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Effect of Temperature Change and Estradiol Levels on Cattle Oocytes Developmental Competency: Comparing X-Sexed and Non-Sexed Sperm Following *in vitro* Fertilization

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Abstract: The study aimed to investigate the effects of temperature change and estradiol (E_2) hormone level on *In Vitro* Maturation (IVM) and fertilization of cattle oocytes using X-sexed and non-sexed sperm. In experiment 1: The oocytes were incubated at various temperatures of 35.5, 36.5, 37.5, 38.5, 39.5 and 40.5°C under similar conditions with 5% CO_2, in humidified air for 22 h. In experiment 2: The IVM medium was supplemented with various concentrations of E_2 (0, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/mL). In experiment 3: The matured oocytes were fertilized *in vitro* using X-sexed and non-sexed frozen-thawed semen. An Analysis of Variance (ANOVA) was performed. Comparisons were considered significantly different at ($p<0.05$) level of significance using Fisher's protected least significant difference test. All the above analyses were performed using SAS 9.2 statistical software, for continuous variables, means and standard errors were employed. The highest percentages of oocyte Polar Body (PB) extrusion were found at 39.5 (63.5±6.22), 38.5 (58.9±5.64) and 37.5°C (54.0±3.68) and differ from other treatments group ($p<0.05$). The oocytes with the first PB extrusion at 0 (2.42±4.39), 0.1 (21.00±7.194), 0.4 (29.90±8.19) and the 0.5 µg/mL (23.40±9.85) revealed significant difference with 0.2 (46.70±14.68) and 0.3 µg/mL (47.30±11.05) concentrations. The presumptive zygotes fertilized using non-sexed sperm had higher fertilization, cleavage, and blastocyst rates compared to the X-sexed sperm. In conclusion, oocytes matured at 39.5°C revealed optimal oocyte PB percentage as compared to other treatments. Oocytes matured at 0.2 and 0.3 µg/mL of E_2 revealed the optimal oocyte PB percentage at 39.5°C. Overall, presumptive zygotes produced at 0.2 and 0.3 µg/mL of E_2 and fertilized using X-sexed sperm provide lower oocyte developmental competency at 39.5°C.

Keywords: Maturation, Cumulus Cells Expansion, Polar Body, Cleavage

Introduction

Heat stress due to global warming has been associated with lower reproductive performance in cattle. The short-term temperature increase during oocyte maturation deeply affects the surrounding cumulus cells and embryo yield and quality (Stamperna *et al*., 2020). These conditions are adverse for agriculture and animal production due to high temperatures. Thus, for decades, *In Vitro* Fertilization (IVF) and embryo transfer techniques have focused on studying the optimal conditions for the production of high-quality embryos to transfer. To date, *in vitro* production and the developmental rate of cattle embryos have attained a great deal of attention (Kidie, 2019;

Sakatani, 2017). However, the impact of temperature and maturation mediums deployed during *in vitro* maturation and fertilization may be one of the greatest threats that affect *in vitro* cattle embryo production. Although the sensitive systems of temperature regulation in living organisms within an *in vitro* setting present a challenging task under different levels of heat stress might reveal any perturbation in oocyte maturation (Picton *et al*., 2008). From a clinical standpoint, the elimination of damaged follicles in cattle previously exposed to heat stress could result in the earlier development of healthy follicles and the production of high-quality oocytes. During preovulatory follicular development, increases in systemic concentrations of estradiol (E_2) in cattle influence a variety of functions including stimulation of estrous behavior, ovulatory gonadotropin surges, and gamete transport. The elevated occurrence of ovulation failure in cattle experiencing heat stress conditions can possibly be attributed to reduced levels of E_2 production, whether during synchronized or spontaneous estrus (López-Gatius and Hunter, 2017). The E_2 levels increase in pre-ovulatory follicles until a required threshold, which plays a role in influencing both estrus behavior and the pre-ovulatory Luteinizing Hormone (LH) surge preceding ovulation (Ben Said *et al*., 2007). Furthermore, exposure of cattle to acute pre-ovulatory heat stress has been linked to a decrease in the expression of genes specific to granulosa cells within follicles of ovulatory size (Vanselow *et al*., 2016). Although the dominant follicle gains the ability to ovulate once it attains a diameter of approximately 10 mm (López-Gatius *et al*., 2022), Ovulation failure has also been linked to the presence of smaller pre-ovulatory follicles (10-15 mm), in monovular cows (López-Gatius *et al*., 2018).

The possibility of sex preselection has always generated great interest among livestock producers and is one of the determining factors in increasing the genetic progress and farmers' profitability of either beef or dairy cattle (Manzoor *et al*., 2017). In the dairy industry, there is an excess production of male calves that are not in high demand; therefore, sexed frozen-thawed semen can be used to generate herd replacements and additional heifers for herd expansion at a faster rate from within the herd, thereby minimizing biosecurity risks associated with bringing in cattle from different herds. Furthermore, the use of sexed sperm/semen can increase herd genetic gain compared with the use of non-sexed sperm. Although sexed frozen-thawed semen offers economic benefits, its widespread adoption has remained limited so far (Butler *et al*., 2014) and there are numerous disadvantages to using sexed semen, which include low fertilization and cleavage rate and no current *In Vitro* Embryo Production (IVEP) conventions developed yet.

Although the commercial use of IVEP embryos has been growing worldwide, there still exists a need to improve yield and quality in order to increase the pregnancy rate and achieve deep freezing for direct transfer (Ferré *et al*., 2020). Therefore, with the increasing commercial implementation of cattle embryo IVEP worldwide, there is an increased focus on optimizing blastocyst yield and quality using sexed frozen-thawed semen. The incubation temperature, development media, and the semen type utilized are the fundamental elements for cattle embryos' success in arriving at the blastocyst stage from IVM, IVF, to *In Vitro* Culture (IVC). consequently, this study is mainly focused on the optimization of the IVEP from cattle oocytes investigating the possible synergistic effect of temperature and E_2 on IVM and IVF in cattle oocytes fertilized using X-sexed or non-sexed frozen-thawed semen.

Materials and Methods

Ethical Approval and Study Site

The study was approved and carried out according to the guidelines of the Agricultural Research Council, Animal Production Ethics Committee (APAEC 2020/04), and Tshwane University of Technology Ethics Committee (AREC 2021/05/004). This research was carried out in the Agricultural Research Council's (ARC) Irene (Pretoria), animal production and Germplasm Conservation and Reproductive Biotechnologies (GCRB) laboratories.

Chemicals and Reagents

Except where otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Co. in St. Louis, MO, USA.

Cattle Ovaries Collection

Heterogeneous cattle ovaries of unknown reproductive status were collected from a local abattoir (Morgan abattoir, springs, RSA) and the ovaries were immediately transported to the GCRB laboratory in 0.9% saline water (SABAX pour saline Adcock Ingram, RSA) in a thermos flask at 37°C. Upon arrival at the laboratory, warmed buffer saline was used to wash ovaries for removal of blood contamination and further sprayed with 70% alcohol for any preclusion of contamination. The ovary's temperature was checked using a thermometer (Pencil LASEC SA LAST12/20110 memmert, RSA). Then, the ovaries were placed in a water bath (B. Owen Jones LTD. Macdonald Adams and Company, RSA) at 37°C.

Oocyte Collection

The aspiration method for oocyte retrieval was carried out using 10 mL disposable syringes (u-life-medical, RSA) and an

18-gauge sterile hypodermic needle (u-life-medical, RSA). The needle was pushed inside the ovaries and sucked out the follicular fluid of visible follicles (2-5 mm in diameter). The recovered follicular fluid was searched for the recovered oocytes under the microscope Olympus CX 23 microscope at 80× magnification (New York Microscope Co, USA).

In vitro Maturation of Cattle Oocytes

Experiment 1: To compare the maturation rate of cattle oocytes under different incubation temperatures (35.5, 36.5, 37.5, 38.5, 39.5, and 40.5°C). The oocytes underwent six rounds of washing before maturation as follows; they were washed three times in Modified Dulbecco's Phosphate Buffered Saline (MDPBS) and three times in M199+10% Fetal Bovine Serum (FBS). The four-well dish (Thermo scientific Nunclon Delta surface) was used for IVM, each well contained 500 µL of maturation medium (Table 1) supplemented with Fetal Bovine Serum (FBS), Tissue Culture Medium (TCM) 199, sodium pyruvate liquid, antibiotic-antimycotic, Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH) and E_2 covered with 250 µL of mineral oil. A total of 150 oocytes with a full or moderate attachment of cumulus cells were used per treatment and were incubated at 6 various temperatures of 35.5, 36.5, 37.5, 38.5, 39.5, and 40.5°C under similar conditions (5% CO_2, humidified air) for 22 h and the treatment was replicated 9 times. The standard E_2 level (0.2 µg/mL) was used when evaluating the temperatures, therefore, we ran with the best temperature on the following treatment with the various levels of the E_2.

Experiment 2: To determine the optimal concentration of E_2 suitable for cattle oocyte maturation rate. The six different dishes containing 500 µL of maturation medium which were supplemented with FSH, LH, and various concentrations of E_2 covered with 250 µL of mineral oil and incubated under similar conditions (5% CO_2, humidified air for 22 h and the experiment was replicated 8 times). The different concentrations of E_2 used were 0, 0.1, 0.2, 0.3, 0.4, and 0.5 µg/mL per experimental dish. A total of 200 oocytes per treatment were incubated at a pre-determined temperature from experiment 1 (39.5°C).

Oocytes Cumulus Cells Expansion

Following 22 h in the maturation medium, the oocytes were checked for the expansion of cumulus cells as a sign of cytoplasmic maturation. The expansion of Cumulus Oocytes Oophorus Complexes (COCs) was evaluated using a modified version of the methodology outlined by Maruska *et al*. (1984). Briefly, COCs with uniformly granulated cytoplasm, not exhibiting degenerative (apoptotic or necrotic symptoms) and surrounded by several compact layers of cumulus cells were classified as expanded COCs. All of the COCs other than expanded COCs such as COCs without cumulus cell expansion (no observable sign of cumulus expansion) were classified as non-expanded COCs. All COCs that were not expanded, as if COCs without any visible evidence of cumulus expansion were categorized as non-expanded COCs. The COCs expansion of oocyte maturation was done by visualization of the expansion of COCs (Şen and Kuran, 2018).

Oocytes First Polar Body Extrusion

The oocytes were then transferred to the Eppendorf tubes (micro, centrifuge tube USA) following 22 h of IVM. Briefly, oocytes were immersed in a 200 µL M199+10% FBS and vortexed for 1 min and 30 sec to remove the cumulus cells surrounding the oocytes. The extrusion of the oocyte's first PB development was examined under the micromanipulation microscope (New York Microscope Co, USA) using the Oosight Imaging USA system at 20×/0.45 Rc^2 magnification. The number of oocytes with the presence and absence of the first PB extrusion was recorded.

Experiment 3: To investigate the fertilization and cleavage rate competency of cattle oocytes fertilized using X-sexed and non-sexed sperm. Prior to IVF, sperm motility was analyzed with the aid of a Computer-Assisted Sperm Analysis (CASA) system known as the Sperm Class Analyzer® (SCA®) system (Microscopic, S.L, Barcelona, Spain). The 5 µL of semen was taken and placed on a warmed micro-scope glass slide (76×26×1 mm wadmar-knittel, Germany) and covered with a microscope glass coverslip (22×22 m Wadmar-Knittel, Germany) and evaluated under ×10 (Nikon, China) magnification of the SCA®. The analysis consisted of the following parameters: Total Motility (TM), Progressive Motility (PM), Static (STC), Rapid (RAP), Medium (MED), Slow (SLW), Curvilinear Velocity (VCL), time-averaged velocity (VAP), Straightness (STR), Linearity (LIN), Wobble (WOB), the Amplitude of Lateral Head displacement (ALH), Beat Cross Frequency (BCF) and Hyperactive (HA).

Table 1: Composition of the medium for IVM of cattle oocytes

Constituent	Concentration (20 mL)
M199+10% fetal bovine serum	20 mL
Follicle-stimulating hormone	10 µL
Luteinising hormone	100 µL
Estradiol	40 µL
Sodium pyruvate liquid	200 µL
Antibiotic-antimycotic	200 µL

Sperm were evaluated for live normal, dead, and live sperm with abnormalities on the head, mid-piece, or tail, which was determined using Nigrosin-eosin stain (Onderstepoort, faculty of veterinary sciences pharmacy and RSA). A 7 µL frozen-thawed semen (X-sexed and non-sexed sperm) sample was mixed with 20 µL of a Nigrosin-eosin staining solution into an Eppendorf tube. A total mixture of 5 µL was smeared across the microscope glass slide and air-dried at room temperature. The stained microscope glass slide was placed on a microscope table and a drop of immersion oil (Merck chemicals, RSA) was placed on the microscope glass slide to evaluate sperm viability and morphology using a microscope (Olympus, corporation BX 51FT, Tokyo, Japan) under ×100 magnification. The live normal sperm did not absorb the stain, which appeared white, and dead sperm had a brown or dark colour as they absorbed the stain. The 200 sperm per slide/treatment was evaluated for sperm viability and morphology traits.

Bull X-sexed and non-sexed frozen-thawed semen with proven fertility were purchased from American Breeders Service Global Inc. Company. The frozen semen straws were removed from the liquid nitrogen tank (-196°C) during thawing. The frozen semen straw was then exposed for 10 sec in the air and then placed for 1 min in warm water at 37°C. The semen straw was dried off the water with a paper towel and cut on both sealed ends and the contents inside the straw were collected into a 15 mL Falcon® tube.

The frozen-thawed semen was mixed together with 6 mL of pre-warmed Bracket and Oliphant (BO) wash medium. The mixture was centrifuged at 1500 rpm for 8 min at 37°C. Following centrifuging a pellet was formed at the bottom of the tube and the top part was removed carefully using the serological pipette without disturbing the sperm pellet, the same level of BO medium was added to the pellet and the mixture was centrifuged for the second time at the same speed. After centrifuging, the supernatant was removed leaving the pellet at the bottom of the tube. The sperm pellet was diluted with 50 µL BO IVF medium depending on the number of drops having oocytes and the concentration of the sperm.

In vitro Fertilization of Cattle Oocytes

Before fertilization, the matured oocytes were first washed in pre-warmed BO IVF wash medium drops (n = 05) which contained sodium chloride, potassium chloride, sodium phosphate, magnesium chloride, calcium chloride, glucose, antibiotic-antimycotic (Gibco, Germany), phenol red, sodium bicarbonate and sodium pyruvate with a total volume of 100 µL which were made in 1008 Falconi® petri dishes and covered with 3 mL of mineral oil. After washing 20-25 oocytes were transferred in each pre-warmed BO-IVF drop (n = 02) which contained essential fatty acid-free, bovine serum albumin and heparin with a volume of 50 µL covered with 250 µL of mineral oil for fertilization, which was then fertilized with 50 µL sperm pellet at a concentration of approximately 1×10^6 sperm/mL. A total of 75 oocytes were *in vitro* fertilized per treatment with X-sexed and non-sexed sperm. The oocytes were incubated together with the sperm for 18 h in 5% CO_2 in humidified air incubated at 39.5°C. The experiment was replicated 10 times.

Fertilization Rate Assessment

Following fertilization (18 h) of oocyte-sperm incubation, a portion of presumptive zygotes were removed from the IVF drops into a 1.5 mL Eppendorf tube containing 200 µL of M199+10% FBS medium and vortexed for 1 min to remove the cumulus cells while other portion were subjected to embryo culture. After vortexing, presumptive zygotes were washed three times in pre-incubated M199+10% FBS solution. Briefly, the denuded zygotes were stained with the 25 mg (0.025 g) Hoechst 33342-dye solution. Glass slide was used where the presumptive zygotes were placed. The 4 small drops of lubricant (Vaseline) were made around the presumptive zygote drop. A minimum volume (2-10 µL) of stock B solution was added to the presumptive zygote drop. The coverslip turned into positioned over the slide and gently squeezed until it touched the zygote drop. Colourless nail polish was used to seal the ends of the coverslip. The slide was allowed to dry for 2 h in a darkish compartment before counting the zygotes with the aid of an inverted microscope. The fertilization status was assessed by investigating the presence and number of Pronuclear (PN). The oocyte with one and two PN was considered as normal fertilization, whereas those with three PN were considered to have undergone abnormal fertilization and those with no PN were defined as unfertilized.

Cattle Embryo Culture

The IVF-derived presumptive zygotes denuded of cumulus cells were cultured in SOF containing cattle serum albumin (SOF-BSA) in 50 µL droplets covered with mineral oil for further embryonic development for 48 h (day 2 of *in vitro* embryo production). The dish containing the presumptive zygotes was placed in a modular chamber, which was filled with a 5% CO_2 incubator that was controlled by placing water in the modular chamber) and they were incubated in the CO_2 incubator. The cleavage rate was recorded 2 days post-fertilization. After IVC for an additional 72 h (at Day 5), the embryos were transferred to a new pre-warmed medium in 20 µL (SOF-BSA) culture medium which was removed and replaced with 20 µL fresh of SOF-BSA per culture drop. At the end of the *in vitro* culture (Day 7) the embryos were evaluated morphologically for morula and blastocyst formation rates.

Statistical Analysis

Data was collected for the oocyte maturation rate (cumulus expansion and first PB extrusion), fertilization rate (one, two pronuclei, and more than two pronuclei) embryonic development status (cleavage, morulae, and blastocyst rate). The data was then analyzed as a stepwise design with experiment 1 replicated 9 times, experiment 2 replicated 8 times and experiment 3 replicated 10 times. An Analysis of Variance (ANOVA) was performed. Comparisons were considered significantly different at ($p<0.05$), level of significance using Fisher's protected least significant difference test. Pearson's correlation coefficients were calculated to test the relationships between sperm motility, velocity, fertilization, and cleavage rate in using X-sexed and non-sexed sperm. All the above analyses were performed using SAS 9.2 statistical software, for continuous variables, means, and standard errors employed.

Results

The results of the effect of incubation temperatures on the maturation rate of cattle oocytes are presented in Table 2. The means of the COCs in 35.5 (79.4±33.7), 36.5 (85.3±6.8), 37.5 (86.9±5.8), 38.5 (90.7±4.4), and 39.5°C (88.8±3.5) was found to be significantly different compared to 40.5°C (56.6±6.6). The highest percentages of oocytes with the first PB extrusion were at 39.5 (63.5±6.2), 38.5 (58.9±5.6), and 37.5°C (54.0±3.6) and differ from other treatment groups ($p<0.05$). The results demonstrate that oocytes incubated at temperatures lower than 37.5°C and higher than 39.5°C show a reduction of the first polar body extrusion.

The results of the effect of E_2 concentrations on the maturation rate of cattle oocytes are presented in Table 3. The expansion of COCs revealed no significant difference in all the treatments besides the control {0 (55.5±3.7)}. The oocyte with the first PB extrusion (Fig. 1) at 0 (2.42±4.39), 0.1 (21.00±7.194), 0.4 (29.90±8.19), and 0.5 µg/mL (23.40±9.85) revealed significant difference with 0.2 (46.70±14.68) and 0.3 µg/mL (47.30±11.05) of E_2 concentrations.

Table 4 indicates microscopic evaluation results of X-sexed and non-sexed frozen-thawed semen on sperm motility and velocity traits. The total motility (52.61±4.26), static (47.53±4.3), and linearity (23.37±5.60) in X-sexed frozen-thawed sperm were observed to be significant differences compared to non-sexed frozen-thawed semen. No significant difference was observed for all other sperm motility and velocity parameters for all treatments.

The results for bull sperm morphology and viability of sexed and non-sexed frozen-thawed semen are presented in Figure 2. The average percentage of sexed (38.00±2.65) and non-sexed live sperm (58.33±7.64) was recorded ($p<0.05$). There was no significant difference in the midpiece and tail percentages in sexed and non-sexed sperm. Curiously, a significant difference was observed for sexed live sperm head (20.00±3.00) abnormality (cracked, loosened, and detached) percentages compared to non-sexed sperm (3.33±1.15).

Table 2: Effect of incubation temperatures (35.5, 36.5, 37.5, 38.5, 39.5 and 40.5°C) on the maturation rate of cattle oocyte (Mean ± SD)

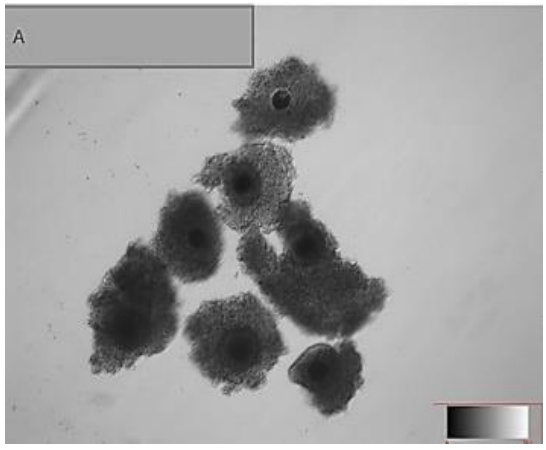
Treatment (°C)	Total number of oocyte matured	COCs expansion %	PB %
35.5	150	79.4±33.7[a]	33.7±4.97[f]
36.5	150	85.3±6.8[a]	47.5±5.3[d]
37.5	150	86.9±5.8[a]	54.0±3.6[c]
38.5	150	90.7±4.4[a]	58.9±5.6[b]
39.5	150	88.8±3.5[a]	63.5±6.2[a]
40.5	150	56.6±6.6[b]	42.1±3.7[e]

[a-f]values within the same column with different superscripts differ significantly ($p<0.05$); COCs = Cumulus Oophorus Complexes and PB = Polar Body

Table 3: Effect of estradiol on the maturation rate of cattle oocytes at 39.5°C (Mean ± SD)

E_2 (µg/mL)	Total number of oocytes matured	COCs expansion %	PB %
0	200	55.5±3.7[b]	2.4±4.3[c]
0.1	200	90.0±1.4[a]	21.0±7.1[b]
0.2	200	89.9±3.4[a]	46.7±14.6[a]
0.3	200	90.3±3.4[a]	47.3±11.1[a]
0.4	200	89.3±3.1[a]	29.9±8.1[b]
0.5	200	87.6±3.4[a]	23.4±9.8[b]

[a-c]Values within the same column with different superscripts differ significantly ($p<0.05$). COCs = Cumulus Oophorus Complexes and PB = Polar Body



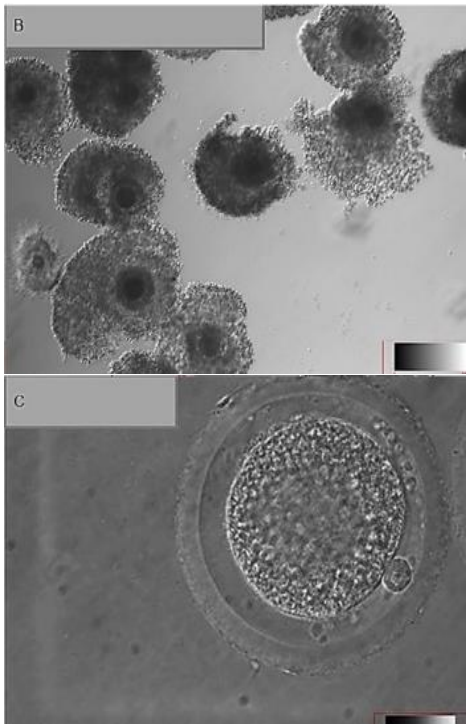


Fig. 1: Immature cattle oocyte; (A) Oocyte cumulus cells expansion; (B) And oocyte polar body; (C) At 40× magnification

Table 4: Microscopic evaluation of X-sexed and non-sexed frozen-thawed semen

Sperm motility parameter	Treatment	
	X-sexed	Non-sexed
TM (%)	52.61±4.26[b]	61.87±2.74[a]
PM (%)	17.56±7.82[a]	24.67±13.03[a]
Static (%)	47.53±4.38[a]	38.13±2.74[b]
RAP (%)	5.26±2.94[a]	10.75±7.83[a]
Medium (%)	25.88±3.88[a]	29.97±9.43[a]
Slow (%)	21.47±9.87[a]	21.15±12.15[a]
VCL (µm/sec)	60.30±13.88[a]	63.53±18.52[a]
VAP (µm/sec)	33.64±6.40[a]	36.77±10.06[a]
STR (%)	62.35±4.99[a]	65.46±3.24[a]
LIN (%)	23.37±5.60[b]	37.17±10.64[a]
WOB (%)	55.43±9.65[a]	59.55±6.79[a]
ALH (µm)	2.05±0.31[a]	1.97±0.26[a]
BCF (Hz)	12.35±2.15[a]	14.00±3.69[a]
HA	5.00±3.46[a]	3.00±3.46[a]

[a-b]Values within the same row with different superscripts differ significantly ($p<0.05$). TM = Total Motility, PM = Progressive Motility, STC = Static, RAP = Rapid, VCL = Curvilinear Velocity, VAP = Average Path Velocity; STR = Straightness, LIN = Linearity, WOB = Wobble, ALH = Amplitude of Lateral Head displacement, BCF = Beat Cross Frequency and HA = Hyperactive

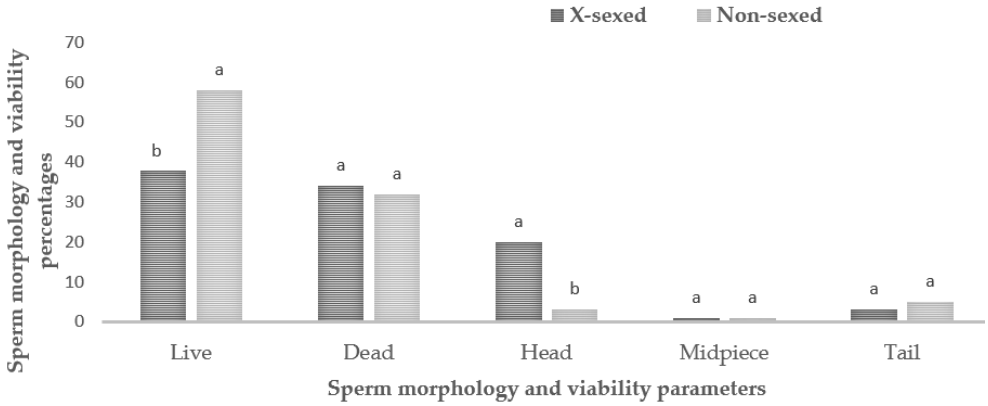


Fig. 2: Bull sperm morphology and viability of X-sexed and non-sexed frozen-thawed semen; [a-b]values with different superscripts across the bars differ significantly ($p<0.05$)

Table 5: Embryo development following *in vitro* fertilization with X-sexed and non-sexed semen frozen-thawed sperm (Mean ± SD)

Semen type	E_2 (µg/mL)	Number of IVF oocyte	Pronucleus (PN) formation% 1PN	2PN	>2PN	No PN	Total fertilization rate %
X-sexed	0.2	75	12.6±1.1[a]	9.0±1.7[b]	1.3±2.3	77.0±2.6[a]	22.9±5.1[b]
X-sexed	0.3	75	9.0±1.4[b]	12.0±0.0[b]	6.0±7.2	73.0±2.0[a]	27.0±8.6[b]
non-sexed	0.2	75	13.3±2.3[a]	22.3±3.5[a]	5.3±6.1	59.6±8.5[b]	40.9±11.1[a]
non-sexed	0.3	75	14.0±0.0[a]	26.3±2.3[a]	2.6±2.3	57.0±6.1[b]	42.9±4.6[a]

[a-b]values within the same column with different superscripts differ significantly ($p<0.05$)

The results of IVF (the PN formation) of oocyte matured at different concentrations of E_2 (0.2 and 0.3 µg/mL) and fertilized using sexed and non-sexed sperm are presented in Table 5. The oocyte matured with 0.2 and 0.3 µg/mL concentrations of E_2 and incubated at 39.5°C were selected for subsequent fertilization rate assessment following higher cytoplasmic maturation and polar body extrusion. There was no significant difference in the formation of 2PN, >2PN, and no PN in presumptive zygotes that were developed from different concentrations of E_2 and fertilized using X-sexed sperm, However, a significant difference was observed in 1PN presumptive zygote formation. Formation of >2PN showed no difference in presumptive zygotes developed from different concentrations of E_2 fertilized using both X-sexed and non-sexed sperm ($p>0.05$). There was a significant difference in the formation of 2PN presumptive zygotes developed from both concentrations of E_2 fertilized using non-sexed sperm. A higher fertilization rate was observed in the oocyte matured at 0.2 (40.9±11.1) and 0.3 (42.9±4.6) µg/mL of E_2 and fertilized using non-sexed sperm compared to X-sexed sperm ($p<0.05$).

The results for embryonic development of presumptive zygotes developed from different concentrations of E_2 (0.2 and 0.3 µg/mL) and fertilized using X-sexed and non-sexed sperm are presented in Table 6. There was no significant difference in the cleavage rate percentage observed in the presumptive zygotes developed at 0.2 (40.00±0.00) and 0.3 (41.33±1.15) µg/mL concentration and fertilized using non-sexed sperm compared to X-sexed sperm. A significant difference was observed between morulae and blastocyst rate derived from X-sexed sperm compared to non-sexed sperm from all the presumptive zygotes developed from all the concentrations of E_2.

Table 7 indicates the X-sexed sperm trait results evaluated by the CASA and the correlation with total fertilization and, cleavage, morulae, and blastocysts rate. There was a negative correlation (r = -0.20) between total sperm motility and total fertilization percentage. Interestingly there was a strong positive correlation (r = 1.00) between total sperm motility and total cleavage percentages. The sperm progressive motility and blastocyst percentage were negatively correlated (r = -0.97).

Table 8 indicates the non-sexed sperm trait results evaluated by the CASA and the correlation with total fertilization, cleavage, morulae, and blastocysts rate. There was a strong negative correlation between total sperm motility and total fertilization rate (r = -0.92). There was a strong negative correlation between total sperm motility, total cleavage, and blastocyst rate (r = -0.74). There was a strong negative correlation between sperm progressive motility and blastocyst rate (r = -0.99).

Table 6: Embryo development following *in vitro* fertilization with X-sexed and non-sexed semen frozen-thawed sperm (Mean ± SD)

Semen type	E_2 (µg/mL)	Number of IVC zygotes	Number of cleaved embryos (%)	Number of morulae (%)	Number of blastocysts (%)
X-sexed	0.2	75	28.00±0.00^{c}	11.00±1.00^{b}	9.66±0.57^{b}
X-sexed	0.3	75	35.00±0.00^{b}	12.00±1.00^{b}	8.66±0.57^{b}
Non-sexed	0.2	75	40.00±0.00^{a}	22.33±3.21^{a}	12.66±2.08^{a}
Non-sexed	0.3	75	41.33±1.15^{a}	20.66±1.15^{a}	13.33±2.08^{a}

$^{a-c}$values within the same column with different superscripts differ significantly ($p<0.05$)

Table 7: Pearson correlation coefficient for X-sexed sperm traits (sperm motility and velocity rate) and the oocyte fertilization (cleavage, blastocyst) rate

Parameters	TM	PM	STC	RAP	MED	SLOW	VCL	VAP	STR	LIN	WOB	ALH	BCF	HA	0 PN	1 PN	>2 PN	TFR	Morulae	BLC	TCR
TM	1.00																				
PM	-0.97	1.00																			
ST	-0.99	0.98	1.00																		
RAP	-0.65	0.80	0.69	1.00																	
MED	-0.94	0.84	0.92	0.35	1.00																
Slow	0.99	-0.99	-0.99	-0.72	-0.90	1.00															
VCL	-0.91	0.98	0.93	0.90	0.72	-0.94	1.00														
VAP	-0.85	0.95	0.88	0.95	0.63	-0.90	0.99	1.00													
STR	1.00	-0.97	-0.99	-0.65	-0.93	0.99	-0.91	-0.86	1.00												
LIN	-0.90	0.97	0.92	0.91	0.70	-0.94	1.00	0.99	-0.90	1.00											
WOB	0.99	-0.95	-0.99	-0.58	-0.96	0.98	-0.87	-0.81	0.99	-0.86	1.00										
ALH	-0.85	0.94	0.87	0.95	0.61	-0.89	0.99	1.00	-0.85	0.99	-0.80	1.00									
BCF	-0.98	0.92	0.97	0.52	0.98	-0.96	0.83	0.76	-0.98	0.82	-0.99	0.75	1.00								
HA	-0.99	0.97	0.99	0.66	0.93	-0.99	0.92	0.86	-1.00	0.91	-0.99	0.85	0.98	1.00							
0 PN	0.01	0.20	0.03	0.74	-0.35	-0.07	0.39	0.50	0.00	0.41	0.09	0.51	-0.17	0.00	1.00						
1 PN	0.29	-0.07	-0.43	0.53	-0.59	0.20	0.12	0.24	0.28	0.14	0.37	0.25	-0.44	-0.27	0.96	1.00					
> 2 PN	-0.25	0.03	0.20	-0.56	0.56	-0.16	-0.15	-0.28	-0.24	-0.18	-0.33	-0.29	0.40	0.24	-0.97	-0.99	1.00				
TFR	-0.20	-0.02	0.15	-0.60	0.52	-0.11	-0.21	-0.33	-0.19	-0.23	-0.28	-0.34	0.35	0.18	-0.98	-0.9	0.99	1.00			
Morulae	0.96	-0.99	-0.97	-0.82	-0.82	0.98	-0.98	-0.96	0.96	-0.98	0.94	-0.95	-0.91	-0.97	-0.24	0.03	0.00	0.05	1.00		
BLC	0.90	-0.97	-0.92	-0.91	-0.70	0.94	-1.00	-0.99	0.90	-1.00	0.86	-0.99	-0.82	-0.91	-0.41	-0.14	0.18	0.23	0.98	1.00	
TCR	1.00	-0.97	-0.99	-0.66	-0.93	0.99	-0.92	-0.86	1.00	-0.91	0.99	-0.85	-0.98	-1.00	0.00	0.27	-0.24	-0.18	0.97	0.91	1.00

TM = Total Motility, PM = Progressive Motility, STC = Static, RAP = Rapid, VCL = Curvilinear Velocity, VAP = Average Path Velocity; STR = Straightness, LIN = Linearity, WOB = Wobble, ALH = Amplitude of Lateral Head displacement, BCF = Beat Cross Frequency, HA = Hyperactive, PN = Pronucleus, TFR = Total Fertilization Rate, BLC = Blastocyst and TCR = Total Cleavage Rate

Table 8: Pearson correlation coefficient for non-sexed sperm traits (sperm motility and velocity rate) and the oocyte fertilization (cleavage, blastocyst) rate

Parameters	TM	PM	STC	RAP	MED	SLOW	VCL	VAP	STR	LIN	WOB	ALH	BCF	HA	0 PN	1 PN	2 PN	> 2 PN	TFR	Morulae	BLC	TCR
TM	1.00																					
PM	0.67	1.00																				
ST	-1.00	-0.67	1.00																			
RAP	0.30	0.90	-0.30	1.00																		
MED	0.99	0.72	-0.99	0.37	1.00																	
Slow	-0.74	-0.99	0.74	-0.86	-0.79	1.00																
VCL	0.38	0.94	-0.38	0.99	0.45	-0.90	1.00															
VAP	0.32	0.91	-0.32	1.00	0.39	-0.87	0.99	1.00														
STR	-0.72	-0.99	0.72	-0.88	-0.76	0.99	-0.91	-0.89	1.00													
LIN	-0.94	-0.88	0.94	-0.60	-0.96	0.92	-0.67	-0.62	0.91	1.00												
WOB	-0.84	-0.96	0.84	-0.76	-0.87	0.98	-0.82	-0.78	0.98	0.97	1.00											
ALH	0.95	0.86	-0.95	0.57	0.97	-0.91	0.64	0.59	-0.89	-0.99	-0.96	1.00										
BCF	0.62	0.99	-0.62	0.93	0.68	-0.98	0.96	0.94	-0.99	-0.85	-0.94	0.83	1.00									
HA	-0.21	0.57	0.21	0.86	-0.14	-0.49	0.81	0.85	-0.52	-0.12	-0.34	0.08	0.62	1.000								
0 PN	0.92	0.91	-0.92	0.65	0.94	-0.94	0.72	0.67	-0.93	-0.99	-0.98	0.99	0.88	0.189	1.00							
1 PN	-0.74	-0.99	0.74	-0.86	-0.78	1.00	-0.90	-0.87	1.00	0.92	0.98	-0.90	-0.98	-0.500	-0.94	1.00						
2 PN	-0.95	-0.42	0.95	0.00	-0.92	0.50	-0.09	-0.02	0.47	0.79	0.63	-0.81	-0.35	0.500	-0.75	0.50	1.00					
> 2 PN	0.74	0.99	-0.74	0.86	0.78	-1.00	0.90	0.87	-1.00	-0.92	-0.98	0.90	0.98	0.500	0.94	-1.00	-0.50	1.00				
TFR	-0.92	-0.91	0.92	-0.65	-0.94	0.94	-0.72	-0.67	0.93	0.99	0.98	-0.99	-0.88	-0.180	-1.00	0.94	0.75	-0.94	1.00			
Morulae	0.74	0.99	-0.74	0.86	0.78	-1.00	0.90	0.87	-1.00	-0.92	-0.98	0.90	0.98	0.500	0.94	-1.00	-0.50	1.00	-0.94	1.00		
BLC	-0.74	-0.99	0.74	-0.86	-0.78	1.00	-0.90	-0.87	1.00	0.92	0.98	-0.90	-0.98	-0.500	-0.94	1.00	0.50	-1.00	0.94	-1.00	1.00	
TCR	-0.74	-0.99	0.74	-0.86	-0.78	1.00	-0.90	-0.87	1.00	0.92	0.98	-0.90	-0.98	-0.500	-0.94	1.00	0.50	-1.00	0.94	-1.00	1.00	1.00

TM = Total Motility, PM = Progressive Motility, STC = Static, RAP = Rapid, VCL = Curvilinear Velocity, VAP = Average Path Velocity; STR = Straightness, LIN = Linearity, WOB = Wobble, ALH = Amplitude of Lateral Head displacement, BCF = Beat Cross Frequency, HA = Hyperactive, PN = Pronucleus, TFR = Total Fertilization Rate, BLC = Blastocysts and TCR = Total Cleavage Rate

Discussion

Effect of Temperatures on the Maturation Rate of Cattle Oocytes

In this study, comparisons of incubation of temperatures were used in order to optimize the suitable temperature for IVM in cattle oocytes. It was found that low temperatures {35.5 (79.4±33.7) and 36.5°C (85.3±6.8)} had higher COC expansion as compared to high temperatures {40.5 (56.6±6.6)}. The higher incubation temperature of 40.5°C resulted in the shape of the oocytes being fragmented leading to the irregularity of the oocytes with the first polar body. The low temperatures of 35.5 and 36.5°C were considered to be the temperature of preovulatory follicles and at which supposedly *in vivo* maturation occurs (Lenz *et al*., 1983). This suggests that a low incubation temperature of 36.5°C during the maturation process does not alter the progress of cattle nuclear maturation.

However, our results show that reduced and elevated temperature also may influence cytoplasmic maturation as revealed by cumulus cell expansion. This study agrees with the results that were found by Lenz *et al*. (1983) who found that low incubation temperature (36.5°C) during IVM did not affect the cumulus cells expansion and although in this study, the oocyte PB formation was found to be low. The results obtained by Ye *et al*. (2007) suggest that the addition of serum in the maturation media may protect oocytes from possible negative effects, if any, of low incubation temperatures. Differently, previous studies have shown that low incubation temperature during IVM did not affect the rate of cumulus expansion (Fortune and Hansel, 1985) the oocytes PB formation, and the proportion of oocytes that reached the metaphase II stage (Ye *et al*., 2007). In the present study, the oocyte's first PB extrusion was recorded to be high in the oocytes that were matured at 39.5°C (63.5±6.2) as compared to other temperatures, although 37.5 (54.0±3.6) and 38.5°C (58.9±5.6) were found to be suitable during IVM cattle oocytes. It has been found that exposure of oocytes during maturation to high temperatures interferes with the process of oocyte maturation and may result in maturation failure.

Effect of Estradiol Concentrations on in vitro Maturation and Fertilization Rate

The present study compared the efficacy of E_2 on IVM on cattle oocytes. In this study, oocytes matured without the supplementation of E_2 {0 (55.5±3.7)} showed low COC expansion compared to the oocytes that matured with the presence of E_2. It is also found that E_2 increased the percentage of oocyte PB extrusion of oocytes matured at {0.2 (46.7±14.6) and 0.3 (47.3±11.1)} µg/mL concentrations compared to other treatments. It is believed that E_2 upholds the atomic development of oocytes during *in vitro* development. This study agrees with the study of Maksura *et al*. (2021) where it was found that supplementation of medium with E_2 increased the COCs expansion rate in oocytes from both river buffalos and Black Bengal goat oocytes. Thus, it shows that E_2 coordinates with oocyte-derived factors in the culture medium, which in turn promotes COC expansion.

Here, higher numbers of oocytes treated with 0.1 (21.0±7.1), 0.4 (29.9±8.1) and 0.5 (23±9.8) µg/mL of E_2 showed reduced oocyte PB extrusion percentages compared to other treatments. This further indicates that low or high concentrations of E_2 inhibit the meiotic maturation of cattle oocytes. It was interesting to note that the low (0.1 µg/mL) or higher concentration (0.4 µg/mL) of E_2 results in a change or the shape of the oocyte, which results in damage to the cytoplasm or shrinkage. Several factors are included in culture media to improve the success rate of IVM. The E_2 works on the cell surface and improves the potential of oocytes for development which improves nuclear and cytoplasmic maturation as well as COC expansion, particularly in cattle. The concentration of 1 µg/mL is commonly used for oocyte maturation medium in bovine and in different species, which mimics the presence of E_2 (1.1±0.06 µg/mL) in the preovulatory follicle *in vivo* (Maksura *et al*., 2021). Previously, it was

reported that a proportion of E_2 was absorbed by mineral oil covering the micro-drops of the maturation medium (Miller and Pursel, 1987). In the present study, each droplet was placed in a separate maturation dish and, thus, E_2 could not move.

Embryo Development Following in vitro Fertilization with Sexed and Non-Sexed Frozen-Thawed Sperm

The present study examined the PN formation and cleavage development of presumptive zygotes developed at different concentrations of E_2 following fertilization using X-sexed and non-sexed frozen-thawed sperm. In this study, it was found that the fertilization rate from presumptive zygotes developed at 0.2 μg/mL of E_2 and fertilized using X-sexed sperm (22.9±5.1) was different compared to the non-sexed sperm (40.9±11.1). A significant difference was also observed between morulae and blastocysts percentages derived from X-sperm compared to non-sexed sperm. The X-sexed sperm revealed a slower growth of embryo development than the non-sexed sperm. Similarly, Bermejo-Álvarez *et al*. (2010) reported that cattle embryos fertilized with sexed sperm presented a slower early cleavage than those with non-sexed sperm. In contrast, a recent cattle study reported that embryos derived from sexed and conventional sperm did not show a difference in the time required to reach an embryonic stage (Steele *et al*., 2020) *in vitro* embryos are particularly sensitive to culture conditions at 2-4 cell and morula stages Bermejo-Álvarez *et al.* (2010) Furthermore, embryo formation is mainly affected by oocyte origin and temperature employed, while cryo survival and gene expression can be altered by culture conditions regardless of the origin of the oocyte (Gad *et al*., 2012).

Our results suggest that reduced fertilization and cleavage rates of X-sexed frozen-thawed sperm are a result of altered sperm morphokinetics. This alteration reduces the probability of sperm successfully reaching and fertilizing the oocyte, leading to abnormal early embryonic development. The results obtained also suggest that X-sexed frozen-thawed semen was relatively less efficacious *in vitro* than *in vivo*. This study agrees with the supported studies that reported that X-sexed sperm produce fewer blastocysts via IVF than non-sexed sperm (Lonergan *et al*., 2003; Lu and Seidel, 2004).

In summary, our study has shown that the sexing of bull sperm results in detrimental effects on various aspects of sperm function, leading to reduced sperm motility, altered sperm morphology, and compromised early embryonic development. Our findings suggest that these effects are cumulative, impacting multiple stages of the reproductive process. Specifically, in terms of sperm morphogenetics, sex sorting results in an increased proportion of immotile sperm and a decreased number of progressive and hyperactivated sperm. This implies that a smaller number of sperm can detach from the tubal sperm reservoir and successfully navigate through the female genital tract (Merton *et al*., 1997).

The sperm motility results are an indication of an active metabolism and are considered to be of great importance for fertilization to take place (Steele *et al*., 2020). Semen quality, especially sperm number and motility, can be affected by bull age (Brito *et al*., 2002) the season of semen collection (Malama *et al*., 2017), and ejaculate number (Everett and Bean, 1982), which may then affect fertilization outcome after IVF. The results for sexed frozen-thawed semen showed a reduced percentage of progressively motile sperm (17.56±7.82) compared to non-sexed frozen-thawed semen (24.67±13.03). Reduced sperm motility leads to a decreased number of sperm reaching the fertilization site *in vivo*, with the propulsion of sperm aided by the smooth muscle contractions in the cattle reproductive tract (Kunz *et al*., 1996). High sperm motility is pivotal to allowing the sperm to escape from the strong turbulences near the cilia of the tubal wall and reach the middle of the lumen of the fallopian tube (Kölle, 2015). However, in this *in vitro* study, sexed frozen-thawed semen also revealed increased percentages of hyperactivated sperm compared to non-sexed frozen-thawed semen. Hyperactivation is defined by the presence of vigorous, uneven flagellar movement (Suarez, 2008) and is crucial for its role in enabling the sperm to detach from the tubal sperm reservoir, migrate through the oviduct and reach the oocyte *in vivo* (Rodriguez-martinez, 2007).

The live percentage of X-sexed sperm (38.00±2.64) was found to be low compared to the non-sexed sperm (58±7.63). Higher sperm head defects were found to be in X-sexed (20.00±3.00) compared to non-sexed sperm (3.33±1.15). The study found that sperm movement was adversely affected, leading to reduced lateral head displacement, decreased average path distance, and a lower average path velocity. These changes had a negative impact on overall sperm velocity, resulting in fewer sperm reaching the oocyte. Moreover, the altered sperm movement could potentially cause delayed arrival at the oocyte, increasing the likelihood of fertilization to be successful. In terms of early embryo development, the observed decline in conception rates in other studies (Steele *et al*., 2020; Sales *et al*., 2011) can be linked to various disruptions in embryonic development. This includes reduced capacity for cleavage and reduced likelihood of reaching the expanded blastocyst stage, as well as an increased occurrence of embryonic arrest, cell shrinkage, cell fusion, and reduced survival time. These alterations may be associated with epigenetic changes, which require further investigation. Overall, the findings of this study emphasize the enduring impact of the sex-sorting process on sperm, extending

beyond fertilization. It emphasizes the crucial role of sperm integrity in determining the quality of the resulting embryo (Steele *et al*., 2020).

A lower pregnancy rate has been continually reported with sexed sperm as compared to conventional sperm (Sales *et al*., 2011; Thomas *et al*., 2014). Part of this fertility impairment is due to premature capacitation of flow cytometry sexed sperm and its reduced capacity to remain bound to oviductal cells (Carvalho *et al*., 2013; De Oliveira Carvalho *et al*., 2018) which compromises sperm longevity. Thus, the reduced sperm longevity of sexed sperm in the female reproductive tract may alter the optimum time for IVF in relation to the occurrence of ovulation.

There is a remarkable knowledge platform of the necessary requirements at several stages of embryo development for achieving optimal developmental rates although much can still be learned from embryo culture systems that provide important insights in order to develop a completely defined and optimized media (Baltz, 2013). Platforms and dynamic systems may offer a pathway toward the optimization of embryo culture conditions, attempting to maximize gamete competence, viability, and pregnancy rate (Zhao and Fu, 2017). Cumulatively, a detailed determination of not only thermo-physical, physicochemical, and hormonal/endocrine conditions but also prevention of hyperthermic conditions, under which IVM of heifer/cow oocytes takes place, might be suitable for enhancements of developmental outcomes noticed for bovine and other mammalian *in vitro*-produced embryos propagated either by classic IVF (Arias *et al*., 2022; Gorczyca *et al*., 2022) or by such inventive methods of reproductive biotechnology as IVF micro surgically assisted by intracytoplasmic sperm injection (Fuentes *et al*., 2022; Menéndez-Blanco *et al*., 2020) and cloning based on the Somatic Cell Nuclear Transfer (SCNT) (Wiater *et al*., 2021; Su *et al*., 2014).

Conclusion

The oocytes matured at 39.5°C revealed optimal oocyte PB extrusion percentage as compared to other treatments. Maturation results show an increase in oocyte PB extrusion at (37.5, 38.5 and 39.5°C) these maturation temperatures can result in embryo production. Oocytes matured at 0.2 and 0.3 µg/mL of E_2 revealed the optimal oocyte PB percentage incubated at 39.5°C. Overall, the presumptive zygote developed at 0.2 and 0.3 µg/mL of E_2 and fertilized using non-sexed sperm incubated at 39.5°C provides better embryo developmental competency compared to X-sexed sperm.

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Author's Contributions

Maleke Dimpho Sebopela, Masindi Lottus Mphaphathi and Sindisiwe Mbali Sithole: Conception, designed, data collection, analysis, interpretation, drafted and reviewed of the article.

Tlou Caswell Chokoe: Conception, reviewed the article, gave final approval of the version to be submitted and any revised version.

Tshimangadzo Lucky Nedambale: Conception, designed, reviewed of the article.

Ethics

The experiments were evaluated and approved by the Animal Production Ethics (APAEC 2020/04) and Tshwane University of Technology Ethics Committee (AREC 2021/05/004).

Conflict of Interest

All authors have reviewed and approved this final version of the manuscript. There is no competing interest in our submission.

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