This results in lower estrogen production by the ovaries, which in turn affects the follicular recruitment, selection, and dominance process and has unfavorable effects on the quality of the oocytes (Roth and Wolfenson, 2016). Different studies have indicated that the bovine oocyte is sensitive to heat stress during maturation, which affects its quality and survival (Roth and Wolfenson, 2016; Rodrigues *et al.*, 2016).

On the other hand, the animal genotype also influences the reproductive behavior. In South America, particularly in Brazil, the production of embryos by *in vitro* fertilization has been promoted in *B. indicus* breeds due to their greater ability to provide viable oocytes (Gimenes *et al.*, 2015). The *B. taurus* criollo breeds, descendants of cattle brought to the New World in the 15th century, can tolerate temperatures from 25 to 40 °C (de Alba-Martínez, 2011). In hot tropical climates, the annual average temperature is greater than 22 °C, and the average of the coldest month is 18 °C. The differential volume of precipitation throughout the year affects the seasonal availability of forages and influences the reproductive behavior of cattle, especially in non-adapted breeds (Rosales-Martínez *et al.*, 2021).

More research and biotechnological improvements are needed for adapted *B. taurus* in hot tropical climates. The criollo Tropical Milking and Romosinuano breeds are

adapted to the hot climates of the Americas, including Mexico. These breeds would benefit from oocyte recovery, *in vitro* fertilization, and cryopreservation to increase their population sizes and participate in conservation and genetic breeding programs (de Alba-Martínez, 2011; Rosendo-Ponce and Becerril-Pérez, 2015). Therefore, the aim of this study was to evaluate the effect of the season of the year on the viability of embryos vitrified *in vitro* of the criollo Tropical Milking and Romosinuano breeds.

MATERIALS AND METHODS

Area of study

The study was carried out in Veracruz, Mexico (18° 58′–19° 11′ N and 96° 16′–96° 20′ W). Altitude varies between 20 and 100 m (SMN, 2020). The predominant climate is hot subhumid (Aw), with high temperatures throughout the year and rainfalls between June and October (García, 2004). For this study, two seasons of the year were defined and used: hot-dry season (HD) from March to May, with mean maximum temperatures of 31.6 ± 1.0 °C and 77.2 ± 1.1 % relative humidity, and hot-humid season (HH) from June to October, with mean maximum temperatures of 31.4 ± 0.4 °C and a relative humidity of 84.7 ± 1.0 % (Rosales-Martínez *et al*., 2021).

Experimental animals

Criollo Tropical Milking (TM; 27) and Romosinuano (RM; 31) cows with up to three calves delivered and the presence of corpus luteum of follicular dynamics were used, aged 54.2 ± 4.9 to 95.9 ± 5.5 months and with a body condition scale from 2.6 ± 0.1 to 2.9 ± 0.1 (scale of 1–5) according to Edmonson *et al.* (1989). The annual vaccination against rabies (Rabisin B®, Merial, Mexico) was also given. Additionally, parasites were removed (1 mL 50 kg^{-1}; Virbamec® Platinum, Virbac, Mexico), and phosphorous was given four times per month (10 mL; Phospho®, Virbac, Mexico), as well as 100 g of mineral salt (Veramin 8 bovines in grazing®, Virbac, Mexico) every day for 30 days. Feeding was carried out at pará grass (*Brachiaria mutica* Forssk) and native grass (*Paspalum* spp.) pastures with free access to water. From each cow, data on the age (EDD), respiratory frequency (RF), cardiac frequency (CF), body temperature (BT), serum glucose (GLU), basal insulin (INS), and blood cortisol (COR) were collected.

In vitro embryo production

During both seasons, the oocytes were obtained using the ultrasound-guided follicular aspiration process (Filipiak and Larocca, 2012). The oocytes were classified by the morphological characteristics of the *Cumulus* (Bó and Mapletoft, 2018). Viable oocytes were selected, placed in maturation media (MIV-Salt Biotech, Salt Biotech®, Brazil), and transported to the lab (Advanced Genetic Reproduction: AGR *In vitro*®, Veracruz). They were incubated (Eve incubator, WTA®, Denmark) for 24 h at 38.7 °C, 20.9 % oxygen, 5.5 % carbon dioxide, 73.6 % nitrogen, and 46 % relative humidity (Bó and Mapletoft, 2018).

For the *in vitro* fertilization, the oocytes (maximum of 28) were placed in 50 µL *in vitro* fertilization medium drops (FIV-Salt Biotech®, Salt Biotech, Brazil). To fertilize the oocytes, semen straws from TM and RM breeds were used. After thawing in a water bath at 36 °C, the straws were released into 1.5 mL Eppendorf tubes that were conditioned using the Percoll gradient method. The number of spermatozoa in each drop varied from 2 to 5 µL, depending on the concentration (at least $25x10^6$ spermatozoa) and individual motility (at least 60 % progressive motility of spermatozoa). After 18 to 20 h, the cumulus cells of the fertilized oocytes were removed using a 100 µL micropipette. Once the fertilized oocytes were denuded, they were placed in Petri dishes (35×10 mm; Corning Incorporated®, USA) with 100 µL of culture medium (CIV-Salt Biotech, Salt Biotech®, Brazil) supplemented with 10 % fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific®, USA), 0.25 µL of antibiotic (Gentamicina, Pfizer®, USA) with pH of 7.2–7.4 and 4 mL of mineral oil (Ovoil, Vitrolife®, Sweden). The fertilized oocytes were incubated (Eve incubator, WTA®, Denmark) for 7 days at 38.7 °C, 5 % oxygen, 5 % carbon dioxide, 90 % nitrogen, and a relative humidity of 46 %. A total of 632 fertilized oocytes were recorded, with structures with 2 to 16 blastomeres found.

Cryopreserved embryos

A total of 109 embryos were cryopreserved in a blastocyst stage with 90 to 100 cells, considered excellent in quality (quality 1) according to their morphology, integrity of the inner cell mass and zona pellucida, homogeneous cytoplasm, blastomeres of uniform size, and no signs of pyknosis. The open pulled straw (OPS vitrification protocol) was used (Romo *et al.*, 2007; Youngs, 2011).

Vitrification

The base medium (BM) was prepared with 6 mL cell culture medium (TCM 199-HEPES: Gibco™ Medium 199 (1X), liquid, with HEPES, Thermo Fisher Scientific®, USA) plus 1.5 mL FBS. For the LL 0.5 M solution, 3 mL of BM plus ethylene glycol (LL; Freeze, ARBiotech, Mexico). The vitrification solution 1 (VS1) consisted of 425 µL of BM, 37.5 µL of dimethyl sulphoxide (DMSO; PRIME-XV FreezIS DMSO-Free, Irvine Scientific, USA) plus 37.5 µL 5-methoxymethylfurfural (MMF, Sigma-Aldrich, Mexico); and the vitrification solution 2 (VS2) consisted of 670 µL of LL 0.5 M solution, 165 µL of DMSO, and 165 µL of MMF.

The selected and identified embryos were placed using the pipette and washed in two drops of 100 µL of the base medium. They were then moved in groups of at least five into 200 µL VS1 drops, where they remained for 3 min. With the micropipette, they were placed into the VS2 drop, where they remained for 15 min. They were then placed in a 0.25 mL straw for embryos cut in the shape of a spear (previously prepared), removing the excess VS2 medium and submerging the spear into the liquid nitrogen (LN2) between 25 and 30 s, from the placement of the embryo into VS2 and vitrification (Romo *et al.*, 2007, Youngs, 2011).

Heating of the vitrified embryos

To heat the embryos vitrified *in vitro*, the media were prepared using the following solution concentrations: 0.5 M LL solution; 0.25 M LL solution; 7.5 mL 0.5 M LL solution plus 7.5 mL of MB; 0.15 M LL solution: 2 mL 0.25 M LL solution plus BM; and transportation medium: 4.5 mL of TCM 199-HEPES, 500 µL FBS, 10 µL pyruvate (Gibco™ Pyruvate Solution, Thermo Fisher Scientific®, USA) plus 25 µL of antibiotic (Gentamicin, Pfizer®, USA). All solutions were adjusted to a pH of 7.1–7.2 to ensure proper heating and manipulation of cryopreserved embryos.

The media were stabilized at room temperature. The spears that contained the embryos were identified and taken out of the LN2 to submerge them in a dish with 0.25 M LL solution for 1 min, raising and lowering the embryos with the pipette for 30 s, and grouping them at the bottom of the dish. Then, the embryos were placed in a 100 µL drop with 0.15 M LL solution, where they were washed and removed for 30 s, and grouped at the bottom for 5 min. Later, they were transferred to 100 µL of the MB medium and placed in Petri dishes (35×10 mm; Corning®, USA) with 3 mL of transportation medium (Romo *et al.*, 2007, Youngs, 2011).

Viability of the cryopreserved embryos

To determine the viability of cryopreserved embryos, as well as the functionality of their structures, techniques such as *in vitro* embryo eclosion, eclosion ability, and cell viability (live and dead cells) were used.

Capacity of *in vitro* eclosion (structural viability)

The heated embryos were planted for 72 h and transferred into Petri dishes with 100 µL drops of culture medium (CIV-Salt Biotech, Salt Biotech®, Brazil), supplemented with 10 % of FBS, 0.25 µg mL^{-1} of gentamicin, and 0.5 % of non-essential amino acids, with a pH of 7.2–7.4 and 4 mL of mineral oil. They were incubated (Eve incubator, WTA®, Denmark) at 38.7 °C, 5.5 % carbon dioxide, 20.9 % oxygen, 76.6 % nitrogen, and 46 % relative humidity. To estimate the structural viability of the embryos, they were counted and their *in vitro* capacity of eclosion (CE) after 72 h was observed, along with the blastocele reconstitution (BL), trophoblast (TR), zona pellucida (ZP), and cell mass (CM). The embryos were evaluated following the technique described by Bó and Mapletoft (2018).

Cell viability, double staining technique with Hoechst/propidium iodide

For the staining with propidium iodide (P-4170, Sigma®, USA) and bisbenzimide (BIS, Hoechst 33342, Sigma®, USA), the media were prepared using the following solution concentrations: bisbenzimide staining: 1 mg of bisbenzimide stock solution plus 1 mL of sodium citrate (2.3 %); bisbenzimide working solution (10 µg mL^{-1}): 750 µL of sodium citrate (2.3 %), 250 µL ethanol (100 %) plus 10 µL of bisbenzimide stock solution; propidium iodide staining: iodide stock solution (5 mg mL^{-1}), 5 mg propidium iodide (P-4170, Sigma®, USA) and 1 mL PBS; iodide working solution

(0.05 mg mL^{-1}): 10 µL of iodide stock solution plus 990 µL of PBS; and washing solution (PBS + 0.025 % PVP): PBS (phosphate buffered saline, Sigma®, USA) 100 mL plus PVP (polyvinylpyrrolidone, Sigma®, USA) 25 mg.

After 24 h of having planted the embryos, they were stained, extracted from the culture medium, and double washed in a saline solution buffered with phosphate with 0.025 % PVP for 1 min. Afterwards, the embryo with a volume of 10 µL of washing medium was placed on a slide. The excess medium was removed and 10 µL of working bisbenzimide solution was added (10 µg mL^{-1}) for 1 min; the sample was kept away from direct light. After the allotted time, the bisbenzimide was removed and washed with 10 µL of solution, pipetting gently without losing sight of the embryo. The washing medium was then removed, and 10 µL of the iodide working solution (0.05 mg mL^{-1}) was added for 1 min. The excess propidium iodide was removed and washed, followed by the addition of 10 µL of antifade solution (S7114, Sigma®, USA) to prepare the slide for the embryo evaluation, with four dots made of a paraffin-petroleum jelly mixture (1:10 p/p) placed in the four corners to create space and prevent the embryo from losing its shape.

Serial sections were observed and obtained using a confocal microscope TCS-SP8+STED (Leica Microsystems GmbH, Wetzlar, Germany), with a HCX PL APO 40x0.75 DRY lens and the use of a filter with the ability to read the intensity of fluorochromes, the Hoechst (excitation at 405 nm and emission at 433–473 nm), and the propidium bromide (excitation at 488 nm and emission at 620 nm). Both fluorochromes were intercalated into the DNA. Hoechst enters all cells, while propidium iodide only enters dead or necrotic cells with membrane damage. The precise count and target of live cells (CVI) and dead cells (CMU) of the embryo was carried out using the software CaptaVision® (version 5.1), acknowledging the state and the viability of the embryo under study (Chatzimeletiou *et al.*, 2021). From the counts, the percentage of live cells (PCVI) and dead cells (PCMU) were calculated.

Statistical analysis

The data were analyzed with a fixed-effect generalized linear model, which included the effects of the season (EST), the breed (RA), the season × breed interaction (EST*RA), the variables of blastocele reconstitution (BL), trophoblast (TR), zona pellucida (ZP), cell mass (CM), live cells (CVI), dead cells (CMU), percentages of live cells (PCVI) and of dead cells (PCMU), and the covariable of age (EDD), respiratory frequency (FR), cardiac frequency (FC), body temperature (TR), serum glucose (GLU), basal insulin (INS), and blood cortisol (COR). A P(λ) distribution of the experimental error was considered, with a logit link function, using the GENMOD procedure of SAS® (SAS Institute, 2013). The comparison of means was performed using Tukey's honest test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Capacity of *in vitro* eclosion

The EST*RA interaction had a significant effect ($p \leq 0.001$) on the evaluated characteristics. In addition, the main effect of EST on the capacity of eclosion (CE) was observed 72 h after devitrification, which favored HD by more than 13 % ($p \leq 0.001$). RA significantly increased TM values by over 16 % ($p \leq 0.001$) (Figure 1).

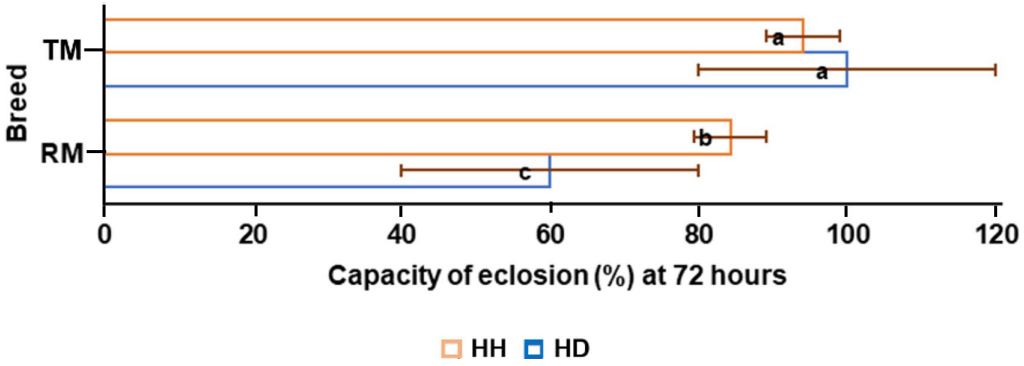

Figure 1. Effect of the hot-dry (HD) and hot-humid (HH) seasons on the capacity of eclosion observed at 72 h of planting after the devitrification of Tropical Milking (TM) and Romosinuano (RM) criollo cows in Veracruz, Mexico. [abc] Different letters indicate statistical differences ($p \leq 0.001$).

For the effects of EST and RA on BL, TR, ZP, and CM (Table 1), BL showed a 30 % reduction for RM in HD and a 12.5 % reduction in HH. The TM breed displayed a similar response to BL in both seasons, with a global difference with the RM breed of over 16 % ($p \leq 0.001$) and between seasons of over 11 % in favor of HH ($p \leq 0.001$). For TR, a 32 % reduction was estimated for RM in HD and 12 % for HH. The TM responded similarly to TR in both seasons, with a 20 % global difference with RM ($p \leq 0.001$) and more than 15 % in favor of HH ($p \leq 0.001$) between seasons.

In ZP, a 25 % reduction was estimated for RM in HD and 12 % in HH compared to TM, which displayed a similar response in both seasons, with a global difference with the RM breed of 17 % ($p \leq 0.001$) and between seasons of 13 % in favor of HH ($p \leq 0.001$). On CM, RM was estimated to be reduced by 25 % in HD and by more than 10 % in HH. The TM breed responded similarly to the CM breed, with a global difference of over 16 % ($p \leq 0.001$) and a seasonal difference of over 10 % favoring HH by 13 % ($p \leq 0.001$). For CE and the reconstitution variables BL, TR, ZP, and CM, the TM breed presented the highest values, which were similar in the HD and HH seasons, indicating that TM embryos are more resistant to seasonal change. Likewise, the RM breed consistently responded positively, moving from a less favorable condition in

Table 1. Functional characteristics of the devitrified embryos of the Tropical Milking (TM) and Romosinuano (RM) breeds during the hot-dry (HD) and hot-humid (HH) seasons in Veracruz, Mexico.

Characteristic	Breed	Season of year		Global
		HD	HH	
BL	RM	62.6 ± 2.0^{c}	82.7 ± 1.3^{b}	75.0 ± 1.1^{B}
	TM	92.6 ± 2.4^{a}	95.2 ± 1.3^{a}	91.1 ± 1.3^{A}
	Global	77.3 ± 1.5^{Y}	88.5 ± 0.9^{X}	
TR	RM	62.6 ± 2.0^{c}	84.0 ± 1.3^{b}	75.8 ± 1.1^{B}
	TM	92.6 ± 2.4^{a}	95.2 ± 1.3^{a}	90.9 ± 1.3^{A}
	Global	77.3 ± 1.6^{Y}	89.1 ± 0.9^{X}	
ZP	RM	67.9 ± 2.1^{c}	85.4 ± 1.3^{b}	78.6 ± 1.2^{B}
	TM	92.6 ± 2.4^{a}	95.8 ± 1.3^{a}	91.9 ± 1.3^{A}
	Global	80.0 ± 1.6^{Y}	90.3 ± 0.9^{X}	
CM	RM	67.9 ± 2.1^{c}	85.9 ± 1.3^{b}	78.9 ± 1.2^{B}
	TM	92.6 ± 2.4^{a}	95.8 ± 1.3^{a}	91.8 ± 1.3^{A}
	Global	80.0 ± 1.6^{Y}	90.5 ± 0.9^{X}	

BL: blastocele reconstitution; TR: trophoblast; ZP: zona pellucida; CM: cell mass. ^{AB} Different letters per row indicate statistical differences ($p \leq 0.001$). ^{XY} Different letters per column indicate statistical differences ($p \leq 0.001$). ^{abc} Different letter per row and column indicate statistical differences ($p \leq 0.001$).

HD to a more favorable one in HH, although at a lower response level than the TM breed.

The impact of the maternal environment on the reproductive behavior of the criollo breeds has been discussed (Guerrero *et al*., 2011; Rosales-Martínez *et al*., 2021). Prostaglandin application has been shown to cause an estrous manifestation of 52–100 % for TM (Guerrero *et al*., 2011; Rosales-Martínez *et al*., 2021). Fertility was similar in TM (81 %) and RM (83 %) (Parra-Cortés *et al*., 2019); the TM breed had gestation percentages between 40 and 77 % (Guerrero *et al*., 2011; Parra-Cortés *et al*., 2019), whereas RM had a value of 85 % (Martínez y Pérez, 2006). In terms of birth rate, TM had 85 % (Parra-Cortés *et al*., 2019), and RM, from 85 to 89 % (Ossa-Saraz, 2013; Parra-Cortés *et al*., 2019). In addition, the RM calving interval ranges from 442 to 553 d, while the TM calving interval ranges from 389 to 455 d (Ossa-Saraz, 2013; Rosendo-Ponce and Becerril-Pérez, 2015; Parra-Cortés *et al*., 2019; Martínez-Rocha *et al*., 2021).

For over 500 years, natural selection and cattle farmer selection during various historical periods have led to the adaptation of the TM and RM Criollo breeds. While the RM has been focused on producing meat, the TM has been more focused on producing dairy (de Alba-Martínez, 2011). In terms of adaptation, it is reasonable to assume that

TM embryos are very well adapted and more stable during the transition from HD to HH season. This may, in turn, have a positive impact on its ability to reconstitute after vitrification. Naranjo-Chacón *et al.* (2016) found that TM cows had 28.6 % ($p \leq 0.5$) more pregnancies with vitrified embryos than crossbred cows, which had only 5.6 %. When analyzing climatology in both seasons, there is no evidence of a significant difference in the mean maximum temperature, with 31.6 °C in HD and 31.4 °C in HH. However, there is a significant difference in relative humidity (RH), with 77.2 % in HD and 84.7 % in HH, as well as annual rainfall (5.4 % in HD and 89.5 % in HH), which is manifested in a larger reproductive response of the cattle (Rosales-Martínez *et al.*, 2021).

The feed of the bovines and their nutritional condition are relevant in the reproductive behavior (Tomita *et al.*, 2023). Malnutrition, understood as a deficient nutritional stage due to scarcity, inadequate absorption, an increase in needs, or an excessive loss of nutrients, is related to a reduction in the number of recruited follicles, a low ovulation rate, a reduction in the duration and return to estrus, a reduction in embryonic development, and an increase in embryonic and fetal mortality, which have a bearing on longer calving intervals and, generally, lower fertility (Henao and Trujillo, 2003; Tomita *et al.*, 2023).

Previous studies found that the percentage of gestation in artificially inseminated heifers decreased during the HD season, which was characterized by lower forage quality than the HH season (50 and 65 %, respectively) (Rosales-Martínez *et al.*, 2021). Bovines with a body condition < 2.5 had a higher embryonic mortality rate of 11 %, compared to bovines with a condition > 2.5, where embryonic mortality was 5 % (Rhind, 2004). These findings suggest that weather and diet have an effect on reproductive behavior and physiological mechanisms.

Cell viability

No effect of RA or EST was observed in CVI, PCVI, or PCMU ($p > 0.05$) (Figure 2). EST reduced HH in CMU by over 38 % ($p \leq 0.04$) (Figure 3). According to de Barros and Paula-Lopes (2018), the embryonic development *in vitro* is susceptible to heat stress before the activation of the embryonic genome. Silva *et al*. (2013) observed that embryos in their first stages displayed alterations in gene expression (caudal-type homeobox transcription factor 2; *CDX2*) associated with embryonic survival, implantation, and placental development. Sakatani (2015) and Paula-Lopes and Hansen (2002) found that expanded embryos undergo DNA fragmentation at 41 °C. Rodrigues *et al*. (2016) reported that blastocysts produced from heat-stressed oocytes had a lower abundance of transcripts related to cell growth and differentiation, whereas Sakatani *et al*. (2013) found a decrease in cell division rate in oocytes exposed to heat stress at 41 °C for 12 h. The increased expression of heat stress protective genes (*HSP70*; heat shock protein 70, *HSP90*; heat shock protein 90, *HSP27*; heat shock protein 27, *HSP60*; heat shock protein 60, *Hsf1*; heat shock factor 1, *BAG3*; and *BCL2*-associated athanogene 3) reduces apoptosis and increases embryonic survival (Silva *et al*., 2013). TM and RM breeds

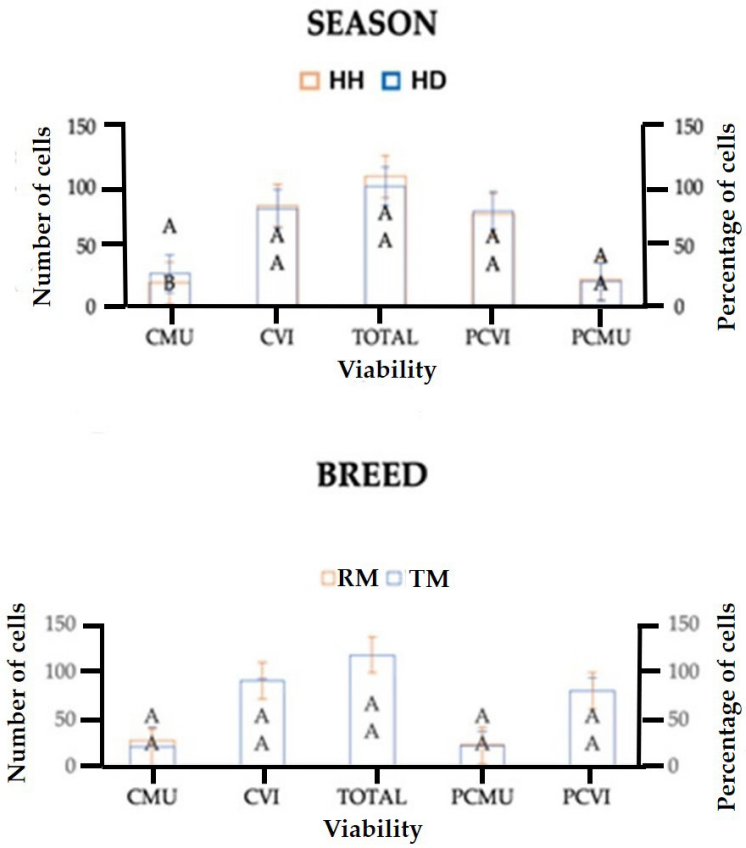

Figure 2. Cell viability of devitrified embryos of Tropical Milking (TM) and Romosinuano (RM) criollo cows in Veracruz, Mexico, under hot-dry (HD) and hot-humid (HH) seasons. CVI: number of live cells; CMU: number of dead cells; PCVI: percentage of live cells; PCMU: percentage of dead cells. ^{AB} Different lines indicate a statistical difference ($p \leq 0.05$).

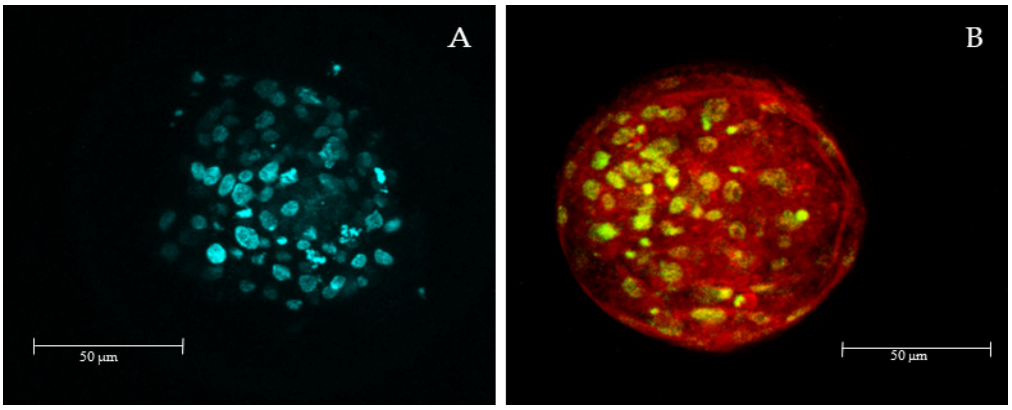

Figure 3. Embryos of the Tropical Milking Criollo breed captured using a TCS-SP8+STED confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the HCX PL APO 40x/0.75 DRY lens. A: Image with a high content of live cells; B: image with a high content of dead cells.

have developed genetic and physiological mechanisms to maintain homeostasis during extreme heat (Martínez-Rocha *et al*., 2021). These adaptation mechanisms assist in their survival and reproduction in high-temperature environments, including a greater resistance to apoptosis caused by heat stress in embryos.

Seasonal effects may have an impact on the viability of cells in vitrified embryos, with a higher number of dead cells during the HD season compared to the HH season. According to Sakatani *et al*. (2015), the critical stage in which heat stress may negatively affect embryonic development in humans is between fertilization and the stage of 4–8 cells, whereas in bovines it is produced at the stage of 8–16 cells. High temperatures induce DNA fragmentation and affect RNA; therefore, if the cumulus-oocyte complexes **(**COCs) are exposed to high temperatures in warm seasons, the number of cells in the few embryos that reach the blastocyst stage is reduced, resulting as well in greater damage in the blastomeres (de Barros and Paula-Lopes, 2018; Paula-Lopes and Hansen, 2002; Rodrigues *et al*., 2016). These damages have an impact of heat stress on embryo survival (Roth and Wolfenson, 2016).

Sakatani *et al.* (2013) observed that the morula stage is more resistant to higher temperatures (40–41 °C) in culture than the two-cell embryo. In addition, Paula-Lopes and Hansen (2002) and Sakatani *et al*. (2015) found an increase in the concentration of intracellular reactive oxygen species (ROS) in the embryo. These studies indicate underlying mechanisms that may contribute to embryo apoptosis caused by high temperatures. One of them is an increase in the production of ROS in embryonic cells; ROSs are highly reactive molecules that may cause oxidative damage to cell structures such as DNA, proteins, and lipids, which leads to the activation of apoptotic signaling pathways and, eventually, cell death.

Heat stress-induced apoptosis may have a significant impact on embryonic viability and development if the transcription of stress-related genes is not carried out correctly during embryonic development, whether *in vitro* or *in vivo*, and may jeopardize the result in the final stages of development and thus affect the embryo after vitrification (Paula-Lopes and Hansen, 2002; Sakatani *et al*., 2015; de Barros and Paula-Lopes, 2018). This indicates the importance of rigorously controlling the temperature during the oocyte recovery, IVF, and embryo cryopreservation in assisted reproduction procedures.

CONCLUSIONS

The effect of the season × breed interaction was observed in the characteristics of the devitrified embryos. In the Romosinuano breed, a greater negative impact of the hot-dry season was observed on the eclosion capacity of the embryos, unlike the Tropical Milking breed, whose embryos had a greater reconstitution and development capacity in both seasons, hot-dry and hot-humid. No main effect of the breed was discovered on embryo viability. These results highlight the importance of considering the effect of the season on embryo vitrification when planning assisted reproduction programs, reproductive technologies and feeding, with special reference to the hot-dry season.

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