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**MOLECULAR CHARACTERISATION OF *IN VITRO*-
PRODUCED BOVINE EMBRYOS EXPOSED TO HEAT STRESS**

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Declaration

I, the undersigned, Nagy Katalin,

author of the dissertation entitled MOLECULAR CHARACTERISATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS EXPOSED TO HEAT STRESS, declare that, in preparing this dissertation, I acted in accordance with the current Code of Scientific Ethics of the Hungarian Academy of Sciences, with knowledge of the research standards applicable to my field and in possession of the necessary permits. Wherever the ideas and results presented in this dissertation are not my own, I have clearly and unambiguously identified their source.

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Doctoral candidate

1. BACKGROUND

The increasing frequency and intensity of extreme weather events resulting from climate change pose a major challenge to animal production. The cattle sector is particularly vulnerable to environmental heat load, as high temperature and humidity adversely affect not only animal production and general physiological status but also reproductive efficiency. The physiological and cellular changes induced by heat stress may affect several stages of female reproduction, including follicular development, oocyte maturation, fertilisation and early embryonic development.

The sensitivity of preimplantation embryos to heat load depends strongly on their developmental stage. Embryos at early developmental stages are particularly susceptible to the adverse effects of elevated temperature, whereas their resistance to heat load gradually increases as development progresses. Both embryonic genome activation and the functioning of cellular stress response mechanisms may contribute to the development of this resistance.

Environmental stressors may induce molecular changes that are not necessarily reflected immediately in the developmental parameters conventionally assessed. In addition to heat shock proteins, genes involved in metabolic processes, the maintenance of cell junctions and epigenetic regulation, as well as microRNAs involved in post-transcriptional regulation, may contribute to the cellular response to heat stress.

In vitro-produced embryos may be exposed to additional stressors during culture and subsequent use, alongside heat load. Of these, cryopreservation, which is widely used in cattle breeding, is particularly important, as it may involve changes in membranes, organelles and metabolic processes, as well as the induction of molecular responses. It is particularly important to determine how environmental exposures before cryopreservation may influence the response of embryos to freezing and thawing, and how the consequences of different stressors may be interconnected at the molecular level.

The assessment of embryonic responses to stressors also depends strongly on the duration of observation. *In vitro* culture of bovine embryos traditionally continues until the blastocyst stage; therefore, later developmental changes and longer-term embryo viability can only be investigated to a limited extent in routine culture systems. Extended *in vitro* culture beyond the blastocyst stage may allow embryo viability to be monitored, thereby enabling investigation of the delayed consequences of previous environmental or technological stressors.

2. OBJECTIVES

This dissertation presents the results of four successive experiments that contributed, from different perspectives, to a comprehensive assessment of the mechanisms underlying the effects of heat treatment.

2.1. Investigation of the effects of controlled heat treatment on embryo development and cryotolerance

Our aim was to investigate the effects of controlled heat treatment applied at the early morula stage on the development of *in vitro*-produced bovine embryos and their survival after cryopreservation.

2.2. Investigation of gene expression changes induced by controlled heat treatment in fresh and cryopreserved embryos

The aim of our investigations was to determine how controlled heat treatment applied at the early morula stage affects the expression of the genes studied in fresh, as well as cryopreserved and thawed, *in vitro*-produced bovine embryos.

2.3. Investigation of the cellular effects of controlled heat treatment

The aim of our investigations was to evaluate the cellular effects of heat treatment, with particular emphasis on the qualitative assessment of apoptosis and necrosis, and the examination of inner cell mass and trophectoderm cells using immunofluorescent labelling.

2.4. Investigation of the effects of controlled heat treatment and cryopreservation during extended embryo culture

We also investigated how heat treatment and cryopreservation affect the viability of *in vitro*-produced bovine blastocysts under long-term *in vitro* culture conditions.

2.5. Hypotheses

H1 – Investigation of development and cryotolerance (Experiments A, B and C).

Controlled heat treatment applied at the early morula stage, with a gradual increase from 38.8 °C to 40.0 °C followed by a two-hour exposure, does not significantly reduce the blastocyst rate of *in vitro*-produced bovine embryos or their survival rate after slow freezing, because the thermotolerance of morula-stage embryos increases following embryonic genome activation (Sakatani, 2017; Oliveira *et al.*, 2021).

H2 – Investigation of the molecular response in fresh embryos (Experiments A and C).

Controlled heat treatment induces measurable changes in the expression of stress-related genes (*HSP90AA1*, *HSPB11*, *HSPA1A*) and miRNAs (miR-191-5p, miR-378a-3p, miR-138-5p) even in the absence of impaired developmental competence; thus, molecular markers are more sensitive indicators of mild heat load than developmental parameters (Sakatani *et al.*, 2013; Sengar *et al.*, 2018).

H3 – Investigation of combined heat and cryogenic stress (Experiments B and C).

Controlled heat treatment applied before cryopreservation modifies the molecular response of embryos to freezing: following thawing, the expression of genes encoding heat shock proteins, particularly *HSPA1A*, is higher in heat-treated embryos than in the control group subjected only to cryopreservation.

H4 – Investigation of long-term viability (Experiment D).

Controlled heat treatment applied at the early morula stage improves the long-term survival of *in vitro*-produced bovine embryos up to day 14 of culture at elevated culture temperatures (39.0 and 39.5 °C) and after cryopreservation, resulting in a measurable increase in thermotolerance.

3. MATERIALS AND METHODS

3.1. *In vitro* embryo production

In vitro embryo production was performed according to the protocol of IVF Limited T/A IVF Bioscience (Cornwall, United Kingdom), with minor modifications. Commercial media produced for cattle by IVF Limited T/A IVF Bioscience were used throughout IVEP: OPU, WASH, OIL, BO-HEPES-IVM, BO-IVM, BO-IVF, BO-SEMENPREP and BO-IVC. All steps were performed using media preheated to 38.8 °C and a heated stage.

3.1.1. Ovary collection

I collected ovaries for *in vitro* embryo production from the cattle abattoir of Tendon Kft. in Gyöngyös, where the vast majority of cattle delivered were Holstein-Friesian. After identifying the breed of the donor animals on the slaughter line, I excised ovaries exclusively from the reproductive tracts of Holstein-Friesian animals within 5 minutes of slaughter. I then collected them in airtight bags containing phosphate-buffered saline at room temperature (PBS, Thermo Fisher Scientific Inc., Waltham, MA, USA) for further laboratory processing and follicular aspiration. Throughout the study, I collected a total of 1067 ovaries, which we processed. We processed 296 ovaries in Experiment A, 265 in Experiment B, 239 in Experiment C and 267 in Experiment D. Transport of the ovaries from the abattoir to the laboratory took approximately 50 minutes. During transport, I placed the bags in a polystyrene box to avoid temperature fluctuations.

3.1.2. Oocyte aspiration and *in vitro* maturation (IVM)

Oocytes were aspirated from thoroughly cleaned ovaries using an 18 G hypodermic needle and a three-part 5 mL syringe. Follicular fluid aspirated from follicles 2–15 mm in diameter was collected in 15 mL centrifuge tubes containing oocyte pick-up medium (OPU) + 10% fetal bovine serum (FBS) (Capricorn Scientific, Ebsdorfergrund, Germany). The contents were examined under a stereomicroscope, and grade I cumulus–oocyte complexes (COCs), as defined by the International Embryo Technology Society (IETS), were selected. In these COCs, the surface of the zona pellucida was covered by more than three complete, compact layers of cumulus cells, and the ooplasm was dense and evenly granulated. Following washing, in Experiments A and B, the cumulus–oocyte complexes (COCs) were placed in cryogenic tubes containing 1 mL of BO-HEPES-IVM medium, which were sealed airtight and placed in a CO₂ incubator for 21–24 hours to allow oocyte maturation. A maximum of 40 oocytes was placed in each tube. In Experiments C and D, oocytes were matured *in vitro* in four-well culture dishes containing 600 µL of equilibrated BO-IVM medium without an oil overlay, under humidified atmospheric gas conditions (5.5–6.5% CO₂ and 21% O₂), with a maximum of 40 oocytes per drop. The BO-IVM medium used contains

phenol red as a pH indicator; therefore, potential pH shifts can be monitored visually through changes in the colour of the indicator. The pH and osmolarity of the medium were not measured instrumentally; consequently, direct measurements were not available to exclude potential evaporation-induced changes in osmolarity or to characterise potential pH changes quantitatively. The use of different IVM systems and maturation vessels was determined by the laboratory infrastructure available at the time of each experiment. The different IVM systems and maturation vessels were not investigated as independent experimental factors.

3.1.3. *In vitro* fertilisation (IVF)

Two hours before the scheduled time of fertilisation, I prepared four-well culture dishes (NUNC®, Thermo Fisher Scientific Inc., Rochester, USA), placing 500 µL of BO-IVF medium in each well under mineral oil. I then placed the dishes in a CO₂ incubator containing a gas mixture of 6% CO₂ and 16% O₂ for equilibration. At the end of the maturation period, I assessed oocyte maturation under a stereomicroscope. I placed a maximum of 40 oocytes in each drop for fertilisation.

The semen used for *in vitro* fertilisation originated from a Holstein-Friesian breeding bull of the same breed as the ovary donors. I performed fertilisation using semen collected on 5 January 2021 from the bull Reform, central registration number 33589. To minimise paternal variability, I used semen from the same bull in all experiments. For thawing, I transferred the 0.25 mL straws containing the semen from liquid nitrogen to a water bath at 37.8 °C for 20 seconds, then emptied them into a 15 mL centrifuge tube containing 4 mL of BO-SEMENPREP medium. Sperm motility was assessed by subjective estimation in a Makler chamber (Sefi Medical Instruments Ltd., Haifa, Israel), using an Olympus BX43 binocular microscope (Olympus Corporation, Tokyo, Japan). Semen samples with a motility of at least 40% were accepted for *in vitro* fertilisation; the motility of the samples used generally ranged from 40–50%. The volume of semen to be added to the fertilisation drops was calculated from the determined sperm concentration and then adjusted for the measured motility. The final sperm concentration in the fertilisation drops containing the oocytes was set to 2.0×10^6 spermatozoa/mL. Fertilisation was performed by co-culturing, or co-incubating, the oocytes and semen for 16–19 hours in a gas mixture of 6% CO₂ and 16% O₂, using an IVFTech six-chamber incubator (IVFTech, Klintehøj, Birkerød, Denmark).

3.1.4. *In vitro* embryo culture (IVC)

A total of 4509 zygotes was placed in *in vitro* culture throughout the experimental series. For embryo culture, I prepared four-well culture dishes containing 500 µL of BO-IVC medium covered with mineral oil. I then kept the dishes overnight in an IVFTech six-chamber incubator with a gas mixture of 5.5–6.5% CO₂ and 5.5–6.5% O₂ for equilibration.

Vortexing was used to remove cumulus cells, i.e. for denudation. The presumptive embryos were then also washed in previously equilibrated BO-IVC medium and transferred to the appropriate drops in four-well culture dishes, with a maximum of 40 per drop. Embryos were cultured for seven days at 38.8 °C in a gas mixture of 5.5–6.5% CO₂ and 5.5–6.5% O₂, using an IVFTech six-chamber incubator.

3.2. Heat treatment experiments

At 116 hours post-insemination (hpi), I selected early morula-stage embryos and allocated them to equally sized groups according to the type of experiment. In all experiments, I cultured five embryos per 50 µL medium drop.

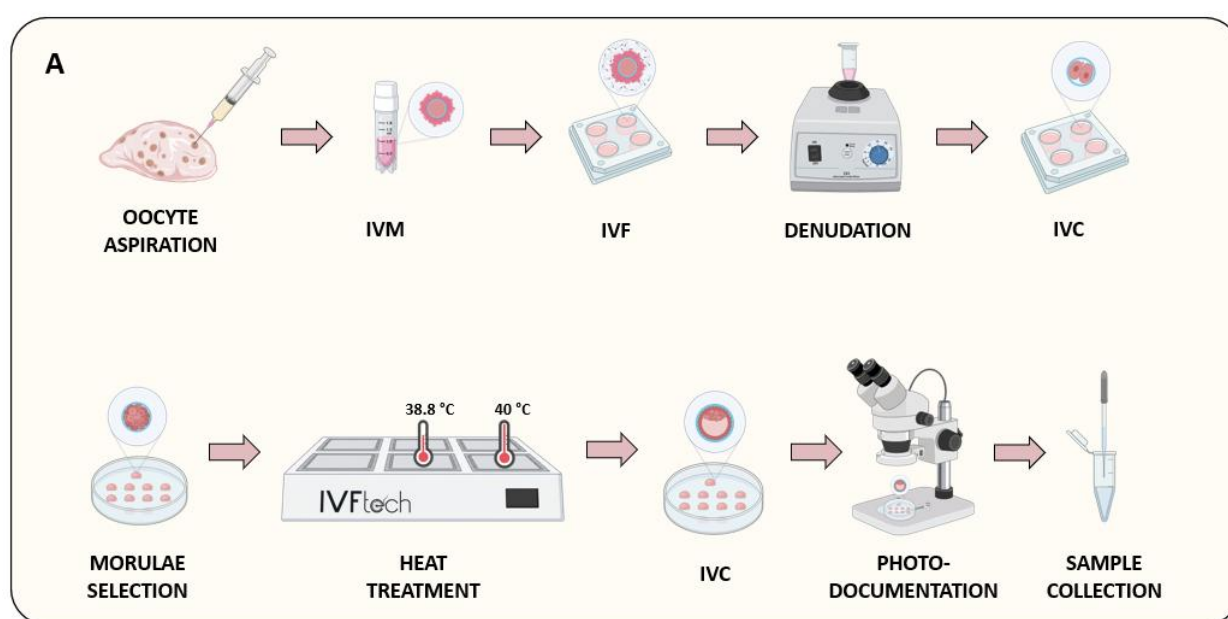
3.2.1. Experiment A – Investigation of fresh embryos

In this experiment, I placed five early morula-stage embryos in each drop of BO-IVC medium. The drops were covered with mineral oil in 35 mm Petri dishes and equilibrated for two hours under the embryo culture conditions before the embryos were introduced. I used two incubator chambers: the control group (K) was cultured in chamber 1, while heat treatment (HK) and heat stress treatment (HS) were performed in chamber 2. Throughout the study, I consistently used the same two incubator chambers and assigned each treatment group to the same chamber. To ensure identical temperature conditions, the temperature of both incubator chambers was calibrated and checked before each experimental replicate. As all chambers of the incubator are supplied simultaneously by a single shared gas-mixing system, identical gas composition was maintained throughout the experiment, allowing chamber-specific differences in gas composition to be excluded. In addition, the frequency and duration of chamber door opening were identical for all treatment groups. It should also be noted that the incubator has no humidification system; therefore, chamber-specific differences attributable to humidification could not occur. I maintained the control group under the culture conditions described above. I increased the temperature of the chamber containing the heat-treated embryos from 38.8 °C to 40.0 °C in increments of 0.1 °C every 7 minutes. Once the target temperature was reached, I also placed the culture dish of the heat shock group in the heat treatment chamber and incubated embryos from both groups at 40.0 °C for two hours. Following the two-hour incubation, I returned the culture dish of the heat shock group to the chamber maintained at the standard culture temperature of 38.8 °C, while gradually restoring the temperature of the chamber containing the heat-treated group to the initial value of 38.8 °C. I performed the experiment in three independent biological replicates. A total of 270 early morula-stage embryos was included in Experiment A. Embryos were allocated equally among the control, heat-treated and heat shock groups (n = 90 per group) in three replicates. The numbers of embryos included in the experiment are summarised in Table 1.

Table 1. Numbers of embryos included in Experiment A

Replicate	Experimental group	Number of embryos examined	Number of embryos collected for gene expression analysis
A1	K	20	10
	HK	20	10
	HS	20	7
A2	K	40	20
	HK	40	20
	HS	40	20
A3	K	30	12
	HK	30	12
	HS	30	12

The workflow of Experiment A is illustrated in Figure 1.

**Figure 1.** Schematic representation of the workflow of Experiment A

3.2.2. Experiment B – Investigation of cryopreserved embryos

In this experiment, I subjected embryos to heat treatment according to the protocol used in Experiment A. I did not apply heat shock treatment in subsequent experiments, because the gene expression response of embryos exposed to heat shock in Experiment A showed extremely high individual variability, limiting the interpretability of the results for this group. After embryos reached the blastocyst stage, I cryopreserved them by slow freezing, using commercially available media from Minitube GmbH (Tiefenbach, Germany). As the first step of freezing, I washed the blastocysts several times in BoviHold medium and then placed them in BoviFreeze medium containing ethylene glycol for eight minutes. I subsequently loaded the embryos, together with the cryoprotectant solution, into straws using a straw-loading pipettor and placed them in the chamber of an EFT-3002 embryo freezer (Beltron Instruments, Longmont, USA) precooled to -6.5°C .

After two minutes, I induced ice nucleation (seeding) using a copper rod cooled in liquid nitrogen. After ten minutes, I started the freezing device; the chamber temperature was then reduced to -34.3°C at a rate of $0.5^{\circ}\text{C}/30$ seconds. When the target temperature was reached, I held the embryos in the chamber for ten minutes before rapidly transferring them to liquid nitrogen.

I stored the embryos in liquid nitrogen at -196°C until thawing. During thawing, I placed the straws containing the embryos in a water bath at 37.8°C for 30 seconds, then emptied their contents into 35 mm Petri dishes. We washed the embryos in several steps using BoviHold medium to remove the cryoprotectant. I then transferred the blastocysts to culture dishes containing BO-IVC medium under an oil overlay. I determined the survival rate 24 hours after thawing using a stereomicroscope (Marsico *et al.*, 2019). In Experiment B, a total of 64 blastocysts was included in the freezing studies, of which 29 belonged to the control group and 35 to the heat-treated group.

Table 2. Numbers of embryos included in Experiment B

Replicate	Experimental group	Number of frozen embryos	Number of embryos collected for gene expression analysis*
B1	K	7	7
	HK	9	7
B2	K	11	8
	HK	10	6
B3	K	11	8
	HK	16	15

* The number of embryos collected for analysis equals the number of viable embryos after thawing.

The workflow of Experiment B is illustrated in Figure 2.

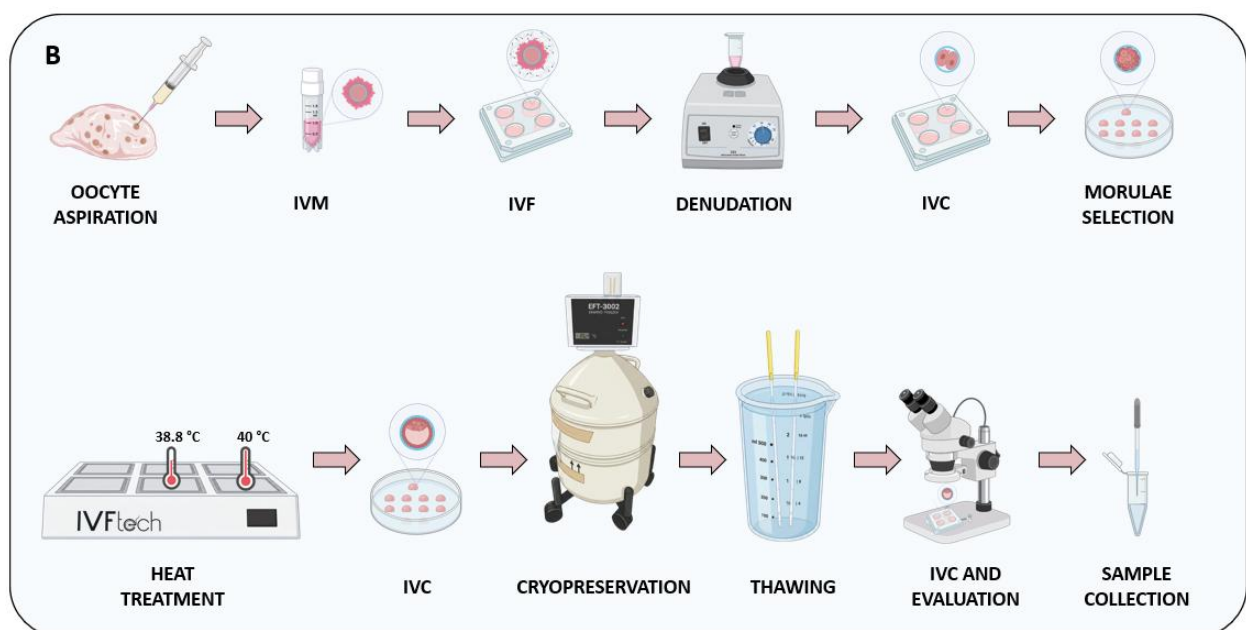


Figure 2. Schematic representation of the workflow of Experiment B

3.2.3. Experiment C – Investigation of fresh and cryopreserved embryos

I conducted Experiment C by combining the experimental designs of Experiments A and B, with an increased sample size to confirm the previous findings. I performed the study in 3 biological replicates, including $n = 160$ early morula-stage embryos per replicate and 480 in total, which I allocated equally between the control (K) and heat-treated (HK) groups ($n = 80$ per group per biological replicate).

I performed heat treatment at 116 hours post-insemination, according to the protocol described for Experiment A. Unlike in Experiments A and B, I assessed developmental competence at two time points: blastocyst rates were determined on both day 7 (D7) and day 8 (D8) of culture. At both time points, I collected $n = 15$ blastocysts per group as pooled samples for gene expression analysis. Some of the embryos that reached the blastocyst stage on day 7 were cryopreserved by slow freezing according to the protocol described for Experiment B; 24 hours after thawing, I likewise collected $n = 15$ surviving blastocysts per group for gene expression analysis. Thus, the experiment yielded four sample groups: fresh control (K), fresh heat-treated (HK), cryopreserved control (KF) and cryopreserved heat-treated (HKF). The workflow of Experiment C is illustrated in Figure 3.

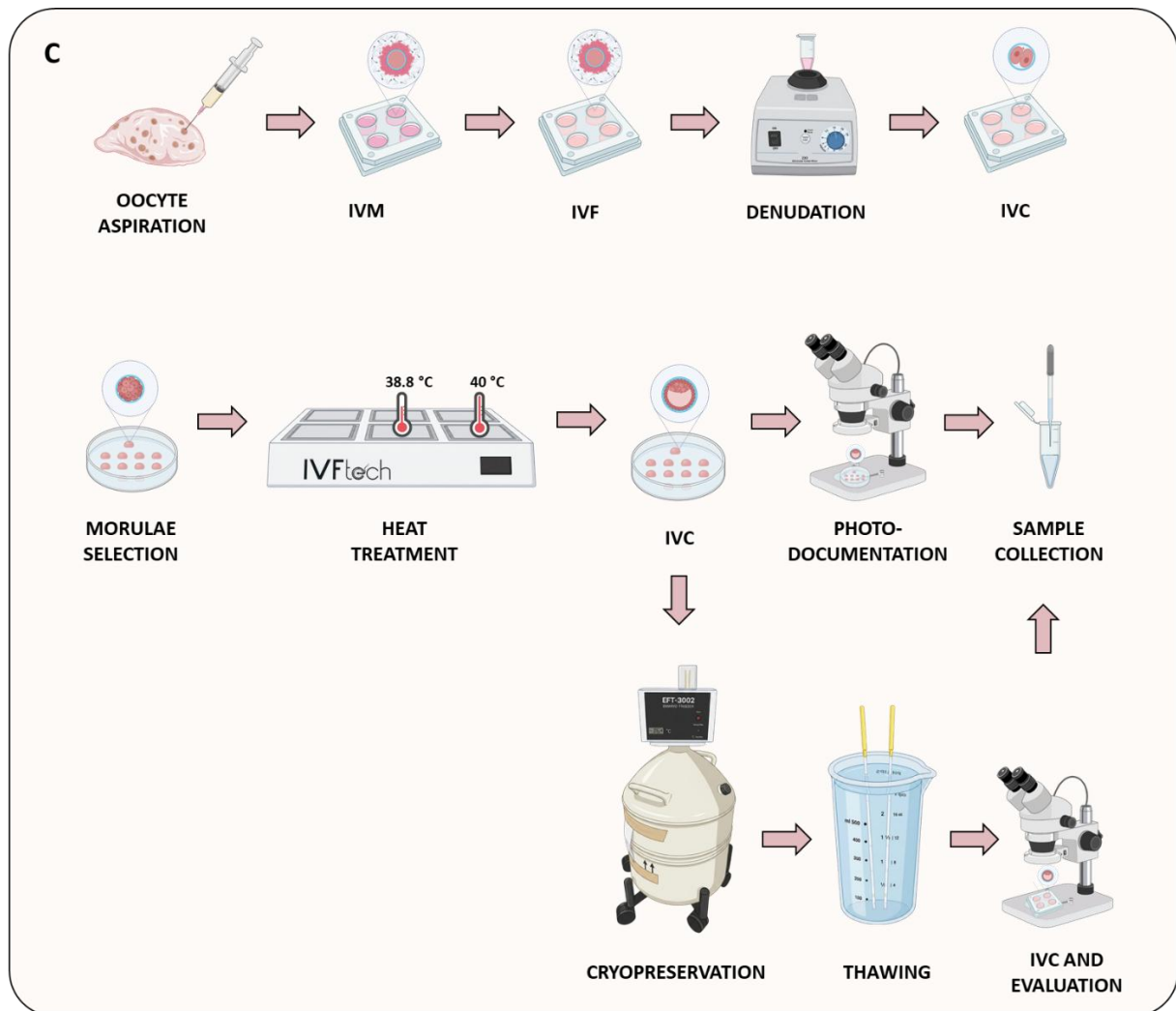


Figure 3. Schematic representation of the workflow of Experiment C

3.2.4. Experiment D – Extended embryo culture

To prepare for long-term embryo culture, I used the embryo production protocol described for Experiment C. We cryopreserved the embryos on day seven of embryo culture and thawed them after immersion in liquid nitrogen, according to the previously described protocol. Following thawing, we transferred the embryos to BO-IVC culture medium. On day eight of embryo culture, I individually transferred fresh blastocysts and embryos surviving cryopreservation into 30 μ L drops of pre-equilibrated BO-IVC medium containing 10% (v/v) FBS under an oil overlay. I placed 10 embryos in each culture dish. Each culture dish contained embryos from only one experimental group (K, HK, KF or HKF). I then placed the culture dishes in a preheated six-chamber incubator, in separate chambers corresponding to the three culture temperatures applied (38.5, 39.0 and 39.5 °C). The culture temperatures used in the study were selected with reference to estimated intrauterine temperature conditions based on published data (El-Sheikh et al., 2013; Lees et al., 2018b; López-Gatius–Hunter, 2020; Vitali et al., 2023). I performed the experiment in three replicates, using 160 early morula-stage embryos per replicate and 480 in total. In each

replicate, I allocated embryos equally between the control and heat-treated groups (n = 80 per group). Following heat treatment and the freezing and thawing procedures, I included a total of 344 viable embryos from the four treatment groups in extended culture. Depending on the number of viable embryos available, I cultured up to 10 embryos from each treatment group per replicate at each temperature; thus, embryos from all four groups were cultured simultaneously in each of the three temperature chambers. The sample sizes of the experimental groups are summarised in Table 3. I assessed embryo survival every other day, on days 8 (D8), 10 (D10), 12 (D12) and 14 (D14) of culture, using morphological criteria under a stereomicroscope.

Table 3. Sample sizes of the experimental groups by replicate

	38.5 °C				39.0 °C				39.5 °C			
	K	KF	HK	HKF	K	KF	HK	HKF	K	KF	HK	HKF
1.	8	10	9	9	9	10	7	10	10	10	9	10
2.	10	10	10	10	9	9	8	9	10	9	9	10
3.	10	10	10	10	10	10	10	10	10	10	10	10
SUM	28	30	29	29	28	29	25	29	30	29	28	30

The experimental design is illustrated in Figure 4.

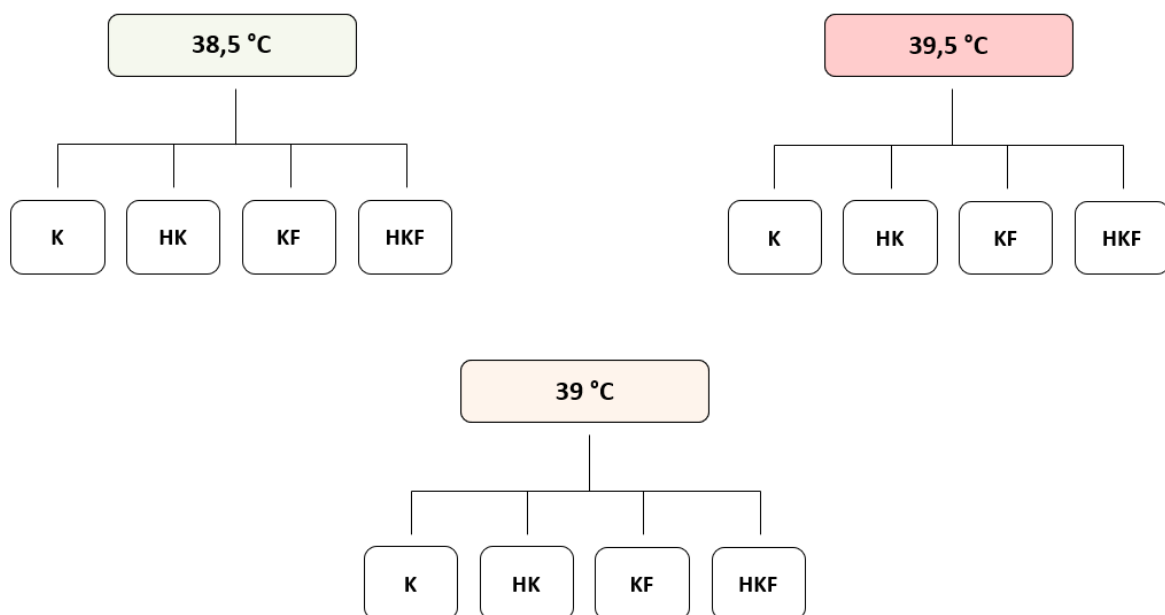


Figure 4. Arrangement of treatment groups in the extended embryo culture experiment at incubation temperatures of 38.5, 39.0 and 39.5 °C

3.3. Sample collection for gene expression analysis

After embryos reached the blastocyst stage, I graded them according to the recommendations of the IETS and Bó and Mapletoft (2013). In Experiment A, I washed the blastocysts in Dulbecco's phosphate-buffered saline (DPBS) and collected them in Eppendorf

tubes containing 100 μ L of lysis buffer from the RNAqueous™ Lysis Buffer Micro Kit (Applied Biosystems™, Thermo Fisher Scientific Inc., Waltham, USA). In Experiment B, blastocysts were collected using the same method after the survival rate had been determined. In Experiment C, I collected $n = 15$ blastocyst-stage embryos per group per biological replicate on days 7 and 8 of culture and 24 hours after thawing for gene expression analysis, and processed them as pooled samples before RNA isolation. I performed the study in three independent biological replicates, with three technical replicate reactions per sample. I stored the collected samples at -70°C until gene expression analysis.

3.4. Gene expression analysis

RNA was extracted using the RNAqueous™-Micro Total RNA Isolation Kit AM1931 (Applied Biosystems™). The amount of isolated RNA was assessed using a NanoDrop instrument (ND-1000, UV-Vis Thermo Fisher Scientific, Waltham, MA, USA). The sample concentration had to reach 25 ng/ μ L for SYBR Green qPCR and 5 ng/ μ L for Advanced TaqMan qPCR. For the analysis of mRNA markers, cDNA was synthesised from total RNA using a reverse transcription cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Waltham, MA, USA). For real-time polymerase chain reaction (qPCR), we used SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Analyses were performed using a Mastercycler® ep realplex instrument (Eppendorf AG, Hamburg, Germany).

The samples were then subjected to qPCR to analyse the expression profiles of genes associated with embryonic development and genes encoding heat shock proteins (*RPS15A*, *HSP90AA1*, *HSPB11*, *HSPA1A*, *GPX1*, *IGF2R*, *CCNB1*, *DNMT3A*, *PLAC8*).

TaqMan Advanced miRNA qPCR technology was used to determine miRNA expression. cDNA synthesis was performed using the Applied Biosystems TaqMan Advanced miRNA cDNA Synthesis Kit (catalogue number: A28007), according to the manufacturer's protocol. Owing to the low initial RNA concentration, samples were used without dilution, with the maximum sample volume permitted for the reaction (2 μ L). cDNA synthesis consisted of four consecutive steps: poly(A) tailing, adaptor ligation, reverse transcription (RT) and miR-Amp preamplification. Undiluted miR-Amp reaction products were stored at -80°C until real-time qPCR analysis. The miRNAs investigated were miR-191-5p, miR-320a-3p, miR-372-3p, miR-378a-3p and miR-138-5p.

A negative control reaction was also run for each assay. miR-92a-3p was used as the endogenous reference miRNA (internal control) for normalising gene expression data.

3.5. Apoptosis and necrosis staining

Apoptosis and necrosis staining of embryos was performed using the commercially available FITC–Annexin V Apoptosis and Necrosis Detection Kit (PromoKine, Heidelberg, Germany; PK-CA707-30018). Embryos were incubated in drops containing 50 µL of staining solution for 15 minutes at room temperature in the dark. Hoechst 33342 was used to label nuclei, FITC–Annexin V to detect phosphatidylserine externalisation during apoptosis, and Ethidium Homodimer III to identify necrotic cells that had lost membrane integrity. Fluorescence imaging was performed using an ImageXpress® Pico Automated Cell Imaging System (Sunnyvale, California, USA) with DAPI/Hoechst, FITC and Texas Red filters. We examined 16 control and 17 heat-treated embryos by apoptosis and necrosis staining.

3.6. Immunostaining

The primary antibodies used were rabbit monoclonal anti-CDX2 (1:100; Abcam, ab76541) and rabbit monoclonal anti-OCT4 (1:100; Abcam, ab181557). Following three washes with PBS containing 0.01% BSA, embryos were incubated with a goat anti-rabbit secondary antibody conjugated to the Abberior STAR RED fluorophore (1:200; Abberior/Unicam) for 1 hour at 37 °C in the dark. Nuclei were counterstained with TO-PRO-3 iodide (1:500; Thermo Fisher Scientific) for 15 minutes at room temperature. Samples were examined using a confocal laser scanning microscope (TCS SP8, Leica Microsystems IR GmbH, Wetzlar, Hesse, Germany). We examined 15 control and 13 heat-treated embryos by immunofluorescence staining.

3.7. Statistical evaluation

Data were analysed using generalised linear mixed models (GLMMs) with a binomial error distribution and a logit link function. The procedures applied are summarised in Table 4. The significance threshold was $p < 0.05$.

Table 4. Statistical and qualitative evaluation procedures applied

Variable examined	Method and model structure	Comparisons and reporting
Blastocyst rate A and C	GLMM; fixed effect: treatment; random effects: biological replicate and culture drop.	Pairwise comparisons with Tukey correction.
Post-thaw survival B and C	GLMM; fixed effect: treatment; random effect: biological replicate.	Pairwise comparisons with Tukey correction.
Long-term survival D, D10–D14 combined	GLMM; fixed effects: culture temperature, heat treatment, cryopreservation and culture day; random effect: biological replicate.	Joint assessment of main effects; D8 was excluded from the model.
Long-term survival D, by day	Separate GLMMs for D10, D12 and D14. Fixed effects: temperature, heat treatment, cryopreservation and all their two- and three-way interactions; random effect: biological replicate.	Type III Wald χ^2 test; estimated marginal means; K–HK comparisons for each temperature–cryopreservation combination, with Holm correction. Estimated survival probability, odds ratio and 95% confidence interval.
Gene expression A and B	Student’s t-test on fold change values relative to the control ($2^{-\Delta\Delta C_t}$).	Relative expression compared with the control group.
Gene expression C	One-way analysis of variance followed by Student’s t-test on ΔC_t values normalised to the reference gene.	Presentation: $2^{-\Delta C_t}$; mean $\pm$ SEM.
Cellular analyses	Qualitative assessment of apoptosis, necrosis and immunofluorescence staining.	Qualitative comparison.

GLMMs were fitted using the `glmer()` function in the R package `lme4`. Proportion data from Experiments A–B were analysed in R 4.6.0 using maximum-likelihood estimation and the Laplace approximation; the `glht()` function in the `multcomp` package was used for Tukey comparisons. In Experiments C–D, R 4.5.0 was used with `Anova()` from the `car` package, `emmeans` and `ggplot2`. Survival rates in Experiment D were calculated relative to the number of embryos alive on D8. D8 was excluded from the models to avoid complete separation due to 100% survival; dead embryos were also counted as dead at subsequent time points. qPCR data were processed using Eppendorf RealPlex and GenEx 7.0 software. NormFinder analysis was used to select

references; the internal controls were *EEF1A1* and miR-92a-3p, respectively. The biological unit of gene expression analysis was the pooled sample per replicate and group (n = 3 biological replicates); the three technical replicates do not constitute independent biological replicates.

Parts of this dissertation were prepared using the author's previously published scientific article. Parts of the Materials and Methods chapter, the results of Experiments A and B, the results of the apoptosis, necrosis and immunostaining analyses, and parts of the discussion were partially reported in the following publication: Nagy *et al.*, 2026.

4. RESULTS AND DISCUSSION

Throughout the study, we ensured that measurements were always performed on blastocyst-stage embryos, and gene expression levels in each treatment were evaluated relative to those of the corresponding control group in every experimental replicate. Control and heat-treated groups were compared within each replicate, because the gene expression profiles of control embryos also differed between experimental replicates, presumably owing to differences in the environmental exposures of donor cows and individual biological variation.

4.1. Analysis of blastocyst and survival rates

4.1.1. Experiment A

The blastocyst rates determined in each replicate are presented in Table 5.

Table 5. Blastocyst rates in individual replicates

Replicate	Experimental group	Number of blastocysts	Blastocyst rate (%)
A1	K	10	50.0 ± 25.8
	HK	10	50.0 ± 20.0
	HS	7	35.0 ± 10.0
A2	K	25	62.5 ± 32.8
	HK	29	72.5 ± 18.3
	HS	27	67.5 ± 26.0
A3	K	12	40.0 ± 28.3
	HK	16	53.3 ± 16.4
	HS	12	40.0 ± 21.9

*±: standard deviation between culture drops within each biological replicate

Pairwise comparisons between groups revealed no statistically significant differences in blastocyst rates for any pair of treatment groups (K–HK: $p = 0.464$, K–HS: $p = 0.988$, HK–HS: $p = 0.381$). The model-estimated probabilities of blastocyst formation were 50.3% (control), 59.9% (HK) and 49.2% (HS).

4.1.2. Experiment B

Post-thaw survival rates varied between experimental replicates in both the control and heat-treated groups. Post-thaw survival rates are summarised in Table 6.

Table 6. Survival rates in individual replicates

Replicate	Experimental group	Number of surviving blastocysts	Survival rate (%)
B1	K	7	100
	HK	7	75
B2	K	8	73
	HK	6	60
B3	K	8	73
	HK	15	95

Post-thaw survival rates ranged from 73% to 100% in the control group and from 60% to 95% in the heat-treated group across biological replicates.

Treatment had no statistically significant effect on post-thaw survival ($p = 0.957$). The model-estimated probability of survival was 79.3% (23/29) in the control group and 80.0% (28/35) in the heat-treated group. Variability between experiments was low (SD = 0.16).

4.1.3. Experiment C

The blastocyst rates determined in each replicate are presented in Table 7.

Table 7. Blastocyst rates in individual replicates

Replicate	Experimental day	Experimental group	Number of blastocysts (n)	Blastocyst rate (%)
C1	7	K	49	61.3 ± 27.5
		HK	54	67.5 ± 30.6
C2	7	K	57	71.3 ± 22.7
		HK	58	72.5 ± 17.9
C3	7	K	58	72.5 ± 19.6
		HK	54	67.5 ± 15.7
C1	8	K	53	66.3 ± 26.6
		HK	61	76.3 ± 22.9
C2	8	K	69	86.3 ± 13.0
		HK	69	86.3 ± 13.7
C3	8	K	68	84.0 ± 13.8
		HK	59	73.8 ± 16.2

The survival rates determined in Experiment C are presented in Table 8.

Table 8. Survival rates in individual replicates

Replicate	Experimental group	Number of surviving blastocysts	Survival rate (%)
C1	K	19	95
	HK	19	95
C2	K	23	100
	HK	24	100
C3	K	25	100
	HK	23	92

Generalised linear mixed model analysis indicated that heat treatment had no significant effect on embryo development. Blastocyst rates did not differ significantly between the control and heat-treated groups on either day 7 (68.3%, 164/240 and 69.2%, 166/240; $p = 0.844$) or day 8 of culture (79.2%, 190/240 and 78.8%, 189/240; $p = 0.910$). Similarly, no significant difference in post-thaw survival rate was detected between the control (98.5%, 67/68) and heat-treated groups (95.7%, 66/69) ($p = 0.340$).

4.1.4. Experiment D

The morphological changes observed during long-term embryo culture are illustrated in Figures 5 and 6.

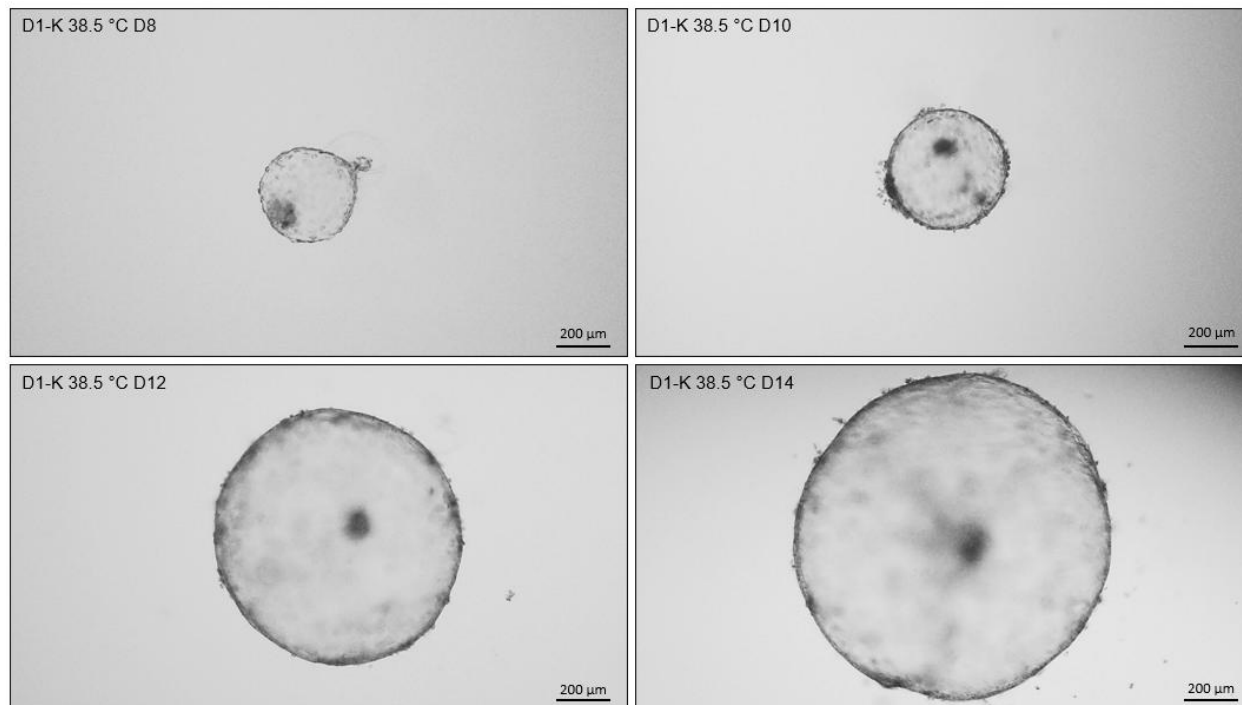


Figure 5. Morphological changes in an *in vitro*-produced bovine blastocyst during extended culture in the control group (K) cultured at 38.5 °C (D8–D14)

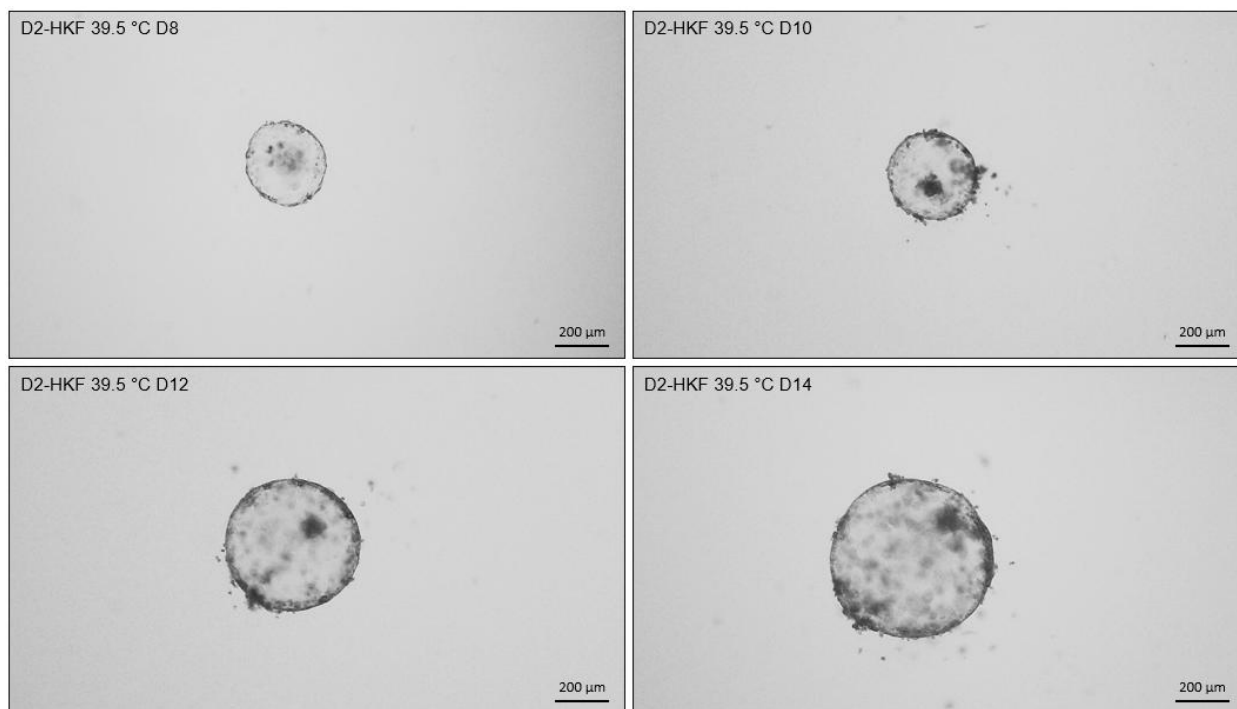


Figure 6. Morphological changes in an *in vitro*-produced bovine blastocyst during extended culture in the heat-treated and cryopreserved group (HKF) cultured at 39.5 °C (D8–D14)

Embryo survival rates gradually decreased with increasing culture duration in all treatment groups (Figure 7).

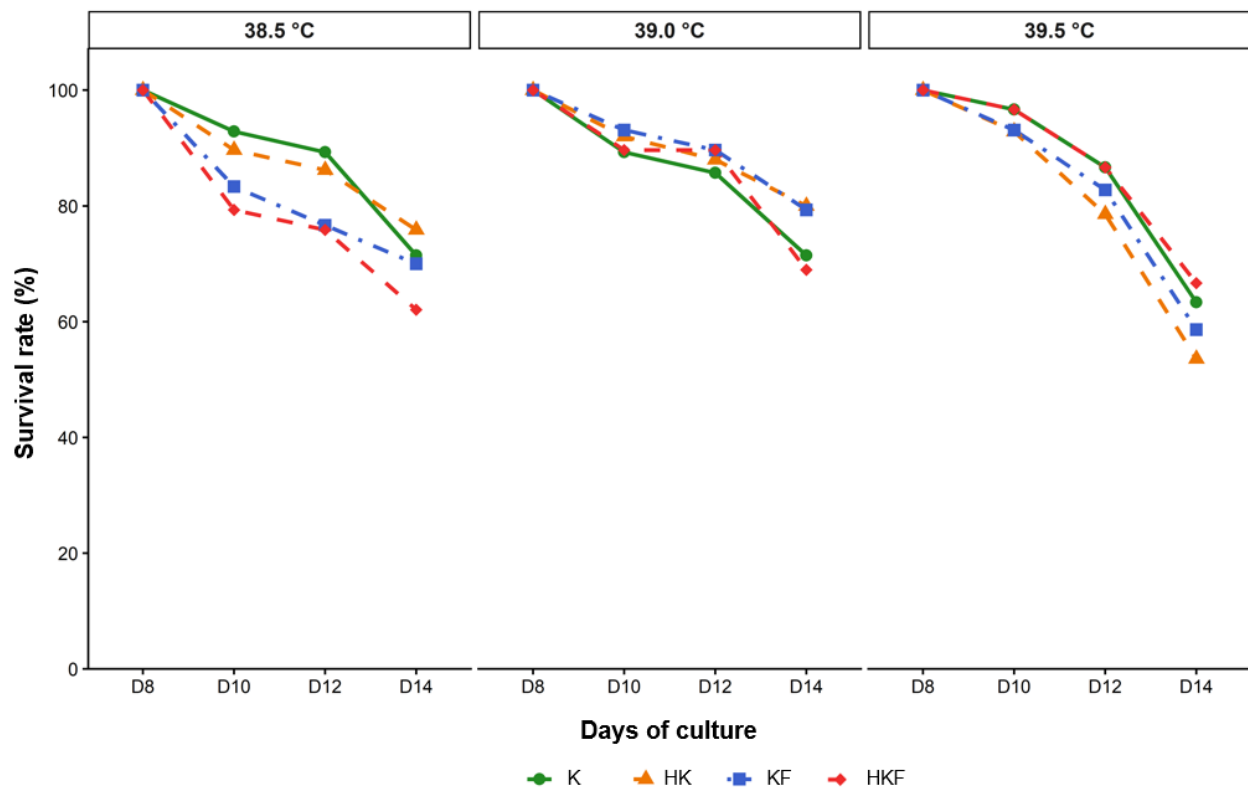


Figure 7. Embryo survival rates during extended culture at different culture temperatures and in different treatment groups. The figure shows survival rates determined at D8, D10, D12 and D14 in the control (K), heat-treated (HK), cryopreserved control (KF) and cryopreserved heat-treated (HKF) groups, separately for culture temperatures of 38.5, 39.0 and 39.5 °C.

The generalised linear mixed model (GLMM) used for the combined analysis of the D10–D14 period showed a significant effect of culture day on embryo survival ($p < 0.001$), confirming that embryo viability declined progressively over time during long-term culture. In contrast, neither culture temperature ($p = 0.134$), heat treatment ($p = 0.631$) nor cryopreservation ($p = 0.319$) had a significant main effect on survival. The interaction between heat treatment and cryopreservation was also not significant ($p = 0.961$). The survival curves showed a similar declining trend in all treatment groups, and none of the treatments conferred a sustained survival advantage during extended culture.

As the primary aim of the study was to determine whether heat treatment improves embryo survival at elevated culture temperatures and after cryopreservation, culture days D10, D12 and D14 were also analysed separately using factorial GLMMs. The interaction between culture temperature and heat treatment was not significant at any time point examined (D10: $p = 0.710$; D12: $p = 0.924$; D14: $p = 0.495$), suggesting that the effect of heat treatment did not depend on culture temperature. Similarly, the interaction between heat treatment and cryopreservation was not significant (D10: $p = 0.889$; D12: $p = 0.786$; D14: $p = 0.472$); thus, heat treatment did not significantly improve the survival of cryopreserved embryos. The three-way interaction among

culture temperature, heat treatment and cryopreservation also failed to reach statistical significance on any culture day (D10: $p = 0.563$; D12: $p = 0.762$; D14: $p = 0.270$), suggesting that the effect of heat treatment did not depend jointly on culture temperature and cryopreservation. Pairwise comparisons with Holm correction revealed no significant differences between heat-treated and non-heat-treated groups in any case (D10: $p = 0.516$ – 0.725 ; D12: $p = 0.405$ – 1.000 ; D14: $p = 0.370$ – 0.702).

4.2. Analysis of gene expression patterns

4.2.1. Experiment A

The relative expression levels of the genes investigated in the two treated groups (HK: heat-treated; HS: heat shock), compared with the control group, are shown in Figures 8a and 8b. Figures 8c and 8d illustrate the expression responses of *HSP90AA1* and *HSPB11* to heat treatment and heat shock. In both cases, heat treatment resulted in a significant decrease in the expression levels of *HSP90AA1* ($p = 0.021$) and *HSPB11* ($p = 0.032$). The gene expression response of embryos to heat stress showed substantial individual variability, resulting in high standard deviations in the groups examined.

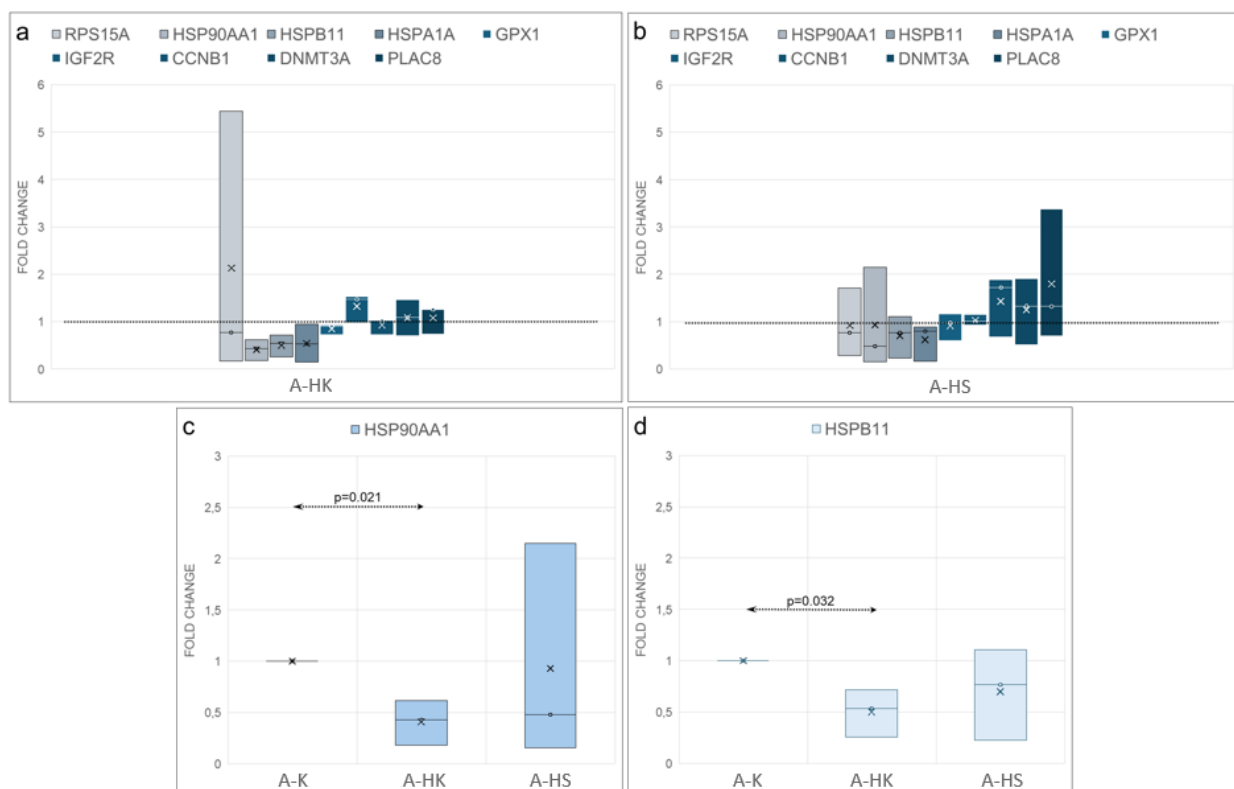


Figure 8. (a) Relative expression levels of the genes investigated in the heat-treated group; (b) relative expression levels of the genes investigated in the heat shock-treated group; (c) relative expression level of *HSP90AA1* ($p = 0.021$); (d) relative expression level of *HSPB11* ($p = 0.032$).

Figure 9 shows the relative expression levels of miR-191-5p, miR-372-3p, miR-378a-3p, miR-138-5p and miR-320a-3p in the treated groups compared with the control group. Among the miRNAs investigated, miR-191-5p expression was significantly lower in both the heat-treated ($p < 0.001$) and heat shock groups ($p < 0.001$) than in the control group (Figure 9a). In contrast, significant decreases in the expression of miR-378a-3p ($p = 0.013$) (Figure 9d) and miR-138-5p ($p = 0.014$) (Figure 9e) were observed only in the heat-treated group.

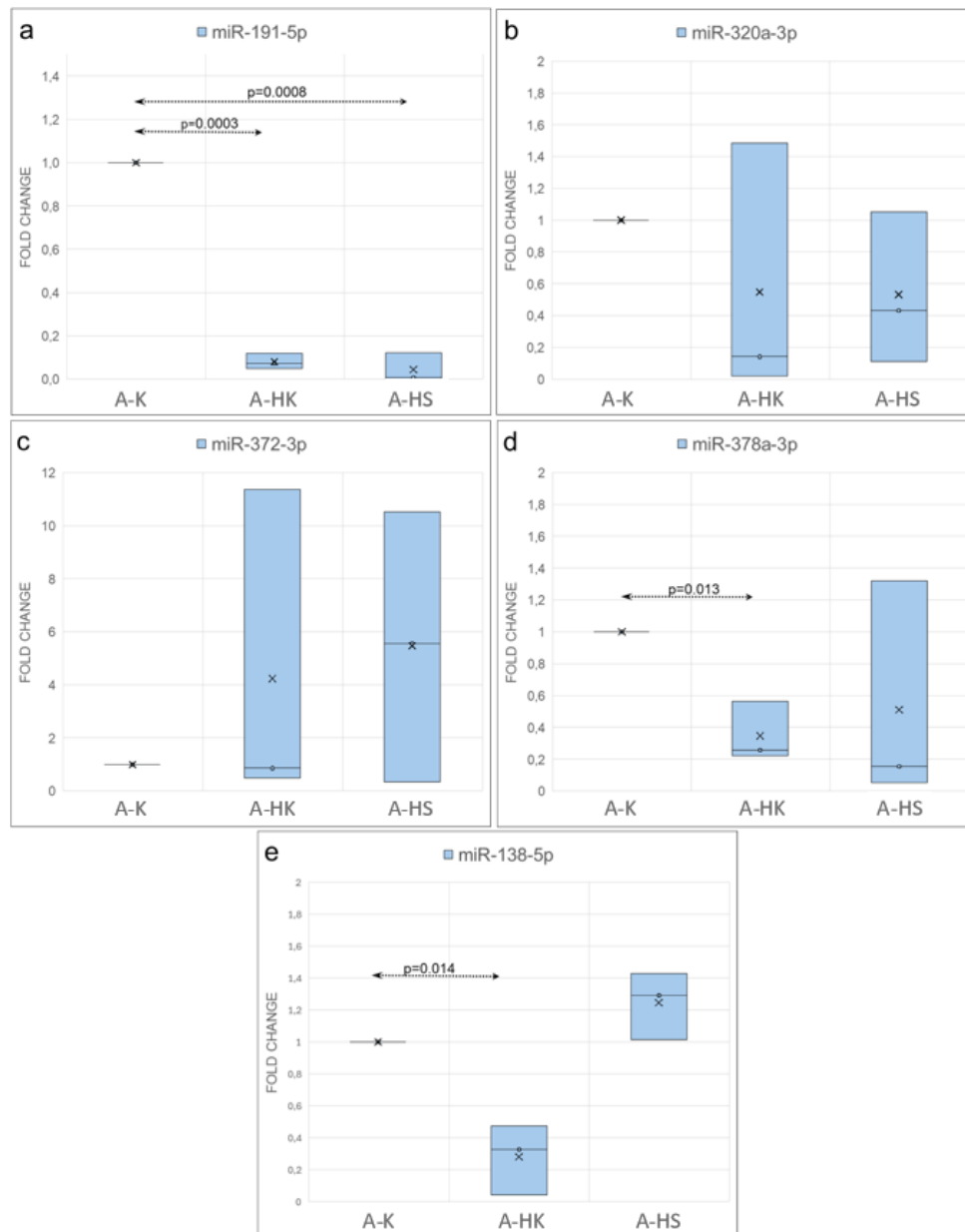


Figure 9. Relative expression levels of the miRNAs investigated. (a) Relative expression level of miR-191-5p; (b) relative expression level of miR-320a-3p; (c) relative expression level of miR-372-3p; (d) relative expression level of miR-378a-3p; (e) relative expression level of miR-138-5p.

4.2.2. Experiment B

The relative expression levels of the genes investigated are shown in Figure 10. As shown in Figure 10a, heat treatment significantly decreased *HSP90AA1* expression ($p = 0.046$), whereas the expression of the other markers investigated remained similar to that of the control group. The decrease in *HSP90AA1* expression was also significant under these experimental conditions (Figure 10b), while *HSPB11* showed a declining trend similar to that observed in Experiment A; however, owing to substantial individual variability, this difference did not reach statistical significance ($p = 0.089$) (Figure 10c).

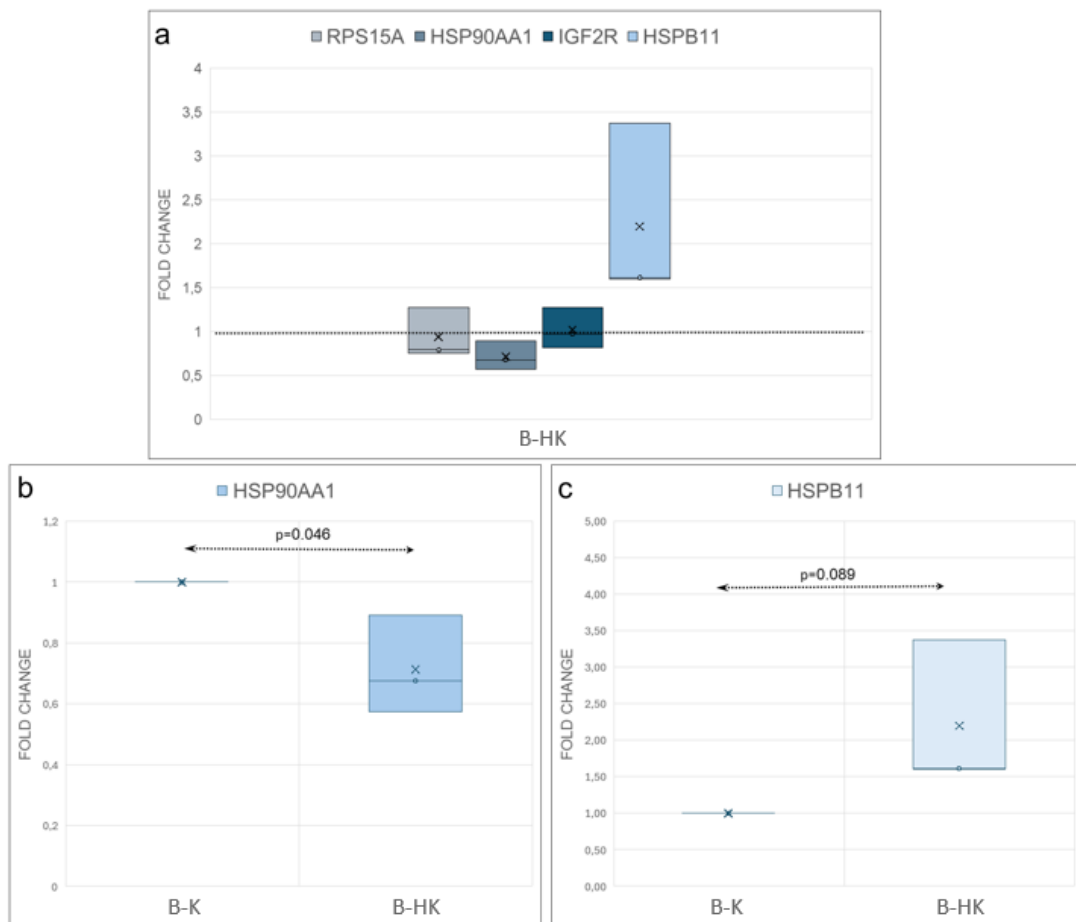


Figure 10. (a) Relative expression levels of the genes investigated; (b) relative expression level of *HSP90AA1*; (c) relative expression level of *HSPB11* after embryo thawing

In this experiment, miRNA expression profiles showed high variability, even within the heat-treated group, which may have been associated with cryopreservation. Consequently, between-sample standard deviations also increased considerably. Among the miRNAs investigated, only miR-378a-3p expression was significantly higher in the heat-treated group than in the control group (Figure 11d; $p = 0.03$). The expression of the other miRNAs that showed significant changes in Experiment A did not differ significantly between the two groups in Experiment B (Figure 11a, b, c, e).

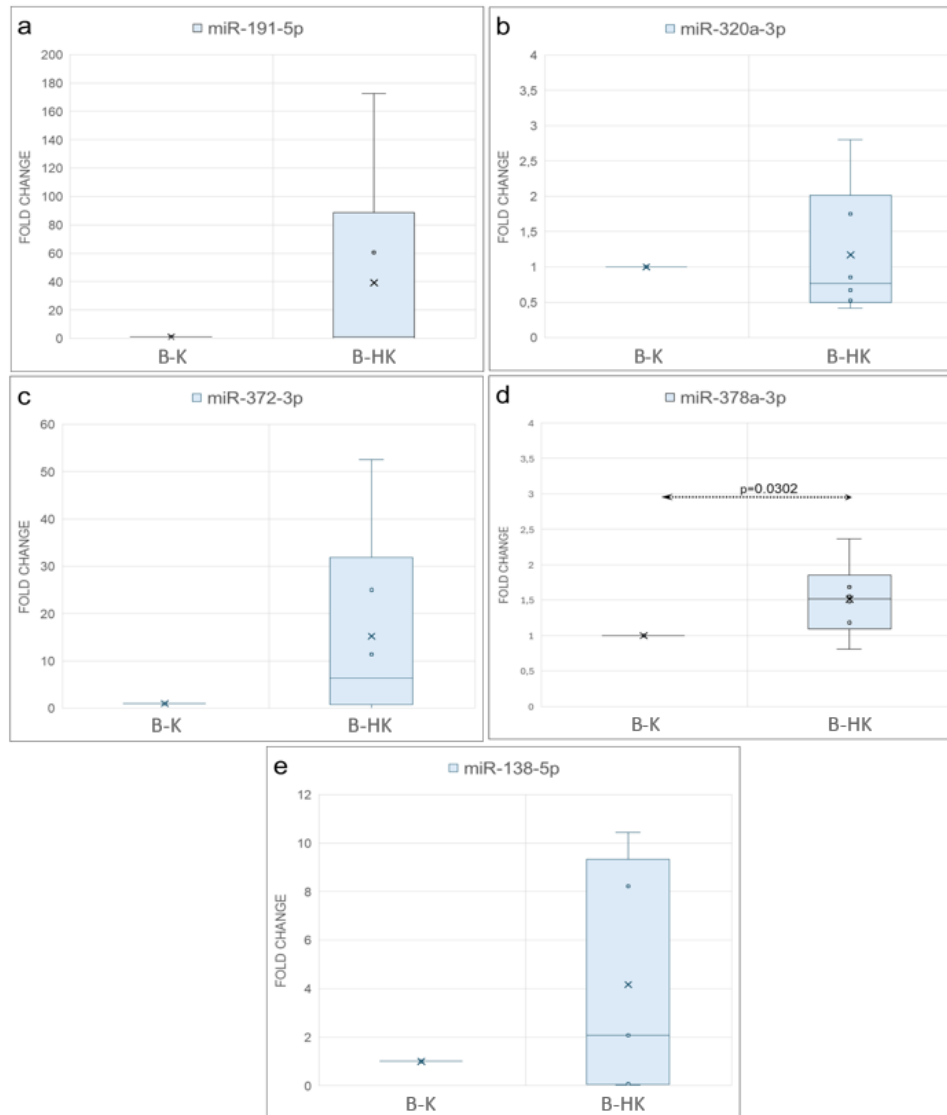


Figure 11. Relative expression levels of the miRNAs investigated in Experiment B. Relative expression levels of (a) miR-191-5p; (b) miR-320a-3p; (c) miR-372-3p; (d) miR-378a-3p; (e) miR-138-5p.

Comparison of *HSP90AA1* and *HSPB11* expression levels measured in the heat-treated (HK) groups of Experiments A and B showed increased expression of both markers following cryopreservation. Relative to Experiment A, this increase was statistically significant for both *HSP90AA1* ($p = 0.01$) and *HSPB11* ($p = 0.036$) (Figure 12). However, as the data originated from two separate experiments, their direct comparability is limited, and the observed difference cannot be attributed unequivocally to cryopreservation alone.

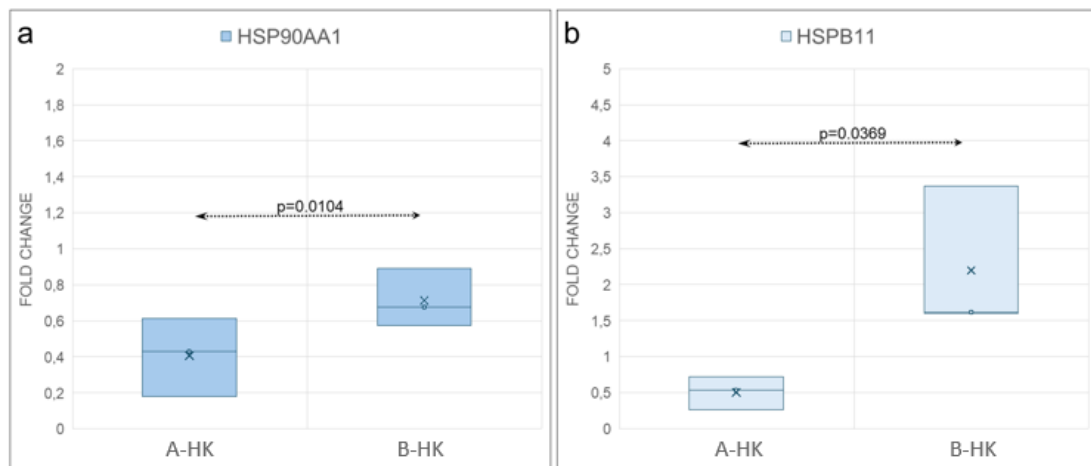


Figure 12. Expression levels of *HSP90AA1* (a) and *HSPB11* (b) in the heat-treated (HK) groups of Experiments A and B

4.2.3. Experiment C

In Experiment C (C_K, C_HK, C_KF, C_HKF), the effect of heat treatment was assessed by examining the expression of three genes encoding heat shock proteins, *HSP90AA1*, *HSPA1A* and *HSPB11*, in fresh blastocysts collected on days 7 and 8 of culture, and following cryopreservation and thawing. The results of gene expression analysis of samples collected on day 7 in Experiment C are summarised in Table 9.

Table 9. Summary of gene expression analysis results in Experiment C

Comparison	Gene	Fold change	p-value	Interpretation
K D7 – HK D7	<i>HSP90AA1</i>	–1.23	0.051	trend
	<i>HSPB11</i>	–1.09	0.097	n.s.
	<i>HSPA1A</i>	+1.09	0.246	n.s.
KF – HKF	<i>HSPA1A</i>	+1.33	0.034	significant
	<i>HSP90AA1</i>	+1.13	0.253	n.s.
	<i>HSPB11</i>	+1.09	0.341	n.s.
K D7 – KF	<i>HSPA1A</i>	+4.33	0.006	significant
	<i>HSPB11</i>	+2.24	0.021	significant
	<i>HSP90AA1</i>	–1.13	0.059	trend
HK D7 – HKF	<i>HSPA1A</i>	+5.27	0.010	significant
	<i>HSPB11</i>	+2.67	0.035	significant
	<i>HSP90AA1</i>	+1.23	0.168	n.s.
K D7 – HKF	<i>HSPA1A</i>	+5.74	0.009	significant
	<i>HSPB11</i>	+2.45	0.051	trend
	<i>HSP90AA1</i>	+1.00	0.498	n.s.

n.s. – not significant

On day 7 of culture, *HSP90AA1* expression (Figure 13a) was lower in the heat-treated group than in the control group (K: 0.0561 ± 0.0026 vs. HK: 0.0454 ± 0.0009 ; $2^{-\Delta\text{Ct}}$, mean $\pm$ SEM), corresponding to a 1.23-fold decrease. The difference was close to the significance threshold ($p = 0.051$) and can therefore be interpreted as a trend. *HSPB11* (Figure 13c) also showed a slight, non-significant decrease (0.000130 ± 0.000010 vs. 0.000119 ± 0.000006 ; $p = 0.097$), whereas *HSPA1A* expression (Figure 13b) remained virtually unchanged (0.0091 ± 0.0017 vs. 0.0098 ± 0.0017 ; $p = 0.246$).

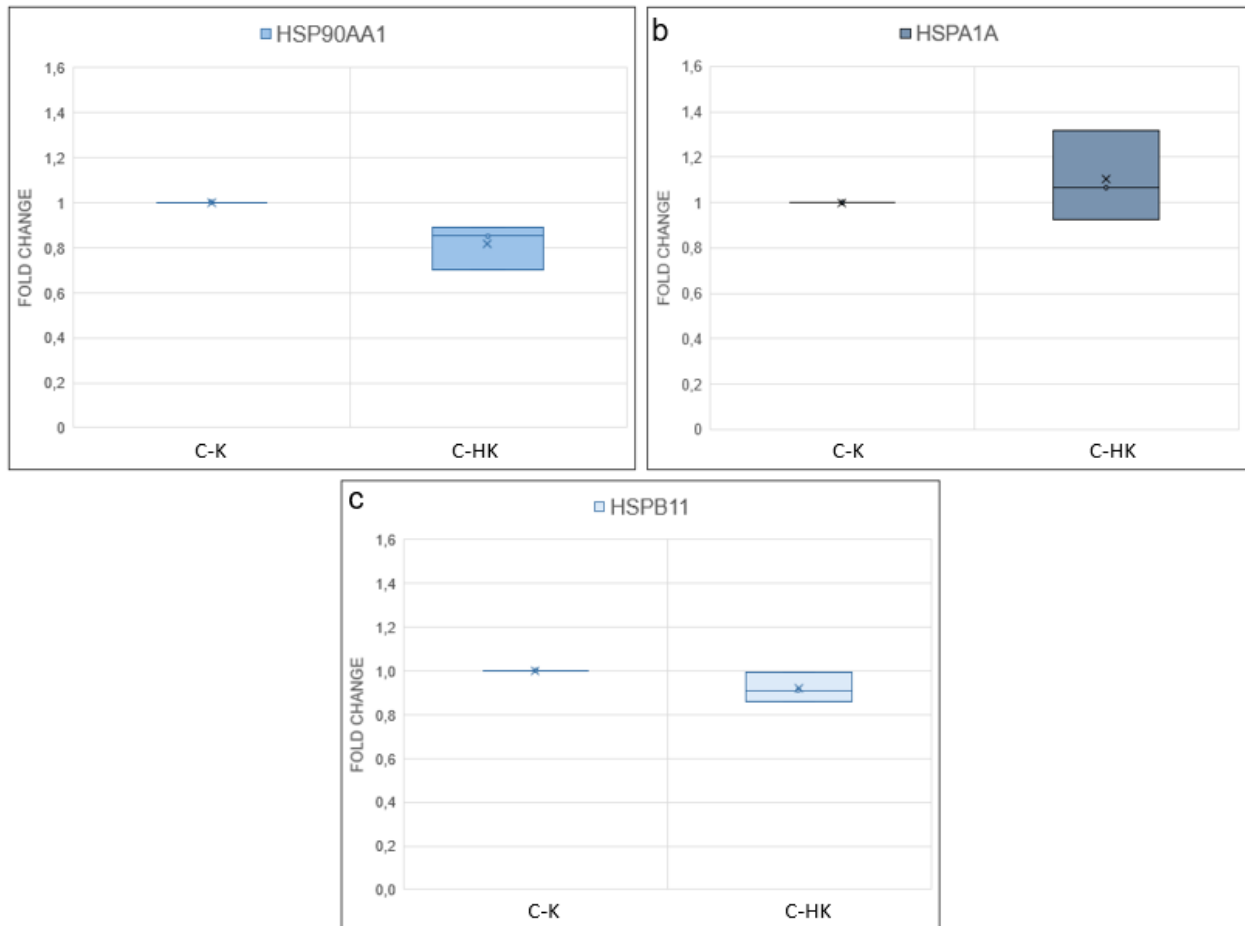


Figure 13. Expression levels of *HSP90AA1* (a), *HSPA1A* (b) and *HSPB11* (c) in samples collected on day 7 of embryo culture in Experiment C

By day 8 of culture, the trend observed on day 7 had disappeared: no differences were detected between the control and heat-treated groups for any of the genes investigated (*HSP90AA1* (Figure 14a): 0.0458 ± 0.0022 vs. 0.0479 ± 0.0039 ; *HSPA1A* (Figure 14b): 0.0121 ± 0.0019 vs. 0.0129 ± 0.0011 ; *HSPB11* (Figure 14c): 0.000208 ± 0.000023 vs. 0.000232 ± 0.000010 ; $p > 0.05$ in all cases). This may indicate that the transcriptional effect of heat treatment applied at the early morula stage is transient and subsides as blastocyst development progresses.

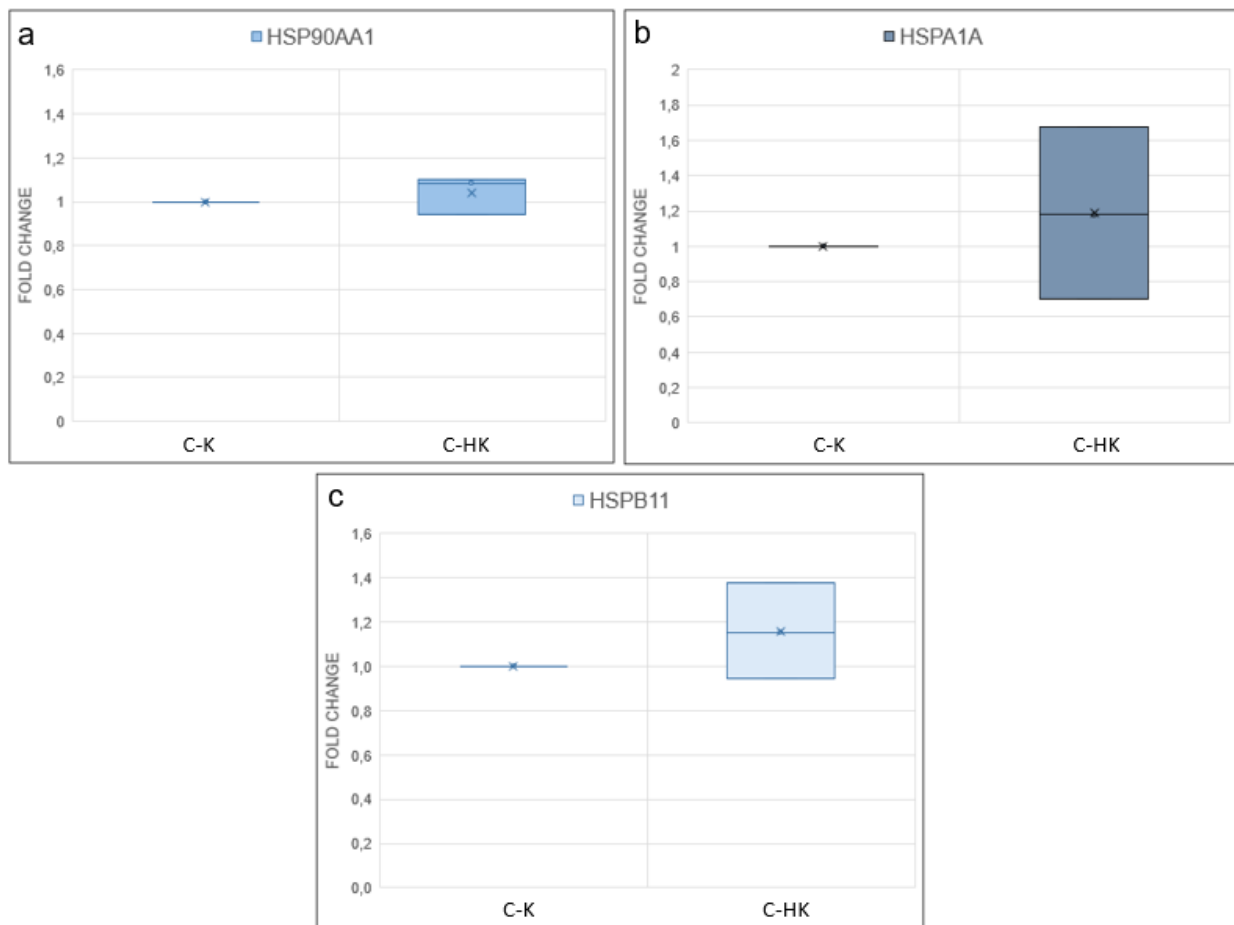


Figure 14. Expression levels of *HSP90AA1* (a), *HSPA1A* (b) and *HSPB11* (c) in samples collected on day 8 of embryo culture in Experiment C

Following cryopreservation and thawing, however, a clear difference emerged between the two groups: *HSPA1A* expression (Figure 15b) was significantly higher in the heat-treated group than in the control group (0.0373 ± 0.0043 vs. 0.0488 ± 0.0029 ; 1.33-fold increase; $p = 0.034$). *HSP90AA1* (Figure 15a) (0.0495 ± 0.0010 vs. 0.0570 ± 0.0058 ; $p = 0.253$) and *HSPB11* (Figure 15c) (0.000294 ± 0.000033 vs. 0.000328 ± 0.000047 ; $p = 0.341$) showed increases in the same direction, but these were not significant.

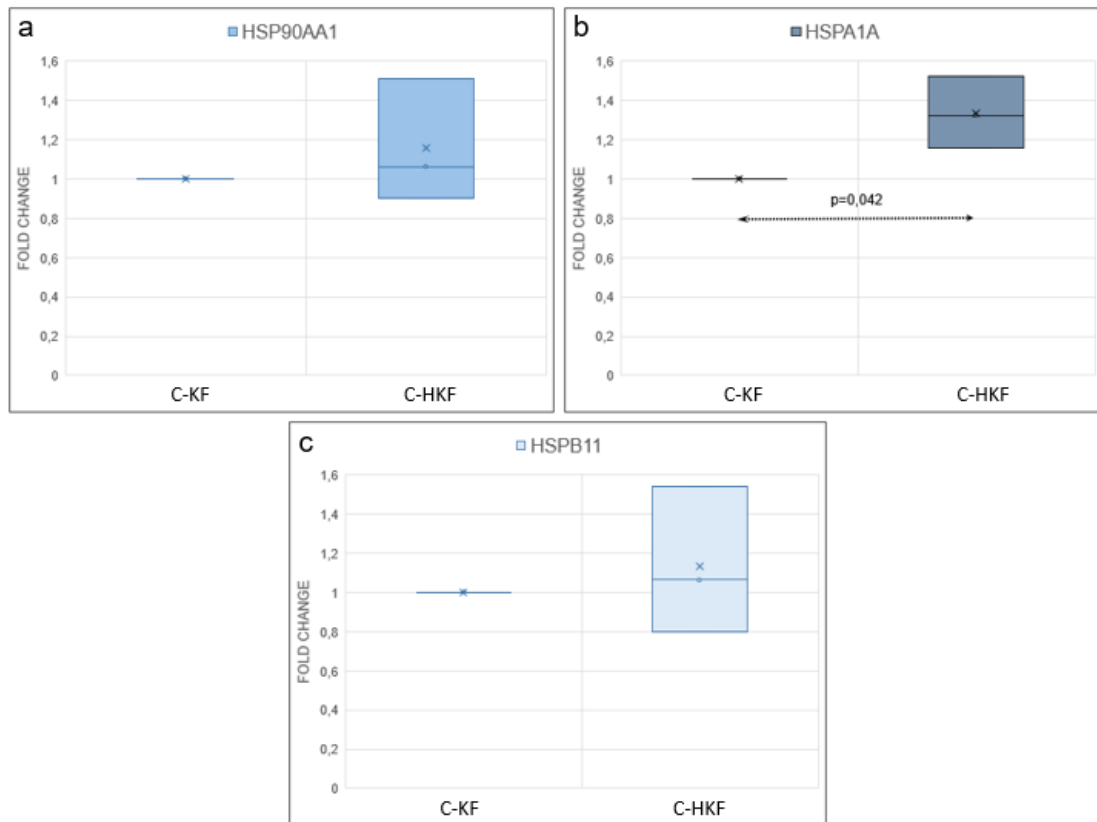


Figure 15. Expression levels of *HSP90AA1* (a), *HSPA1A* (b) and *HSPB11* (c) in samples collected 24 hours after embryo thawing in Experiment C

Cryopreservation alone also substantially altered the expression of the genes investigated. Comparison of fresh day 7 samples with thawed samples showed a 4.33-fold increase in *HSPA1A* expression ($p = 0.006$) and a 2.24-fold increase in *HSPB11* expression ($p = 0.021$) in the control group. These increases were greater in the heat-treated group: 5.27-fold for *HSPA1A* ($p = 0.010$) and 2.67-fold for *HSPB11* ($p = 0.035$). *HSP90AA1* expression did not change significantly in response to freezing in either group (control: $p = 0.059$; heat-treated: $p = 0.168$).

The combined effects of the two treatments are illustrated by comparing the fresh control and cryopreserved heat-treated groups: in this comparison, *HSPA1A* expression increased 5.74-fold (0.0091 ± 0.0017 vs. 0.0488 ± 0.0029 ; $p = 0.009$), while *HSPB11* showed a 2.45-fold increase close to the significance threshold (0.000130 ± 0.000010 vs. 0.000328 ± 0.000047 ; $p = 0.051$). In contrast, *HSP90AA1* expression remained virtually unchanged ($p = 0.498$).

Overall, the results of Experiment C suggest that controlled heat treatment applied at the early morula stage was associated with a trend towards transient transcriptional changes in fresh blastocysts that subsided by day 8; following cryopreservation, however, heat-treated embryos showed a more pronounced *HSPA1A* response than the control group. This may indicate that prior heat treatment modifies the molecular response of embryos to cryopreservation. Nevertheless, as

no differences were detected in developmental or survival parameters (Section 4.1.3), these changes alone do not demonstrate improved cryotolerance.

4.3. Apoptosis, necrosis and immunofluorescence staining

To illustrate the effects of treatment, we used apoptosis and necrosis staining (Figure 16), as well as immunofluorescence staining (Figure 17), for the qualitative assessment of embryo morphology and the localisation of the markers investigated. Apoptosis and necrosis staining revealed viable embryos in both the control and heat-treated groups, with no appreciable qualitative differences between the two groups. Immunofluorescence staining showed that CDX2 was localised in the trophectoderm in all embryos, whereas OCT4 expression was detected in the inner cell mass; thus, no qualitative differences in the localisation of the markers investigated were observed between the control and heat-treated groups.

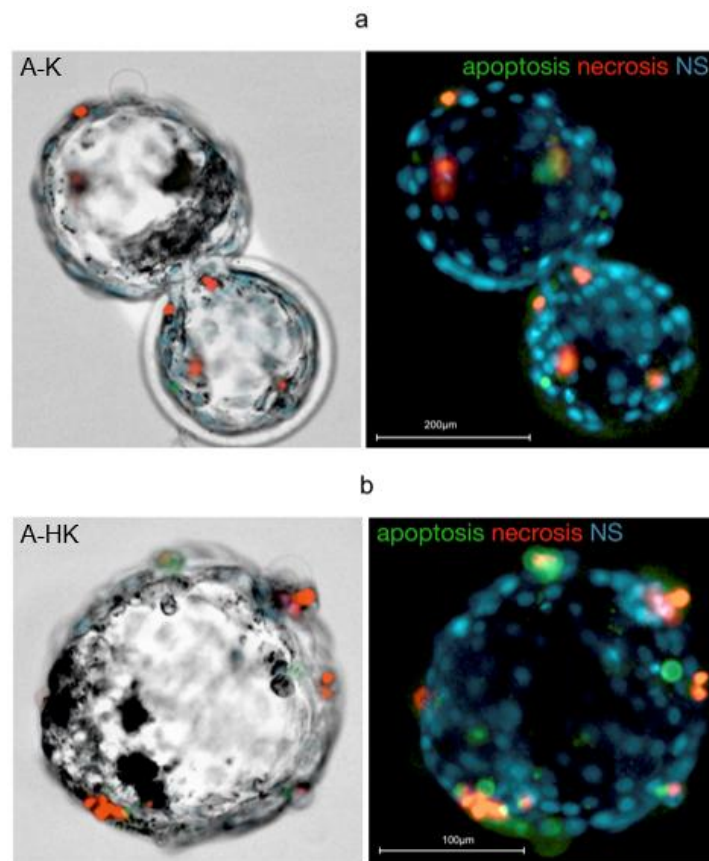


Figure 16. Apoptosis and necrosis staining of control (K) (a) and heat-treated (HK) (b) embryos.

Green indicates apoptotic cells, red indicates necrotic cells, and blue indicates nuclei.

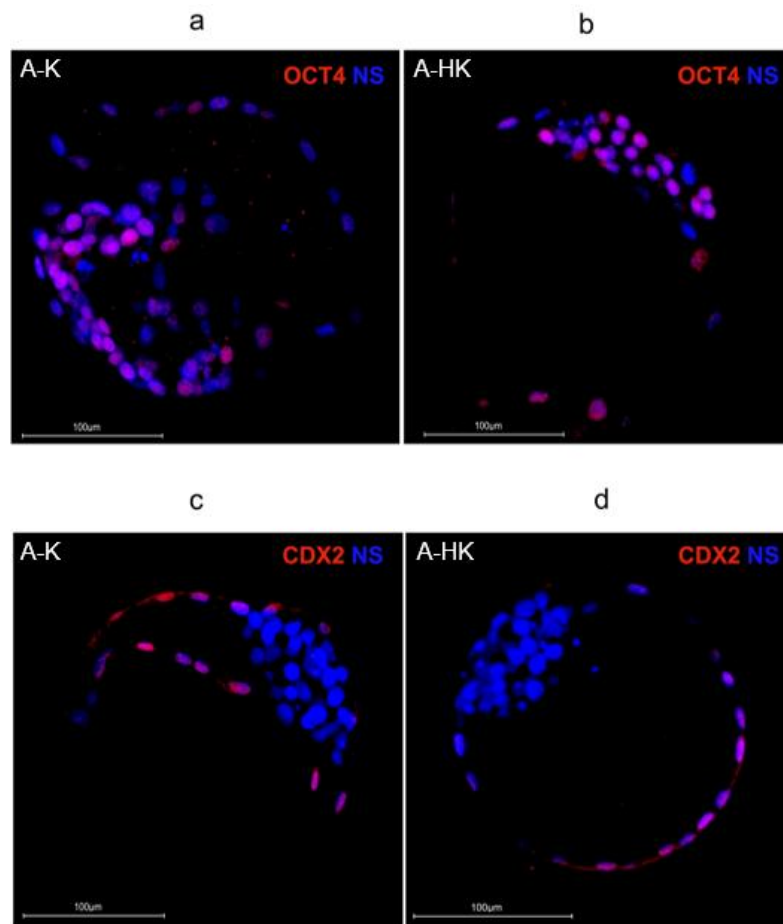


Figure 17. Immunofluorescence staining of OCT4 in control (K) (a) and heat-treated (HK) (b) embryos, and immunofluorescence staining of CDX2 in control (c) and heat-treated (d) embryos.

Red fluorescence indicates CDX2-positive cells, while blue fluorescence indicates OCT4-positive cells.

5. CONCLUSIONS AND RECOMMENDATIONS

According to the literature, heat stress generally reduces the proportion of embryos developing to the blastocyst stage (Amaral *et al.*, 2020; Kasimanickam–Kasimanickam, 2021). In our study, however, heat treatment did not significantly affect blastocyst formation, in agreement with the observations of Oliveira and colleagues (2021). Our results suggest that the applied heat treatment induced mild stress and did not constitute a severely detrimental environmental challenge to embryonic development. Furthermore, heat treatment did not reduce the post-thaw survival rate of frozen embryos. Our results suggest that heat-treated embryos retained their capacity for further development and their suitability for freezing despite the combined heat and cryogenic stress. In Experiment C, embryo survival rates 24 hours after thawing exceeded 90% in all groups and reached 100% in half of the groups, which was higher than the values (66%) reported in previous *in vitro* studies using slow freezing (Arshad *et al.*, 2021; Inaba *et al.*, 2016). These results support hypothesis H1.

In contrast to previous studies (Miętkiewska *et al.*, 2022; Sakatani *et al.*, 2013), which reported increased or unchanged *HSP90AA1* expression following heat stress, the present study showed decreased expression. This discrepancy may be attributable to differences in embryo developmental stage, duration of stress exposure, embryo quality or experimental conditions. The significantly lower *HSP90AA1* expression observed in heat-treated embryos and in embryos subjected to cryopreservation may suggest that the applied heat treatment did not induce a level of cellular stress requiring increased activation of stress response signalling pathways. However, the functional significance of reduced *HSP90AA1* expression cannot be clearly established from the present study. Despite the decrease in *HSP90AA1* expression observed here, neither blastocyst formation nor post-thaw embryo survival deteriorated significantly. In addition to *HSP90AA1*, *HSPB11* expression was significantly lower in Experiment A, while a similar trend was observed in Experiment B; however, owing to substantial individual variability, this did not reach statistical significance. Although the precise role of *HSPB11* during bovine preimplantation development remains incompletely understood, previous studies suggest that it may have a cytoprotective function by stabilising mitochondrial membranes and activating cell survival mechanisms associated with HSP90 and PI3K–Akt signalling pathways (Török *et al.*, 2012). The lower *HSPB11* expression observed may therefore further support the assumption that the treatment conditions did not elicit a severe cellular stress response. On day 7 of culture, *HSP90AA1* expression showed a decreasing trend in the heat-treated group ($p = 0.051$), but this trend disappeared by day 8, suggesting that the transcriptional effect of controlled heat treatment may be transient.

Following cryopreservation, *HSPA1A* expression was significantly higher in heat-treated embryos than in the control group subjected only to cryopreservation ($p = 0.034$), and showed a 5.74-fold increase relative to the fresh control group ($p = 0.009$). As the increase in *HSPA1A* expression was greater in the heat-treated group (5.27-fold) than in the control group (4.33-fold), prior heat treatment modified rather than attenuated the molecular response to freezing. Nevertheless, the results of the present study alone do not allow an unequivocal determination of whether increased *HSPA1A* expression reflects an adaptive cytoprotective mechanism or results from an enhanced cellular stress response induced by the combined effects of heat treatment and cryopreservation. A more precise interpretation of the biological significance of the observed gene expression changes requires further investigations, including measurement of HSP70 protein levels and functional assessment of whether controlled heat treatment increases the tolerance of thawed embryos to subsequent heat stress. Given that no differences in developmental or survival parameters were detected in any experiment, these molecular changes alone do not demonstrate improved cryotolerance. My results thus support both hypotheses H2 and H3.

The applied heat treatment did not affect the expression of development-related markers in any experiment. We subsequently examined in greater detail the potential relationships among markers showing significant changes in expression in response to heat treatment. We hypothesised that these markers may contribute to improved stress tolerance through regulatory signalling pathways. Publicly available databases were used to investigate this hypothesis. Analysis of the miRBase database (Kozomara *et al.*, 2019) indicated that miR-378a-3p, which showed a significant expression change in the present study, has a predicted binding site in the 3' untranslated region (3' UTR) of *AHSA1*, suggesting that it may play a role in regulating its expression. *AHSA1* is a co-chaperone that facilitates and activates *HSP90AA1* function (Agarwal *et al.*, 2015; McGeary *et al.*, 2019). The latest version of the TargetScan database (v8.0; targetscan.org) was used to predict miRNA target genes (Figure 18).

Human AHSA1 ENST00000216479.3 3' UTR length: 225

1	10	20	30	40	50	60	70	80	90
Human	GGCCAGCGGC-AGG-GGAC	UCCA--G	CCUGCU-GGACA	CUUCAGUCCAGCU-C-UCUCCU	GAC-UGGGGC-UUGCGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Chimp	GGCCAGCGGC-AGG-GGAC	UCCA--G	CCUGCU-GGACA	CUUCAGUCCAGCU-C-UCUCCU	GAC-UGGGGC-UUGCGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Rhesus	GGCCAGCGGC-AGG-GGAC	UCCA--G	CCUGCU-GGACA	CUUCAGUCCAGCU-C-UCUCCU	GAC-UGGGGC-UUGCGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Squirrel	GGCCAGUAGC-AGG-GGAC	UCCA--G	CCUCC-UGACA	CUUCAGUCCAGCU-C-UCUCCU	GAC-UGGGGC-UUGCGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Mouse	GGCCAGCAAC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Rat	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Rabbit	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Pig	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Cow	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Cat	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Dog	GGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Brown bat	AGCCAGCAGC-AG-GGGU	UCUG--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Elephant	GGUAGCAGCUGGG-AGGC	CAUA--G	CCUGU-UGAAA	CUUAGUCCAGCC--CUCUC	GCA-AU-GGC-UUGUGACUC	ACAGGAUUGCAUCU-CCCAG-CUG			
Opossum	UGUUAACUUCUACA-GGGC	CACG--G	CCACC-UGGCA	GCUCAGUCCAGCU-C-UCUACU	GUC-UGCAGC-U-GUGACUG	GCAGGAUUGCAUCU-CCCAG-CUG			
Macaw	UACUGGUUUC-UGG-GGG	C--G	UUUGUC-UAAUCU	UCUCA--CCUGUC-C-CUUUCU	AAUCUUGGCC-CUGCUGCG	AGUAAAGUGGG--AU-GACAG-GUG			
Chicken	UAAUGGUUUC-UGG-GGA	C--G	GCUGCC-UGACU	UCUCA--CCUGUC-C-CUUUCU	AAUCUUGGCC-CUGCUGCG	AGUAAAGUGGG--AU-GACAG-GUG			
Lizard	CACUGGCCAA-UGU-GGA	G--U	CUUGAC-CGAUC	UAACG--					
X. tropicalis	AUUUUGCUGG-GGG-G								
Con	GGCCAGCaG.aGG.GGGC	U.Ca..G	CCUGCC.cGACA	CUUCAGUCCAGCC.C.UCUCU	GAC.UGGGGC.UUGuGACUC	ACAGGAUUGCAUCU.CCCAA.CCG			

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P _{CT}	Predicted relative K _D
Position 38-44 of AHSA1 3' UTR	5' ... CCUCUGGACACUUCAGUCCAGC ...							
hsa-miR-378a-3p	3' CGGAAGACUGAGGU-UCAGGUCA	7mer-m8	-0.23	92	-0.23	1.768	N/A	-2.765

Figure 18. Putative binding site of miR-378a-3p as predicted by the TargetScan database (based on Agarwal *et al.*, 2015 and McGeary *et al.*, 2019).

miR-378a may play a protective role in embryonic responses to stressors. The decreased expression observed following heat conditioning may suggest that embryos with enhanced adaptive capacity require less activation of stress-related regulatory mechanisms. In contrast, the increased expression observed following combined heat treatment and cryogenic stress may reflect the activation of compensatory mechanisms intended to counteract cellular stress.

Further studies are required to elucidate the mechanisms underlying the molecular changes induced by controlled heat treatment before cryopreservation. As the regulatory roles of several stress response-related miRNAs identified in the present study remain insufficiently characterised during bovine preimplantation embryonic development, further investigation of miRNA-mediated signalling pathways and miRNA–mRNA interactions may contribute to a deeper understanding of the molecular mechanisms of the embryonic stress response.

Qualitative assessment of apoptosis and necrosis staining revealed no appreciable differences between control and heat-treated blastocysts, suggesting that controlled heat treatment applied at the morula stage did not induce cellular damage detectable by the method used.

CDX2 is a key marker of trophectoderm differentiation, whereas OCT4 is expressed primarily in the pluripotent cells of the inner cell mass (Aguila *et al.*, 2022). The immunofluorescence localisation observed in our study was consistent with this lineage-specific expression pattern. Our observations suggest that heat treatment did not affect the differentiation

or spatial organisation of blastocyst cell lineages, nor did it induce developmental abnormalities indicative of impaired segregation of the trophectoderm and inner cell mass.

Several methodological limitations must be considered when interpreting my results. The study was conducted exclusively under *in vitro* conditions and therefore provides no information on the effects of heat treatment on *in vivo* development or pregnancy outcomes. Furthermore, the cellular analyses (apoptosis and necrosis staining, immunostaining) were based on qualitative assessment, which did not allow smaller differences to be detected.

During extended culture, embryo survival declined progressively with increasing culture duration, confirming that long-term culture beyond 8 days poses a considerable challenge to maintaining embryo viability (Martínez de Los Reyes *et al.*, 2026). Nevertheless, we successfully established a culture system that allowed the viability of fresh and cryopreserved *in vitro*-produced bovine embryos to be monitored up to day 14 of embryo culture. The results showed that neither controlled heat treatment applied at the morula stage, cryopreservation nor the culture temperatures used significantly affected embryo survival during long-term culture. The absence of interactions between heat treatment and culture temperature, and between heat treatment and cryopreservation, suggests that the heat treatment protocol used did not confer a detectable adaptive response either during long-term culture at elevated temperatures or in maintaining viability after cryopreservation. Although higher survival rates were observed in heat-treated embryos for some treatment combinations, these differences were neither consistent nor statistically significant.

The survival rate observed 24 hours after thawing in the present experiment also exceeded the 24-hour re-expansion rates generally reported in the literature following slow freezing (Arshad *et al.*, 2021). Do and colleagues (2017) reported a hatching rate of 66% after 48 hours of post-thaw culture. As the frozen embryos in the present study included blastocysts that had already hatched, post-thaw development was evaluated by observing embryo morphology rather than by determining the hatching rate. Accordingly, survival rates ranged from 79.3% to 96.7% across treatment groups, with a mean of 90.1% across all groups.

To our knowledge, this is the first study to compare extended culture of control and heat-treated, as well as fresh and cryopreserved, *in vitro*-produced embryos at three different culture temperatures within a unified experimental design. Although previous publications reported long-term *in vitro* culture of thawed embryos, they monitored development only up to day 12 of embryo culture (Nicacio *et al.*, 2012), whereas the present study assessed embryo viability up to day 14.

The primary aim of our study was to determine embryo survival; therefore, late developmental events, such as embryonic disc formation, were not investigated. However, as the extended culture system proved capable of maintaining embryo viability up to day 14 of culture,

it may provide a suitable basis for future studies assessing late embryonic development and the morphological and molecular changes that determine it.

Overall, the heat treatment protocol investigated did not produce a detectable increase in thermotolerance during extended culture; therefore, the experimental results do not support hypothesis H4. Nevertheless, the experimental model established provides a new opportunity to investigate bovine embryo development after hatching from the zona pellucida. It also offers a suitable experimental system for evaluating the long-term developmental effects of heat stress treatments of varying intensity, and may thus contribute to a more detailed understanding of the mechanisms determining subsequent embryo development and viability.

6. NEW SCIENTIFIC FINDINGS

In my investigations, I used an integrated experimental system to comprehensively assess the effects of controlled heat treatment applied at the early morula stage on in vitro-produced bovine embryos. The successive experiments enabled a unified investigation of the developmental, molecular, cellular and cryobiological effects of heat treatment, as well as long-term viability after cryopreservation during extended embryo culture. Based on the results, I formulated the following new scientific findings.

1. I demonstrated that controlled heat treatment applied at the early morula stage induces changes at the mRNA and miRNA levels in in vitro-produced bovine embryos without significantly impairing their developmental potential or survival after cryopreservation. My results establish that the molecular markers investigated are more sensitive indicators of changes induced by controlled heat load than the developmental parameters assessed.
2. I identified specific changes in miRNA expression associated with controlled heat treatment in bovine embryos. miR-191-5p expression decreased significantly in response to both controlled heat treatment and heat shock, whereas significant decreases in miR-378a-3p and miR-138-5p expression were detected only following controlled heat treatment. After cryopreservation, miR-378a-3p was the only miRNA investigated to show a significant change in expression, suggesting a potential role for this miRNA in the response to combined stressors.
3. I demonstrated that cryopreservation induces a selective, chaperone-specific heat shock protein response in bovine embryos. *HSPA1A* and *HSPB11* expression increased significantly in both control and previously heat-treated embryos, whereas *HSP90AA1* expression did not change significantly. Post-thaw *HSPA1A* expression was significantly higher in embryos previously exposed to controlled heat treatment than in the control group subjected only to cryopreservation, suggesting that prior heat treatment modifies the molecular stress response to cryopreservation.

4. I successfully adapted the extended in vitro culture system for bovine embryos. I demonstrated that neither culture temperature, controlled heat treatment nor cryopreservation significantly affected embryo survival, and that the interactions among the factors investigated were also not significant.
5. Using qualitative cellular analyses, I demonstrated that, following application of the controlled heat treatment protocol, no differences in the expression of early embryo-specific markers could be identified using the methods employed, and no increase in cellular damage was observed in bovine embryos compared with the control group.

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7. LIST OF PUBLICATIONS

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