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Effect of Embryonic Development Speed and Temperature-Humidity Index on the Pregnancy Rate of Dairy Cattle Embryos Produced *in vitro*

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ABSTRACT

Background: *In vitro* production of dairy cattle embryos, complemented by embryo transfer, is one of the main reproductive biotechnologies employed globally. However, factors such as the embryonic development rate (EDR) and the temperature-humidity index (THI) affect their effectiveness. The aim of this study was to evaluate the influence of EDR, represented by blastocysts obtained on *in vitro* culture days 7 and 8 and THI on pregnancy rate (PR) in Holstein cows using the embryo transfer technique.

Methods: An 800 recipient Holstein cows, which received a single quality 1 expanded blastocyst. The recipient cows were classified by the embryonic development (ED) day (day 7, n=356; day 8, n=444) and thermal conditions at the time of transfer, defined by THI as normal (≤72) or heat stress (HS) (>72). The PR was determined through ultrasonography 60 days after embryo transfer. To evaluate the association between the variables considered, a Pearson's chi-squared test was performed. The association between the variables was estimated using the odds ratio and Cramer's V. A logistic binomial regression model was adjusted to evaluate the individual effects of the variables and their interaction on the probability of pregnancy in cows.

Result: The blastocysts transferred on the 7th day reported a higher PR than those transferred on the 8th day (52.0 vs 44.6%; P=0.038). Furthermore, cows exposed to a normal THI showed a higher PR than those under HS (64.7 vs 41.7%; P<0.001). The interaction between variables was significant, revealing that under HS conditions, blastocysts transferred on the 7th day showed a higher PR than those transferred on the 8th day (52.2 vs 30.9%; P<0.001). The early developmental blastocysts exhibited a greater capacity to maintain pregnancy under HS conditions, suggesting that EDR may serve as a complementary criterion for morphological evaluation to identify embryos with a higher likelihood of establishing pregnancy.

Key words: Blastocysts, Dairy cattle, Embryo transfer, Heat stress.

INTRODUCTION

The heat stress (HS) identified as a major external factor affecting dairy cattle (DC) productivity (Fernandez-Novo *et al.*, 2020). In this context, negative effects have been documented of HS on DC productivity (Winton *et al.*, 2024). These include reproductive disorders, which decrease fertility and, consequently, the reproductive efficiency of dairy systems (Cavestany *et al.*, 1985). It has been reported that HS reduces gestation percentages and disrupts follicular development, oocyte quality, uterine blood flow and progesterone secretion, leading to increased embryonic mortality both *in vitro* and *in vivo* (Silva *et al.*, 2013). However, its severity is determined by a combination of environmental factors, including temperature, relative humidity, solar radiation and wind (Herbut *et al.*, 2018). In this sense, to quantify the intensity of HS across animals in relation to the environment, Hahn *et al.* (2009) developed a temperature-humidity index (THI) based on calculations of wind temperature and relative humidity; considering a normal THI≤72.0.

One of the reproductive biotechnologies used by genetic improvement programs is *in vitro* embryo production *via* ultrasound-guided follicular aspiration (Cardoso-Consentini

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et al., 2021). However, it's been reported that HS affects embryo development in DC (Hansen, 2019). Regarding the negative effects of HS on DC embryos, it has been reported that susceptibility is higher during early embryonic development (ED), whereas after the 7th day, embryos exhibit greater thermotolerance (Hansen, 2019). Biggers *et al.* (1987) reported a negative association between HS and pregnancy rate (PR) in bovine embryos during the 16 day of development. Which suggest that, despite the embryos' apparent acquired tolerance during the 7th day, HS may still affect their viability and reproductive performance. Hence, the objective of this study was to evaluate the influence of embryonic development rate (EDR), as represented by blastocysts obtained on the 7th and 8th days of *in vitro* culture and THI on PR in Holstein cattle using the embryo transfer technique. The objective above may contribute to improving the DC's reproductive success and, consequently, the income of cattle-milk-producing families; furthermore, it will contribute to global efforts to meet the goals of the 2030 Agenda for Sustainable Development (Navarrete-Molina *et al.*, 2019; Autukaitë *et al.*, 2021; Pampori *et al.*, 2025; Armijo-Nájera *et al.*, 2026).

MATERIALS AND METHODS

Animal management

All animals involved in the study were managed in accordance with national and international guidelines for research ethics, care and animal welfare (FASS, 2010; NAM, 2011). This study received institutional approval with number UAAAN-UL-25-1824. The study period spanned from January to December 2025. A total of 800 recipient and 200 donor Holstein cows, free from diseases and reproductive problems, were used in the study, with a body condition score of 3.0±0.25 units. Each bovine recipient's uterus received a single expanded blastocyst-stage embryo at 7 or 8 days of development.

Ovum pick-up

Each female bovine donor underwent a unique follicular aspiration session, performed using a guide connected to a cannula and a vacuum pump (WTA® 240V, USA). To recollect the aspirated oocytes from both ovaries of the donor cattle, 50 mL centrifuge tubes previously prepared with 400 µL of heparin and 5 mL of Phosphate Buffered Saline were employed (Solís-Corrales *et al.*, 2012).

Oocyte selection

The oocytes were filtered through a 75-micron filter (WTA®, USA) until an impurity-free sample was obtained. The oocyte selection was done through direct observation with a stereo zoom microscope (Motic® SMZ-171 TLED, Canada), taking into account the morphologic characteristics of the cumulus-oocyte complex, specifically, the quantity, integrity and compaction of the cumulus cells that surrounded the oocyte (Stringfellow and Seidel, 2010).

Oocyte maturation and fertilization

To complete the nucleus maturation, the oocytes were subjected to an *in vitro* maturation process, following the procedure described by Paula-Lopes and Hansen (2002). Mature oocytes were fertilized according to the method described by Paula-Lopes and Hansen (2002).

Embryo cultivation and cryopreservation

Zygotes were distributed in groups of 25 to 30 embryos in 50 µL drops and supplemented as described by Paula-Lopes and Hansen (2002) until develop to the blastocyst stage they reached the blastocyst stage. The embryos were cryopreserved *via* slow freezing, following the procedure described by Zárate-Guevara *et al.* (2018).

Female bovine recipient synchronization

To synchronize the female recipients, the protocol described by Zárate-Guevara *et al.* (2018) was used.

Embryonic transfer and pregnancy diagnosis

Every recipient cow received a single quality 1 expanded blastocyst-stage embryo, according to the classification by Stringfellow and Seidel (2010), with 7 or 8 days in development. The procedure and technique described by Naranjo-Chacón *et al.* (2016) were used for the transfer. The pregnancy diagnosis was performed 60 days after the transfer by ultrasonography (Mindray®, 2200, USA).

Meteorological data and calculation of the temperature-humidity index

The meteorological data included the maximum daily temperature (°C) and the maximum daily relative humidity (%), obtained from the Meteoblue platform for Gómez Palacio, Durango, México (Meteoblue, n.d.). For each embryonic transfer date, the THI was calculated using the equation proposed by Mader *et al.* (2006):

$$THI = (0.8 \times T) + (RH/100 \times [T - 14.4]) + 46.4$$

Where,

THI = Temperature-humidity index.

T = Maximum daily temperature (°C).

RH = Maximum daily relative humidity (%).

Subsequently, the transfers were classified into two categories based on the THI: normal conditions (THI ≤72.0) and heat stress (THI > 72.0).

Experimental design and statistical analysis

A total of 800 Holstein DC were used as recipients, each receiving a single quality 1 expanded blastocyst-stage embryo. The recipient animals were divided into two groups based on the embryonic development day (EDD) at the time of transfer: 356 received embryos at 7 days of development and 444 received embryos at 8 EDD. The response variable was the PR as determined by ultrasound diagnosis 60 days after embryo transfer (Mindray®, 2200, USA), using the equation:

Pregnancy rate (PR; %) =

$$\frac{\text{Total pregnant cows that received an oocyte at } n \text{ days of development}}{\text{Total cows that received an oocyte at } n \text{ days of development}}$$

A Pearson's chi-squared test was performed to evaluate the association between the PR and the EDD (7 or 8 days) and between the PR and the THI categories (normal ≤72.0 and heat stress >72.0). The association between the variables was estimated using the odds ratio and Cramer's V. A logistic binomial regression model was adjusted to evaluate the effect of the EDD, THI and their interaction on the probability of pregnancy; Nagelkerke's R^2 was calculated to assess the proportion of variance explained by the predictors. All statistical analyses in this study used a 95% confidence interval, with P<0.05 as the threshold for statistical significance and were performed using IBM SPSS Statistics v. 23 (IBM Corp. Armonk, NY, USA).

RESULTS AND DISCUSSION

Blastocyst transfer with different developmental rates and their effect on pregnancy rate

Table 1 shows the results for PR after ET in quality 1 blastocyst expanded stage for different days of development (*i.e.*, 7 or 8). It can be observed that blastocysts transferred on the 7th day had a PR higher than 7.4%, compared with those transferred on the 8th day (52.0% and 44.6%, respectively). These results are consistent with other research that has reported that blastocysts produced *in vitro*, with an early formation (6 or 7 days), show pregnancy rates higher than those with a late formation (8 or 9 days), suggesting that the EDR may serve as an additional indicator of embryonic competence and reproductive potential (Ferré *et al.*, 2020; Kim *et al.*, 2025).

Regarding embryo morphology, a result consistent with those observed in Hanwoo cattle, where blastocysts developed at the 7th day reached a higher PR in comparison to late development blastocysts (*i.e.,* days 8 and 9) (Park *et al.*, 2006). These results may be attributed to the fact that blastocysts with a late developmental rate exhibit lower intrinsic quality, even though their external morphology may be classified as "normal" according to conventional criteria (Rabel *et al.*, 2023). It's been reported that the use of ET during day 7 allows to avoid major thermal sensibility periods in oocytes and early embryos (Gendelman and Roth, 2012; Baruselli *et al.*, 2020).

Temperature-humidity index and its effect on pregnancy rate

The obtained results about the THI and PR (Table 2) demonstrate that, under normal THI conditions, the PR was higher that the recipient DC's uterus under HS (P<0.05), with a calculated difference of 23.0%, by going from a 41.7% in DC under HS, to a 64.7% in DC under normal THI. Likewise, cattle in a normal thermal environment had a 2.55-fold higher probability of achieving pregnancy than those under HS. In this sense, Cramer's V showed a moderate correlation strength for these variables. The PR reduction observed in recipients exposed to HS was consistent with Schüller *et al.* (2014), who reported that cattle exposed to HS had a significantly lower probability of pregnancy than those kept below the HS threshold. Similarly, Kasimanickam and Kasimanickam (2021) reported that cattle exposed to HS had a PR of 31.0%, whereas those not exposed had a PR of 52.0%, indicating a 21-point decrease associated with HS.

In DC, HS not only directly affects the embryo but also alters females' physiological functions, including their reproductive processes. It has been reported that exposure to heat increases circulating concentrations of cortisol, prolactin and prostaglandin metabolites (Jerome and Srivastava, 2012), thereby compromising folliculogenesis, oocyte competence and corpus luteum function. Reducing, as a result, the progesterone production, creating a less favorable uterine environment for the ED and the establishment of pregnancy (Kasimanickam and Kasimanickam, 2021).

From a metabolic perspective, an increase in THI could lead to decreased dry matter intake and a negative energy balance (Rodriguez-Venegas *et al.*, 2023). These systemic changes, together with the aforementioned negative effects on reproduction, may increase early embryonic loss (Kasimanickam and Kasimanickam, 2021). All the information above can contribute to a better understanding of the causes of the low PR obtained under HS conditions (Table 2). However, it should be noted that the present study did not evaluate other factors associated with PR, such as feeding, management and housing conditions of recipient cattle, among others, which also have the potential to affect the results.

Effect of the interaction between the temperature-humidity index and blastocyst transfer at different development rates on pregnancy rate

Table 3 describes the interaction among THI, transfer timing and pregnancy success. It can be observed that, under

Table 1: Association between the day of embryonic development and pregnancy rate in Holstein dairy cows.

Day of embryonic development	N	Non-pregnant; n (%)	Pregnant; n (%)	Odds ratio (95%CI)[1]	P-value	Cramer's V
7	356	171 (48.0)	185 (52.0)	1.35 (1.02-1.79)	0.038	0.07
8	444	246 (55.4)	198 (44.6)			
Total	800	417	383			

Pearson Chi-Square value: $\chi^2(1) = 4.303$; [1]95%CI: 95 % confidence interval.

normal THI conditions, the embryos transferred on the 8th day achieved a PR of 69.9%, higher than the 50.8% recorded on the 7th day. However, a reversal in the results was observed under HS conditions, where PR remained stable on day 7, whereas on day 8 it dropped to 30.9%, indicating a statistically significant association stronger than that observed under normal THI conditions. The effect size, calculated with Cramer's V, reveals that the influence of EDD on PR intensifies with increasing THI (Table 3).

The observed differences in PR percentages across different THI levels provide evidence that HS exerts an effect in accordance with the ED chronology. For example, under thermal neutrality, the higher efficiency on day 8 compared with day 7 suggests optimal synchronization with the recipient's uterine environment (Silva *et al.*, 2013). However, the drastic reduction in PR on day 8 (from 69.9% to 30.9%) under HS may indicate that embryos are highly vulnerable to the mother's physiological alterations (Hansen, 2019). In this sense, it has been reported that maternal hyperthermia decreases uterine blood perfusion and increases intrauterine temperature, thereby compromising the viability of embryos that already have altered metabolism (Silva *et al.*, 2013; Winton *et al.*, 2024).

The stability of the PR during day 7, despite the increase in the THI, supports the thesis that the embryo transfer allows the omission of the major thermic sensibility stages, which take place between days 1 and 6 post-fecundation (Kasimanickam and Kasimanickam, 2021). In that sense, Hansen (2019) highlights that the first seven days of the ED are particularly susceptible to the effects of HS and that embryonic resistance increases progressively as development advances. In this context, it has been demonstrated that the exposure of cattle to HS during the first day after estrus diminished the percentage of embryos that reached the blastocyst stage during day 8, whereas the exposure to HS after days 3, 5 and 7 post-estrus showed a considerably smaller effect (Silva *et al.*, 2013).

The logistic regression model used to estimate the probability of pregnancy was significant (Table 4). The three predictors included in the analysis showed a significant effect on the dependent variable (P<0.001). The results for the EDD factor revealed that, when the other variables were held constant, embryos transferred on day 7 had a 2.44-fold higher probability of pregnancy than those transferred on day 8. For its part, the odds ratio, indicated that the recipients, during normal temperature conditions had a probability 5.18 times higher to achieve pregnancy compared to recipients under HS conditions.

The significance of the logistic regression model confirms that the probability of success in embryo transfer

Table 2: Association between the temperature-humidity index (THI) and the pregnancy rate in Holstein dairy cows.

THI	N	Non-pregnant; n (%)	Pregnant; n (%)	Odds ratio (95%CI)[1]	P-value	Cramer's V
Normal (≤72.0)	215	76 (35.3)	139 (64.7)	2.55 (1.82-3.57)	<0.001	0.204
Heat stress (>72.0)	585	341 (58.3)	244 (41.7)			
Total	800	417	383			

Pearson Chi-square value: $\chi^2(1) = 33.159$; [1]95%CI: 95% confidence interval.

Table 3: Association between the temperature-humidity index (THI), embryonic development day (EDD) and pregnancy rate in Holstein dairy cows.

THI	EDD	Non-pregnant; n (%)	Pregnant; n (%)	Total	Pearson Chi-square value	P-value	Cramer's V
Normal (≤72.0)	7	29 (49.2)	30 (50.8)	59	6.78	0.009	0.178
	8	47 (30.1)	109 (69.9)	156			
Heat stress (>72.0)	7	142 (47.8)	155 (52.2)	297	27.25	<0.001	0.216
	8	199 (69.1)	89 (30.9)	288			
Total	7	171 (48)	185 (52)	356	4.3	0.038	0.073
	8	246 (55.4)	198 (44.6)	444			

Table 4: Logistic regression model for the probability of pregnancy as a function of the embryonic development day (EDD), temperature-humidity index (THI) and their interaction in Holstein dairy cows.

Predictor	Coefficient	Standard error	Wald	P-value	Odds ratio (OR)	OR (95%CI)[1]
Constant	-0.805	0.128	39.818	<0.001	0.447	
EDD 7 vs EDD 8	0.892	0.172	26.757	<0.001	2.441	1.74-3.42
Normal (THI ≤72.0) vs Heat stress (THI >72.0)	1.646	0.216	57.991	<0.001	5.186	3.40-7.92
EDD*THI Interaction	-1.7	0.358	22.563	<0.001	0.183	0.09-0.37

Pearson Chi-square value: $\chi^2(3) = 67.575$, $P < 0.001$; Nagelkerke's $R^2 = 0.108$; [1]95%CI: 95% confidence interval.

programs depends on the interaction between embryo vigor and maternal environmental conditions (Baruselli *et al.*, 2020). In this respect, it has been reported that EDR is a biomarker of biological competence (Kim *et al.*, 2025). Blastocysts that reach the transfer stage early have an inner cell mass with more cells and a higher expression of pluripotency transcription factors (Kim *et al.*, 2025). In contrast, the observed delay in embryos on the 8th day is associated with an increase in the cellular apoptosis index, which reduces their ability to be implanted (Yang and Rajamahendran, 2002). These results also suggests that the biologic advantage of 7th day embryos manifests itself differentially according to the THI; however, under HS, the embryo's intrinsic competence becomes critic, as only those with active thermotolerance mechanisms manage to survive in a uterine environment with reduced levels of progesterone and a compromised blood flow (Silva *et al.*, 2013; Winton *et al.*, 2024).

CONCLUSION

The EDR and thermal conditions at the time of transfer influence pregnancy establishment, as blastocysts transferred on days 7 and 8 showed similar morphological characteristics but differed in PR. In addition, the observed interaction between EDD and THI indicates that the embryos' response to environmental conditions may vary depending on their intrinsic developmental capacity. This suggests that EDR can serve as a complementary criterion in morphologic evaluation to identify embryos with a higher likelihood of achieving a successful pregnancy. It's recommended that the transfer of embryos at the blastocyst stage, around the 7th day of development, constitutes a strategy to reduce the negative effects of HS on fertility. This may allow ET programs for *in vitro*-produced embryos to select high-quality embryos, aiming to mitigate low seasonal fertility in Holstein dairy cows.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

Informed consent was obtained from the owner of the animals involved in this study. All experimental procedures and animal management used in this study were conducted in accordance with international and national standards for research ethics, care and animal welfare. Additionally, this investigation received institutional approval, with reference number UAAAN-UL-25-1824.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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