Theriogenology 141 (2020) 16–25

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Exposing dairy bulls to high temperature-humidity index during spermatogenesis compromises subsequent embryo development *in vitro*

Núria Llamas Luceño [a, *], Daniel de Souza Ramos Angrimani [a, e], Luana de Cássia Bicudo [a, e], Katarzyna J. Szymańska [b], Mario Van Poucke [c], Kristel Demeyere [d], Evelyne Meyer [d], Luc Peelman [c], Erik Mullaart [f], Marleen L.W.J. Broekhuijse [f], Ann Van Soom [a, **]

[a] Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[b] Department of Basic and Applied Medical Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium
[c] Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[d] Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[e] Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, Brazil
[f] CRV BV, Arnhem, the Netherlands

ARTICLE INFO

Article history:
Received 23 April 2019
Received in revised form
9 August 2019
Accepted 28 August 2019
Available online 29 August 2019

Keywords:
Heat-stress
Sperm fertility
Embryo quality
Bovine
Gene expression
Seasonal effects

ABSTRACT

The possible impact of natural heat stress on animal fertility is currently a major concern for breeding companies. Here, we aimed to address this concern by determining the effects of natural heat stress on the fertility of Holstein bulls located in the Netherlands. Semen samples were collected from six bulls at two locations in March 2016 (low temperature-humidity index (THI) group; maximum THI of 51.8 and 55 at their respective locations) or August (high THI group; maximum THI of 77.9 and 80.5 during meiotic and spermiogenic stages of spermatogenesis, 42 to 14 days prior to semen collection). The effect of heat stress on semen quality was assessed by sperm morphology, motility, reactive oxygen species production, lipid peroxidation, viability, and DNA fragmentation. Moreover, we evaluated the development of embryos generated *in vitro* by low and high THI semen, and determined inner cell mass/trophectoderm ratio, apoptotic cell ratio, and embryonic gene expression in day-8 blastocysts. An increase in cell death (propidium iodide-positive cells; $P = 0.039$) was observed in the high THI group (31.5%) compared to the low THI group (27.6%). Moreover, a decrease ($P < 0.001$) was observed in the total blastocyst rates at day 7 post-insemination (15.3 vs 20.9%) and day 8 (23.2 vs 29.6%) in the high THI compared to the low THI group, respectively. There were no differences in the relative abundance of candidate transcripts examined. In conclusion, sperm samples from dairy bulls obtained during a period with higher THI had reduced viability and led to a decrease in blastocyst development and delayed hatching, compared to semen collected during a period with low THI.

1. Introduction

Heat stress has been established as a cause of subfertility in animals, having detrimental effects on the formation and function of gametes, and embryonic and fetal development (reviewed by Ref. [1]). Elevations in temperature associated with global climate change are concerning for animal agriculture, particularly in countries with a moderate climate, and especially when it relates to bovine fertility given the economic importance of cattle in modern farming systems. The Netherlands has a mild maritime climate, i.e., summers are typically highly humid and winters are typically relatively mild. In view of climate change, the dairy industry in the Netherlands is implementing preventive measures, such as

* Corresponding author. Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium.
** Corresponding author.
E-mail addresses: nuria.llamasluceno@ugent.be (N. Llamas Luceño), ann.vansoom@ugent.be (A. Van Soom).

https://doi.org/10.1016/j.theriogenology.2019.08.034

ventilation systems, to minimize the effect of heat stress on cow fertility, based on knowledge obtained in countries with extreme temperatures, such as Australia. Australia has a significant portion of its cattle production under an arid climate, which has a great impact on cattle fertility [2]. This impact has been mainly studied in cows, as female physiology is crucial for correct oocyte production, fertilization, embryo development and to produce healthy offspring (reviewed by Ref. [3]). Moreover, the oocyte plays a decisive role in embryo development. This imbalance leads the focus of research towards the female, thus neglecting the male. The effect of temperature on cows has been extensively studied, resulting in a metric based in the temperature-humidity index (THI) [4–6]. This metric correlates temperature with physiological responses and is presently used as a measure for temperature-wellbeing of cows [7,8]. However, the physiological impact of THI on the bull has not been established to the same extent, and the molecular mechanisms of heat stress-induced sperm damage have not been entirely characterized [9]. In humans, genital heat stress is a substantial risk factor for male infertility [10–14]. At present, it is known that bulls exposed to natural heat stress experience a decrease in sperm quality, even in regions with temperate climates [15]. Natural heat stress has been found to induce variations in lipid composition and reduce normal morphology in fresh bovine semen [16], and reduce very-low-density lipoprotein receptor (VLDLr) mRNA expression, sperm motility, progressive motility and velocity, and to alter both the concentration and composition of fatty acids in frozen bovine sperm [17,18]. In a recent study performed on dairy bulls in Northern Spain [19], higher sperm quality, in terms of kinetics, plasma membrane integrity, acrosome status, mitochondrial membrane potential, and reactive oxygen species (ROS) production, was observed in semen samples collected in spring compared to other seasons.

In a previous study from our group [20], artificial scrotal insulation was performed to detect at which period sperm is mostly affected by heat. This insulation mimicked abnormal high temperatures for the scrotum, which had dramatic consequences on the sperm quality. Significantly higher abnormal morphology, chromatin protamination, and nuclear shape and a decrease in motility and viability of sperm were detected in semen ejaculated between 14 and 42 days after scrotal insulation. Spermatogenesis requires approximately 61 days in total to be complete in the bull: 21 days of spermatocytogenesis, 23 days of meiosis, and 17 days of spermiogenesis. Therefore, increased testicular temperature affected mostly those sperm cells that were at meiotic and the beginning of spermiogenic stages of development at the time of scrotal insulation.

In summer, ROS production significantly increases in sperm, leading to lipid peroxidation (LPO) and major sperm defects [21]. During normal cell metabolism, ROS are produced by mitochondrial respiration [22,23]. Sperm cells naturally produce ROS either during their stay in the male and female reproductive tract [24] or by being handled during assisted reproductive technologies (ART), such as semen cryopreservation or sperm centrifugation [25,26]. Unsaturated fatty acids present in the sperm plasma membrane are vulnerable to ROS, initiating a lipid peroxidation cascade and restricting the fluidity needed to participate in the membrane fusion events associated with fertilization [27–30]. Hydrogen peroxide has been identified as one of the major ROS involved in LPO, impairment of protein function, apoptosis, and DNA damage [31,32]. Reactive oxygen species are not only responsible for DNA fragmentation, but may also cause lower sperm DNA methylation [33], implying that DNA fragmentation leads to global DNA demethylation. Epigenetic modifications, such as DNA methylation, are involved in either enhancing or inhibiting gene expression by opening or condensing chromatin configuration respectively (reviewed by Ref. [34]). Oocytes have a limited degree of DNA repair mechanisms that can restore sperm DNA from fragmentation and give rise to a competent embryo [35], depending on their size [36,37]. However, severe sperm DNA fragmentation induces early embryo apoptosis and, consequently, halt further embryo development [31,36]. Genetic and epigenetic alterations produced by ROS in sperm may compromise the correct transcription of the necessary genes for embryo development [38].

In this study, we aimed to identify the effect of high THI on bull fertility and how such effect compromises embryo development. For this purpose, we combined the assessment of selected sperm quality parameters to detect possible alterations by elevated temperatures and subsequent embryo quality parameters to determine the effect on early embryo development. In contrast to previous studies, we have compared the quality of bovine sperm during low and high THI periods and related it to the quality of the subsequent embryos, by assessing quality parameters: embryo development rates, apoptotic ratio, ICM/TE ratio, and total embryo cell number. Moreover, the expression of pluripotency, DNA methyltransferases (DNMT), imprinted, and interferon tau genes was evaluated because of their importance in early embryo development and maternal recognition of pregnancy [39–42]. In addition, heat shock protein and apoptotic genes were included due to their involvement in the response to thermal shock [43–45].

2. Materials and methods

2.1. Experimental design

Frozen bovine semen samples were obtained from six Holstein-Friesian dairy bulls, one to eight years old, housed in two collection barns in the Netherlands (CRV, Arnhem, the Netherlands). Ejaculates were collected from the same six bulls with an artificial vagina between the 8th and the 22nd of March 2016 (low THI group) and between the 1st and the 30th of August 2016 (high THI group). Semen from one ejaculate per bull and period was used for these experiments. The bulls were housed indoors; however, no cooling facilities were present; therefore, temperatures were similar inside and outside of the stables. Only fresh semen samples with normal morphology (≥70% normal) were cryopreserved according to the company's routine practice, using an animal protein-free extender (OPTIXcell; IMV Technologies, L'Aigle, France). Plastic straws of 0.25 ml were filled with the extended semen samples and cooled to 5 °C in a cooling cabinet for 2–4 h prior to cryopreservation. A programmable freezing machine, DigitCool (IMV Technologies), was used to cryopreserve the semen samples, for subsequent storage in liquid nitrogen. Only frozen/thawed straws were retained with motility ≥30% and progressive motility ≥20%.

Air temperature and relative humidity are factors that determine the level of environmental heat to which that animals are exposed. The THI is a metric that combines those factors and has been used for heat stress measurement [8]. Temperature and relative humidity data were obtained from The Royal Netherlands Meteorological Institute (KNMI) [46]. Consequently, the THI was calculated per hour considering the meiotic and spermiogenic stages of spermatogenesis, i.e., from 14 to 42 days before the ejaculates were collected.

The following equation was used to calculate the THI, where T is the temperature in degrees Celsius and RH is relative humidity:

$$THI = (0.8 \times T\,(^{\circ}C) + (RH\,(\%) / 100) \times (T\,(^{\circ}C) - 14.4) + 46.4)$$

The maximum THI was selected as the highest daily value, whereas the mean THI was calculated as the daily average of the hourly THI values. In the low THI group, the maximum THI

oscillated between 38 and 55, whereas in the high THI group, the maximum THI oscillated between 60 and 81.

We evaluated sperm motility, reactive oxygen species generated by sperm, sperm lipid peroxidation, sperm viability and sperm DNA fragmentation on Percoll®-purified frozen-thawed semen; we chose to only focus on such sperm so as to be representative of live sperm cells that were capable of fertilization. Moreover, we evaluated the development of embryos generated *in vitro* by low and high THI semen after Percoll®-purification and assessed inner cell mass (ICM)/trophectoderm (TE) ratio, apoptotic cell ratio, and embryonic gene expression in day-8 blastocysts.

2.2. Reagents and media

Unless otherwise stated, all media and reagents were purchased from Thermo Fisher Scientific (Merelbeke, Belgium).

Culture and handling media were prepared as described by Ref. [47]. All media were filtered through a sterile 0.22 μm filter (Millipore Corporation, USA) prior to use. Unless otherwise stated, all other components were obtained from Sigma (Diegem, Belgium).

2.3. Sperm motility

In order to assess the effect of heat stress on viable sperm, frozen/thawed sperm were passed through a discontinuous Percoll® gradient (45:90% (v:v); GE Healthcare Biosciences, Uppsala, Sweden) and washed in sperm Tyrode's albumin lactate pyruvate (TALP) for semen quality analysis. Sperm motility was evaluated using computer-assisted sperm analysis (CASA). A semen sample of 10 μl was placed on a clean warm (37 °C) disposable counting chamber (ISAS® D4C20, Proiser, Paterna, Spain). The sperm motility was assessed using a 10x negative phase contrast microscope objective connected to an ISAS® v1 CASA system (Proiser, Paterna, Spain). The software settings specific for bulls were used for sperm motility analysis. The parameter settings were: number of frames captured per second = 25; minimum surface that an object is recognized as a sperm = 5 μm; low VAP cut-off = 10 μm/s; medium VAP cut-off = 25 μm/s; low VSL cut-off = 10 μm/s; medium VSL cut-off = 25 μm/s; STR cut-off = 70%. Static sperm, total and progressive motility data were collected.

2.4. Reactive oxygen species, lipid peroxidation and sperm viability

Flow cytometry and fluorescent staining techniques were used to determine ROS, LPO and sperm viability. Frozen-thawed sperm were passed through a discontinuous Percoll® gradient (45:90% (v:v)) and adjusted to a final concentration of 2.5×10^6 cells/ml in PBS without calcium and magnesium. For ROS evaluation, two assays were performed to measure H_2O_2 and total ROS present in sperm. For this purpose, 100 μM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) or 5 μM CellROX Green Reagent was added to 200 μl of the sperm suspensions (0.5×10^6 cells) and incubated at 37 °C for 30 min in the dark. The sperm suspension was washed twice in PBS at 270 g for 5 min, and 1.5 μM of PI was added to assess sperm viability. All samples were analyzed using a Cytoflex 3 laser flow cytometer. For LPO assessment, 10 μM of BODIPY 581/591 C_{11} (further referred as BODIPY) was added to the Percoll® purified sperm samples and incubated at 37 °C for 15 min in the dark before flow cytometry analysis. Three replicates were performed. Overall 10,000 sperm were screened per sample and replicate.

Cell fluorescence was exited at 488 nm. The fluorescence intensity of the PE (PI and BODIPY) and FITC (CellROX, DCFH-DA and BODIPY) channels was measured to analyze the percentage of cells positive for cell death (PI), ROS production (CellROX and DCFH-DA) and LPO (BODIPY) in low and high THI semen samples. The emission of the PE channel was 585/42 nm and FITC channel was 525/40 nm. The acquisition velocity was 30–60 μl/min. All data were corrected for autofluorescence. The single sperm population was gated on FSC-H vs. FSC-A dotplot to exclude aggregates, and on FS/SS dotplot to exclude debris. All acquired data were processed using CytExpert v2.0.0.153 software (Beckman Coulter, Inc., California, USA). The percentage of DCFH-DA$^+$ and CellROX$^+$ cells was calculated from the viable (PI$^-$) sperm population.

2.5. Sperm DNA fragmentation TUNEL assay

Terminal deoxynucleotidyl transferase fluorescein-dUTP nick-end labeling (TUNEL) assay was used to detect the presence of free 3′-OH termini in single and double-stranded sperm DNA, using the *In Situ* Cell Death Detection Kit (Roche, Brussels, Belgium) following the methods described by Rahman et *al.* [37] with a single modification. Frozen/thawed sperm were passed through a discontinuous Percoll® gradient (45:90% (v:v)) and adjusted to a final concentration of 5×10^6 cells/ml in sperm TALP. Sperm were incubated in 2 mM DTT in Sp-TALP for 45 min at room temperature (RT) as described by Mitchell et al. [48], which breaks the disulphide bridges between protamines and DNA, and allows the enzyme terminal deoxynucleotidyl transferase to access the chromatin structure. Sperm cells were centrifuged and washed in Sp-TALP. After this step, in contrast to the previously mentioned protocol, sperm samples were directly fixed with 4% paraformaldehyde (PFA). After fixation and washing in PBS, the samples were permeabilized and incubated with TUNEL mixture (terminal deoxynucleotidyl transferase and fluorescein-dUTP 1:10). The samples were stained with 5 μg/ml of Hoechst 33,342, smeared onto a poly-L-lysine-coated slide and examined by fluorescence microscopy (Leica, DMR; magnification 400x, oil immersion). At least 200 sperm from each sample were analyzed randomly per replicate to evaluate the percentage of TUNEL-positive cells. Both positive (1000 U/ml DNAse I) and negative (nucleotide mixture in the absence of transferase) controls were included in each replicate. Three replicates were performed.

2.6. Embryo production

Bovine blastocysts were produced *in vitro* as described by Wydooghe et al. [47]. *In vitro*-produced bovine blastocysts were derived from immature oocytes that were collected from slaughterhouse ovaries obtained from October to March. Cumulus-oocytes complexes (COCs) were recovered from follicles with a diameter of 2–8 mm using an 18-gauge needle attached to a 10 ml syringe. Oocytes with homogeneous dark cytoplasm and compact cumulus cells were selected and matured in groups of 60 in 500 μl of bicarbonate-buffered TCM199 medium (Life Technologies, Ghent, Belgium) supplemented with 50 mg/ml gentamycin and 20 ng/ml of epidermal growth factor. Oocytes were matured for 22 h at 38.5 °C in 5% CO_2 in the air. Frozen-thawed low and high THI sperm from six bulls were passed through a discontinuous Percoll® gradient (45:90% (v:v)) and adjusted to a final concentration of 1×10^6 cells/ml in IVF TALP medium for fertilization. An extra group of oocytes fertilized with a control bull of known fertility was always added in every replicate. The matured COCs were washed in 500 ml IVF-TALP and co-incubated with sperm for 21 h. After fertilization, presumptive zygotes were first vortexed to remove the excess of the sperm and cumulus cells, and then cultured in groups of 25 in 50 μl droplets of SOF medium at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. Six replicates were performed. Cleavage rates were determined after 48 h post-insemination and blastocyst rates at 168 and 192 h post insemination (day 7 and 8 respectively) as the

percentage of initial number of COCs. We grouped early and non-expanded blastocysts together as 'early blastocysts', and expanded, hatching and hatched blastocysts as 'advanced blastocysts'.

2.7. Differential blastocyst staining

In vitro-produced day-8 blastocysts were fixed in 4% PFA for 20 min at RT and stored at 4 °C until use. Differential staining was performed as described by Wydooghe et al. [49]. Blastocysts were permeabilized with 0.5% Triton X-100 + 0.05% Tween for 1 h at RT and DNA of the blastomeres was denatured by exposure to 2 N HCl for 20 min followed by 100 mM Tris-HCl (pH 8.5) for 10 min at RT. Blocking solution consisting of 10% goat serum and 0.5% BSA prepared in PBS was added and the embryos were incubated at 4 °C overnight. The embryos were washed and incubated in the ready-to-use primary CDX2 antibody (Biogenex, San Ramon, USA) at 4 °C overnight. The test embryos were washed and incubated overnight at 4 °C in rabbit active caspase-3 antibody (0.768 μg/ml in blocking solution, Cell Signaling Technology, Leiden, the Netherlands). After another wash step, test embryos and negative controls (not exposed to CDX2 and Caspase-3 antibodies) were transferred to goat anti-mouse Texas Red secondary antibody (20 μg/ml in blocking solution, Molecular Probes, Merelbeke, Belgium) for 1 h at RT and to goat anti-rabbit FITC antibody (10 μg/ml in blocking solution, Molecular Probes) for 1 h at RT. Next, blastocysts were rinsed three times in PBS, counterstained with Hoechst 33,342 (50 μg/ml in PBS/BSA, Molecular Probes) for 20 min at RT, and mounted with DABCO mounting medium. 3D images were reconstructed after acquiring Z-stack images using a 63× water immersion objective on a Leica TCS-SP8× confocal microscope (Leica Microsystems, Weltzar, Germany).

2.8. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Blastocysts were produced as described above, after fertilization with low and high THI semen from six experimental bulls. All the embryos reaching blastocyst stage were washed three times in 3 ml of RNase-free PBS, transferred in groups of maximum five blastocysts to 1 μl/blastocyst of lysis buffer consisting of 5 mM Dithiothreitol (DTT; Promega, Leiden, the Netherlands), 4 U/μL RNasinPlus RNase inhibitor (Promega), and 0.64 μM Igepal in RNase-free water, and immediately stored at −80 °C until use for a maximum of two months. The total RNA was extracted from pools of day-8 blastocysts from the same replicate for gene expression analysis using the RNeasy Micro kit (Qiagen, Antwerp, Belgium) following the manufacturer's instructions, eluting the extracted RNA in 15 μL RNase-free water passed through the column twice. Minus-reverse transcriptase control was performed to detect DNA contamination as described by Goossens et al. [50]. A PCR reaction using DNA polymerase in 1 μl of RNA sample was performed. Ubiquitin-C primers -a highly conserved and ubiquitously expressed gene-were used for analysis [51]. Due to the limited amount of samples, concentration and integrity analysis were not assessed. After confirming no presence of genomic DNA in the samples, reverse transcription was performed with the 14 μl left from the RNA extraction procedure. iScriptcDNA synthesis kit (Bio-Rad, Temse, Belgium) was used according to the manufacturer's instructions, and cDNA was diluted 10-fold for downstream PCR.

We selected 25 genes for their importance in early embryo development (pluripotency, DNA methyltransferases and imprinted genes), maternal recognition (interferon tau gene), and stress response (heat shock protein and apoptotic genes). Pluripotency genes are involved in establishing gene regulatory networks and early embryonic dynamics necessary for embryonic development (reviewed by Ref. [39]). DNA methyltransferases (DNMTs) establish and maintain DNA methylation, a key function especially during early embryogenesis epigenetic reprogramming [40]. Imprinted genes are differentially methylated genes according to their parental origin, which cause severe fetal growth abnormalities when they are deregulated (reviewed by Ref. [41]). Establishment of early pregnancy depends on interferon tau (IFN-τ), the signal of the embryo to the mother to prevent luteolysis and maintain the production of progesterone during pregnancy [42]. Heat shock proteins are a family of highly conserved proteins produced for protection against environmental, physical or chemical stress (reviewed by Refs. [43,44]), while programmed cell death or apoptosis is an essential pathway for the correct functioning of an organism [45].

The primers for these genes were designed using Primer-BLAST tools from NCBI based on bovine mRNA sequences, avoiding secondary structures as indicated by MFold [52]. All the amplicons were validated by sequencing and aligned using Bioedit and BLAST tools from NCBI. All PCR reactions were performed in a volume of 10 μl using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), according to the manufacturer's instructions, using 2 μL of 10 X diluted cDNA template and 5 μM of forward and reverse primers. A CFX Connect system (BioRad) was used and the PCR program consisted of initial denaturation at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at a range between 59 °C and 65 °C according to the annealing temperature of the primers, and plate reading step. A final 5 s elongation at 72 °C was performed, followed by a melting curve from 72 °C up to 95 °C, with a temperature increase of 0.5 °C and a plate reading step every 5 s. Two replicates were performed for each gene. Negative (water) and positive (testis cDNA) controls were included in each replicate. The stability of all the reference genes described by Goossens et al. [50] was tested and analyzed for normalization using the GeNorm program. Then, the three most stable genes were selected (GAPDH, SDHA and ACTB).

2.9. Statistical analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) v25. The data were analyzed using Paired-samples *t*-test, Wilcoxon Signed Rank Test and GLM procedure ($p \leq 0.05$). The dataset was checked for normal distribution by Shapiro-Wilk test and Kolmogorov-Smirnov ($p \leq 0.05$). All data are expressed as mean ± SEM. qbasePlus v1.2 software (Biogazelle, Zwijnaarde, Belgium) was used for gene expression analysis. GeNorm was used for the reference genes selection and Wilcoxon Signed Rank Test ($p \leq 0.05$) was performed to analyze the qPCR results.

3. Results

For the low THI semen group, the maximum THI observed during the period of meiosis and spermiogenesis was 51.8 and 55 at the two different locations, whereas the mean THI was 40 and 40.6. For the high THI semen group, the observed maximum THI was 77.9 and 80.5, while the mean THI was 61.6 and 64.3, at the respective locations. The maximum and mean THI for the meiosis and spermiogenesis period of the assessed samples is represented in Fig. 1.

Data from sperm analysis are summarized in Table 1. Low and high THI sperm groups did not differ significantly in the percentage of normal sperm morphology and sperm abnormalities (data not shown). Motility in Percoll®-purified semen was not affected by heat stress although the oscillation index (WOB) was significantly lower in high THI semen (47.5%) compared to low THI (50.7%).

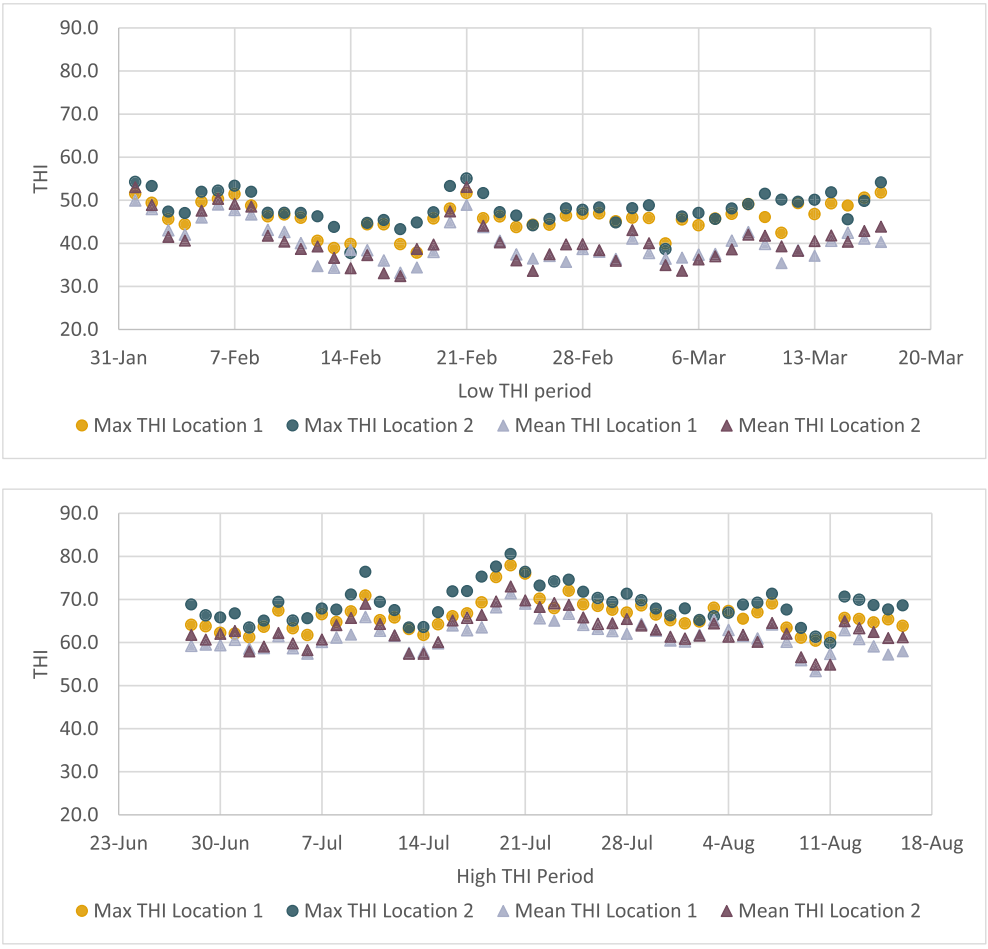

Fig. 1. Daily maximum and mean THI in both locations of the collection barns in the Netherlands, for the meiosis and spermiogenesis period of the low and high THI semen samples. Low THI period from the 1st of February 2016 to the 17th of March of 2016. High THI period from the 28th of June 2016 to the 16th of August of 2016. Data provided by The Royal Netherlands Meteorological Institute (KNMI).

A greater percentage of dead cells (PI^+ cells; $P = 0.039$) was observed in the high THI group (31.5%) compared to the low THI group (27.6%). Although no significant differences ($P > 0.05$) in the two groups were found for ROS production ($DCFH\text{-}DA^+$ and $CellROX^+$ cells), LPO ($BODIPY^+$ cells) and DNA fragmentation ($TUNEL^+$ cells), a tendency towards lower sperm motility ($P = 0.089$), higher LIN ($P = 0.078$) and higher total ROS production ($P = 0.087$) was observed in high THI semen compared to low THI semen.

No differences were observed in the cleavage rates of low (78.3%) and high (74.8%) THI groups. Remarkably, a significant decrease ($P < 0.001$) was observed in the total blastocyst rates at day 7 post-insemination in the high THI group (15.3%) compared to the low THI group (21%), as well as in the total blastocyst rates at day 8 post-insemination in the high THI group (23.2%) compared to the low THI group (29.6%) (Fig. 2). When comparing the developmental stage of the blastocysts, we observed a significant reduction of early and advanced stage blastocysts at day 7 and advanced stage blastocysts at day 8 of the high THI group (Fig. 2). Moreover, hatching was delayed in the high THI group since only the low THI group showed hatched blastocysts at day 7 post insemination.

The higher THI did not equally affect all bulls, as individual variability had a great impact on the blastocyst rates ($P < 0.05$) (Table 2). The bulls were not selected for their fertilizing capacity; therefore, 3 bulls had good *in vitro* fertility (40% or more blastocyst rates) and 3 had poor *in vitro* fertility (less than 25% blastocyst rates). However, all bulls consistently showed decreased blastocyst rates in the high THI group.

No differences ($P > 0.05$) were observed in the total number of cells, ICM cell number and rate (%), and apoptotic cell number and rate (%) in blastocysts generated by low and high THI sperm (Figs. 3 and 4). However, the percentage of ICM and the ICM/TE ratio in the high THI group (34.1% and 0.565, respectively) tended to be lower ($P = 0.090$ and $P = 0.087$, respectively) than in the low THI group (36.0% and 0.603, respectively).

Table 1
Sperm analysis from Percoll®-purified frozen-thawed low and high THI semen groups; including sperm motility (CASA), DNA fragmentation (TUNEL), H_2O_2 production (DCFH-DA), total ROS production (CellROX), LPO (BODIPY) and cell death (PI).

Semen characteristics	Low THI - March	High THI - August	P-value
Total motility (%)	68.9 ± 2.0	68.0 ± 1.4	0.446
Progressive motility (%)	57.4 ± 1.7	55.3 ± 1.4	0.199
$TUNEL^+$ (%)	3.43 ± 0.72	3.17 ± 0.75	0.713
$DCFH\text{-}DA^+$ (%)	15.8 ± 2.75	14.2 ± 3.39	0.474
$CellROX^+$ (%)	70.2 ± 4.67	74.6 ± 3.61	0.087
$BODIPY^+$ (%)	0.25 ± 0.06	0.25 ± 0.08	0.999
PI^+ (%)	27.6 ± 2.76^b	31.5 ± 3.09^a	0.039

Values with different letters in superscripts in the same row express differences which were significant ($P < 0.05$). Data are expressed as mean ± SEM.

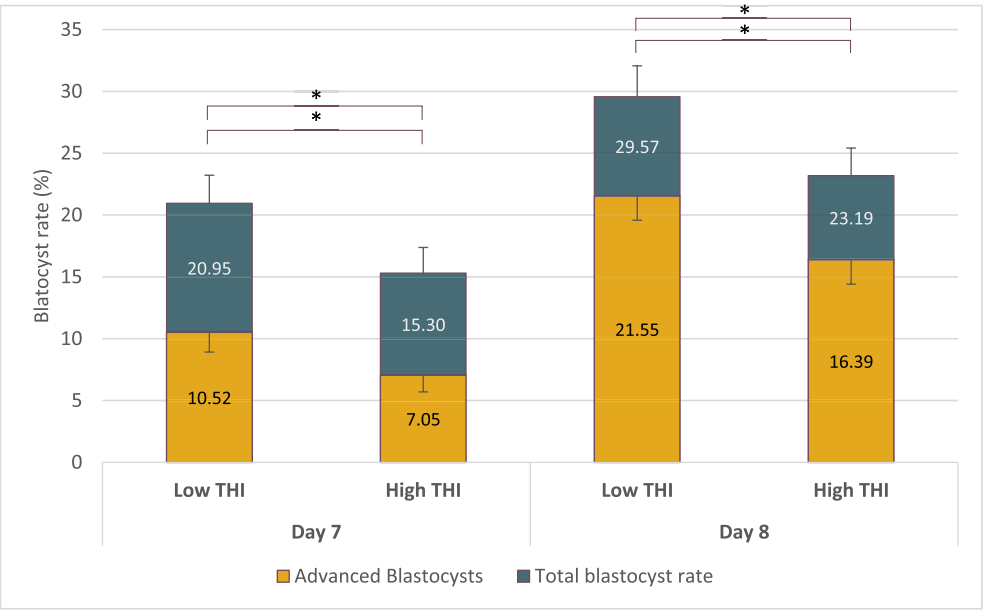

Fig. 2. Total blastocyst development rates of day 7 and 8 after fertilization and percentage of 'advanced blastocysts' of embryos derived from low and high THI semen groups. Significant differences ($P < 0.05$), marked with an asterisk, were found between the two experimental groups for total blastocyst development rates of day 7 and 8, and percentage of 'advanced blastocysts'.

Table 2
Total blastocyst development rates at day 7 and 8 after fertilization of embryos derived from low and high THI semen by individual bulls.

Bull	Number of oocytes		Day 7 blastocyst rate		Day 8 blastocyst rate	
	low THI	high THI	low THI	high THI	low THI	high THI
1	295	306	32.4 ± 6.8	21.9 ± 3.0	44.7 ± 4.5^{a}	27.8 ± 4.2^{b}
2	301	295	26.4 ± 3.3	22.6 ± 5.4	38 ± 1.9	35.9 ± 4.0
3	302	298	16.2 ± 3.5	10.7 ± 1.6	22.4 ± 4.1	16.4 ± 1.6
4	300	294	6 ± 1.3^{a}	0.3 ± 0.3^{b}	10 ± 2.0^{a}	6.1 ± 1.7^{b}
5	305	304	30.8 ± 4.3	27.6 ± 5.2	40.7 ± 2.1	35.4 ± 4.5
6	300	302	14 ± 4.0	8.6 ± 2.4	21.6 ± 5.0	17.6 ± 3.0
AVERAGE	**300.5**	**299.8**	**21 ± 2.3^{a}**	**15.3 ± 2.1^{b}**	**29.6 ± 2.5^{a}**	**23.2 ± 2.2^{b}**

p-value of the influence of individual bull on day 7 blastocysts: 1.545×10^{-06}.
p-value of the influence of individual bull on day 8 blastocysts: 6.816×10^{-11}.
Values with different letters in superscripts in the same row express differences which were significant ($P < 0.05$). Data are expressed as mean ± SEM. Six replicates were performed for each bull. Significant effect of individual bulls on blastocyst development rates of day 7 and 8 ($P < 0.05$).

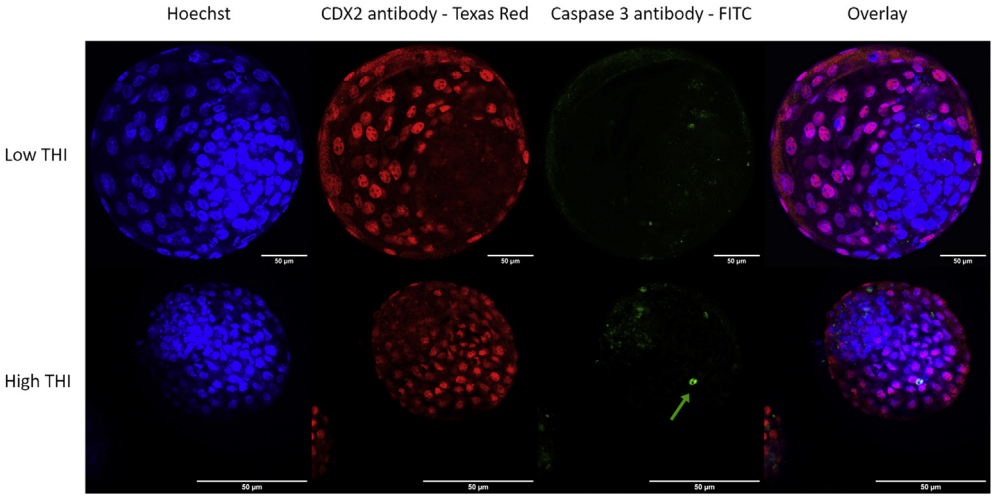

Fig. 3. Differential apoptotic staining of representative bovine blastocysts derived from low and high temperature humidity index (THI) semen groups: both trophectoderm (TE) and inner cell mass nuclei were stained with Hoechst. The CDX2 antibody was indirectly labeled with Texas Red resulting in a red fluorescent signal in TE cells only. The apoptotic cells show a green fluorescent signal because the anti-caspase-3 antibody was indirectly labeled with FITC. An overlay of the three stainings is given.

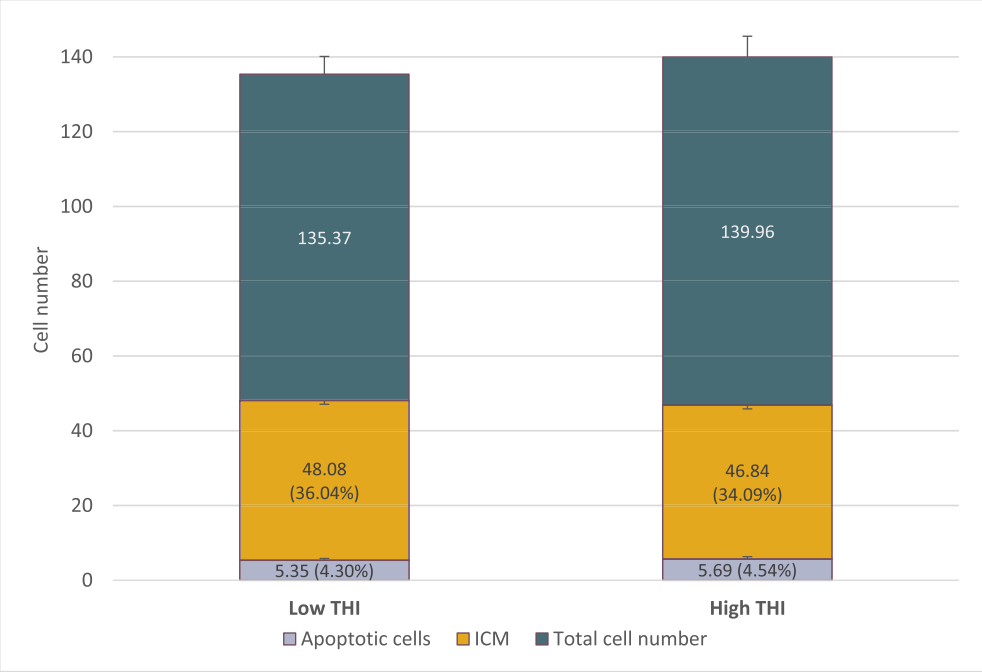

Fig. 4. Total cell number, ICM cell number and rate (%), and apoptotic cell number and rate (%) of differentially stained day-8 blastocysts derived from low and high THI semen groups. No differences ($P > 0.05$) were found between the two experimental groups.

No significant differences were observed in the gene expression of blastocysts at day 8 for any of the assessed target genes (Table 3).

4. Discussion

The main objective of this study was to investigate the existence and degree of damage in sperm cells produced by bulls exposed to natural heat stress conditions from 14 to 42 days before semen collection. Artificial insemination centers select ejaculated semen for cryopreservation according to morphology, kinetics and number of cells in an ejaculate (volume and concentration). In the present study, we investigated the impact of increased THI on sperm and subsequent embryo quality of Holstein bulls housed in the Netherlands and provided evidence that semen classified as good quality and collected some weeks after higher THI had, in fact, inferior quality and fertility which was only apparent after *in vitro* embryo production. In contrast to other studies, we looked at Percoll®-separated semen, as we aim to investigate possible damages that might exist in living sperm that can contribute to fertilization. By separating the living sperm with Percoll®, we looked at the influence of possible sperm damage on resulting embryo development and quality.

In order to assess the damage caused by climate factors on viable sperm, we examined sperm motility, ROS production, LPO, cell membrane damage, and DNA fragmentation after a Percoll® gradient, which selects the motile sperm that could potentially traverse the female reproductive tract and reach the site of fertilization under physiological conditions. By doing that, we expected that major sperm defects would be removed. In agreement with the findings of Argov et al. [17], a significant reduction in sperm viability, assessed by PI staining and likely caused by climate factors, was observed in sperm collected in August at higher THI compared to March at lower THI. We speculate that this reduction, assuming a similar efficiency of Percoll® in all the samples, is due to an increased sensitivity of the viable sperm cells selected with Percoll® in the high THI group compared to the low THI group. Although no significant differences were observed in any other

Table 3
Gene expression of day-8 blastocysts derived from low and high THI semen groups. A total of 25 genes were analyzed. The ratio is based on the comparison high/low THI. Four pairs were analyzed for IGF2 gene due to low expression. Six pairs were analyzed for the rest of the genes.

Target gene	Ratio	P-value	95% CI low	95% CI high
Pluripotency genes				
NANOG	1.102	0.75	0.899	1.352
POU5F1	1.104	0.825	0.921	1.323
SOX2	1.553	0.625	0.755	3.194
DNMT genes				
DNMT1	1.213	0.825	0.477	3.087
DNMT3A	0.953	0.825	0.744	1.221
DNMT3B	0.977	0.825	0.903	1.058
Imprinted genes				
H19	4.146	0.375	1.489	11.542
IGF2	2.426	0.625	0.947	6.219
IGF2R	1.034	1	0.477	2.239
MEG3	1.314	0.825	0.592	2.917
MEG9	1.263	0.825	0.47	3.397
MEST	1.591	0.825	0.406	6.239
PEG10	1.224	1	0.476	3.143
PHLDA2	0.869	0.825	0.434	1.743
PLAGL	1.138	0.964	0.555	2.335
SNRPN	1.035	0.825	0.763	1.405
Interferon TAU gene				
IFNT2	1.608	0.625	0.72	3.589
Heat shock protein genes				
HSF1	0.611	0.5	0.379	0.984
HSPA1A	1.272	0.825	0.557	2.908
HSPA2	2.536	0.375	1.698	3.786
HSPA8	0.931	1	0.554	1.563
HSP10	0.874	0.825	0.528	1.447
HSP60	1.301	0.825	0.797	2.124
HSP90	1.145	0.825	0.648	2.023
Apoptotic gene				
BAX	1.536	0.438	0.485	4.863

High/low THI gene expression ratio, P-value, and low and high confidence intervals are described.

sperm quality parameters (in terms of morphology, kinetics, ROS production, LPO and DNA fragmentation), tendencies towards higher total ROS production ($P = 0.087$) were observed in high THI sperm (74.61%) compared to low THI sperm (70.19%, respectively). In response to heat stress, ROS can be involved in two different pathways: oxidation of important molecules such as membrane lipids and activation of death pathway though the caspase cascade [53].

Sperm cells are not transcriptionally active and, therefore, cannot repair DNA damage [54]. Functional sperm with damaged DNA have the ability to fertilize the oocyte without a significant effect on the first cleavage, only affecting the embryo after the embryonic genome activation at 8 to 16 cell stage, when the damage in paternal DNA can be either repaired or can activate the apoptotic machinery of the early embryo, thus stopping further development [36,55–57]. According to Karoui et al. [58], sperm DNA fragmentation values of 7–10% might indicate low AI success. In our study, minor LPO and DNA fragmentation were present, indicating no severe damage of Percoll®-purified sperm cells by ROS, which might not affect the membrane fusion events associated with fertilization. However, in this study, an inferior blastocyst rate was observed and the growth of those embryos was delayed, as no hatched blastocysts were observed 7 days post-insemination in the high THI group, indicating that molecular mechanisms for advanced blastocyst development were affected.

When bovine sperm heat stress is induced *in vitro*, plasma membrane integrity and mitochondrial membrane potential were significantly reduced [9]. Moreover, incubation of sperm at physiological temperature (38.5 °C) did not impact fertilization and cleavage rates, but embryo development was compromised. In contrast, incubation at a hyperthermic temperature (41 °C) seriously compromised fertilization, cleavage rates and embryo development. The mitogen-activated protein kinase (MAPK14) pathway was activated, which generally misfolds and aggregates proteins which leads to cell apoptosis. Moreover, this MAPK14 pathway has been observed in heat-induced testicular germ cell apoptosis in rats [59]. Similarly, in the present study, we observed that warmer climate conditions significantly affect sperm plasma membrane, causing elevated sperm mortality; however, the role of MAPK14 in naturally heat-stressed sperm has to be determined.

Our study shows for the first time that exposure of bulls to a period of elevated THI has a negative impact on blastocyst development after *in vitro* fertilization. The paternal contribution to the zygote consists of the safe delivery of the paternal genome, epigenetic marks, RNA and centriole into the oocyte for correct embryo development [60–63]. We did not observe substantial damage in the paternal DNA, as no high incidence of DNA breaks were observed in our samples. Therefore, an aberrant DNA methylation or damage of the RNA or centriole could lead to the subsequent embryo development delay or failure. As previously described, global DNA demethylation of the paternal pronucleous can be blocked in heat-stressed sperm when IVF is performed [64].

Although the quality of the embryos that reached the blastocyst stage was similar in both groups, as no significant differences were observed in the embryo quality parameters assessed with the differential staining, tendencies to have smaller ICM% and ICM/TE ratio ($P = 0.090$ and 0.087, respectively) were observed in high THI semen compared to low THI semen. However, apoptosis rate, the total number of cells and gene transcription were similar in the two studied groups; therefore, we infer that the damage produced in the sperm cells by a higher THI was not severe and repaired by the oocyte machinery.

Moreover, we investigated the impact of sperm heat stress on the expression of several important genes for early embryo development. The selected target genes did not differ in expression between the low and high THI embryo groups, leading us to conclude that those gene pathways were not involved in the observed delay of embryo development in the high THI group. Environmental factors modulate the cell behavior thought epigenetics [65]; therefore, we speculate that epigenetic modifications could be contributing to this delay. DNA methylation and histone modification are epigenetic mechanisms involved in DNA transcription [66,67]; therefore, they regulate the quantity of RNAs present in the cell. However, miRNAs are another epigenetic mechanism, involved in mRNA translation, which could be blocking the presence of proteins in the cell [68]. Investigating the gene expression with Next Generation Sequencing techniques can give us an overview of the RNAs present in the embryos, and possibly help understand the molecular mechanisms involved in the heat-stress-induced delay of embryo development [69].

In agreement with Roth [3], we presume that the effect of heat stress might be less drastic in the male compared to the female. However, a decrease in male fertility caused by heat stress when using sperm for *in vitro* techniques could generate an important economic loss. Companies that produce embryos for farmers, who want to obtain embryos from their own top animals, older cows that are difficult to get pregnant, or purchase foreign genetics to transfer into recipients, are susceptible to a decrease in embryo development rates caused by male heat stress.

5. Conclusions

In conclusion, dairy bull sperm viability and *in vitro* fertility are affected by climate conditions in the Netherlands. Sperm samples obtained at higher THI have reduced *in vitro* fertility compared to the samples collected at a low THI. Although we did not observe severe damage of Percoll®-purified sperm, the decrease in blastocyst rates and the delayed hatching observed in embryos produced with higher THI semen indicates that molecular mechanisms for advanced blastocyst development were affected. However, those mechanisms did not involve our target genes. Further studies are required for better understanding the mechanisms compromising the quality of sperm samples obtained at seasons with high THI and of the subsequent blastocyst development.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

NLL performed the laboratory work for all the experiment and wrote the first draft of the manuscript. DSRA and LCB assisted with the sperm analysis. KD and EvM assisted with the flow cytometer. KJS performed the confocal microscope analysis. MVP and LP assisted with the genetic design and analysis. ErM and MB provided the samples for the experiments. NLL, ErM, MB and AVS designed the experiment. AVS provided the funding and helped with the drafting of the manuscript. All authors read the final draft and agreed to its submission for publication.

Acknowledgments

The authors wish to thank Petra Van Damme for her excellent technical assistance, Linda Impe, Dominique Vander Donckt and Xiaoyuan Lin for their support in the genetic analysis, Mar Batlle Pérez for her contribution in primer validation and Prof. Patrick Lonergan, who carefully read the manuscript and provided us with

constructive comments. This project is funded by “European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2019.08.034.

References

[1] Hansen PJ. Effects of heat stress on mammalian reproduction. Philos Trans R Soc Lond B Biol Sci 2009;364:3341–50. https://doi.org/10.1098/rstb.2009.0131.
[2] Dairy Australia. Cool Cows. National services body for the dairy industry. http://coolcows.dairyaustralia.com.au/. [Accessed 19 April 2019].
[3] Roth Z. Effect of heat stress on reproduction in dairy cows: insights into the cellular and molecular responses of the oocyte. Annu Rev Anim Biosci 2017;5:151–70. https://doi.org/10.1146/annurev-animal-022516-022849.
[4] Armstrong DV. Heat stress interaction with shade and cooling. J Dairy Sci 1994;77:2044–50. https://doi.org/10.3168/jds.S0022-0302(94)77149-6.
[5] Mader TL, Davis MS, Brown-Brandl T. Environmental factors influencing heat stress in feedlot cattle. J Anim Sci 2006;84:712–9. https://doi.org/10.2527/2006.843712x.
[6] Dikmen S, Hansen PJ. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? J Dairy Sci 2009;92:109–16. https://doi.org/10.3168/jds.2008-1370.
[7] Polsky L, von Keyserlingk MAG. Invited review: effects of heat stress on dairy cattle welfare. J Dairy Sci 2017;100:8645–57. https://doi.org/10.3168/jds.2017-12651.
[8] García-Ispierto I, López-Gatius F, Bech-Sabat G, Santolaria P, Yániz JL, Nogareda C, et al. Climate factors affecting conception rate of high producing dairy cows in northeastern Spain. Theriogenology 2007;67:1379–85. https://doi.org/10.1016/j.theriogenology.2007.02.009.
[9] Rahman MB, Vandaele L, Rijsselaere T, El-Deen MS, Maes D, Shamsuddin M, et al. Bovine spermatozoa react to in vitro heat stress by activating the mitogen-activated protein kinase 14 signalling pathway. Reprod Fertil Dev 2014;26:245–57. https://doi.org/10.1071/RD12198.
[10] Durairajanayagam D. Lifestyle causes of male infertility. Arab J Urol 2018;16:10–20. https://doi.org/10.1016/j.aju.2017.12.004.
[11] Rao M, Zhao XL, Yang J, Hu SF, Lei H, Xia W, et al. Effect of transient scrotal hyperthermia on sperm parameters, seminal plasma biochemical markers, and oxidative stress in men. Asian J Androl 2015;17:668–75. https://doi.org/10.4103/1008-682X.146967.
[12] Thonneau P, Bujan L, Multigner L, Mieusset R. Occupational heat exposure and male fertility: a review. Hum Reprod 1998;13:2122–5. https://doi.org/10.1093/humrep/13.8.2122.
[13] Sas M, Szöllösi J. Impaired spermiogenesis as a common finding among professional drivers. Arch Androl 1979;3:57–60. https://doi.org/10.3109/01485017908985049.
[14] Jung A, Schuppe HC. Influence of genital heat stress on semen quality in humans. Andrologia 2007;39:203–15. https://doi.org/10.1111/j.1439-0272.2007.00794.x.
[15] Sabés-Alsina M, Lundeheim N, Johannisson A, López-Béjar M, Morrell JM. Relationships between climate and sperm quality in dairy bull semen: a retrospective analysis. J Dairy Sci 2019;102:1–11. https://doi.org/10.3168/jds.2018-15837.
[16] Argov-Argaman N, Mahgrefthe K, Zeron Y, Roth Z. Season-induced variation in lipid composition is associated with semen quality in Holstein bulls. Reproduction 2013;145:479–89. https://doi.org/10.1530/REP-12-0498.
[17] Argov N, Sklan D, Zeron Y, Roth Z. Association between seasonal changes in fatty-acid composition, expression of VLDL receptor and bovine sperm quality. Theriogenology 2007;67:878–85. https://doi.org/10.1016/j.theriogenology.2006.10.018.
[18] Orgal S, Zeron Y, Elior N, Biran D, Friedman E, Druker S, et al. Season-induced changes in bovine sperm motility following a freeze-thaw procedure. J Reprod Develop 2012;58:212–8. https://doi.org/10.1262/jrd.10-149N.
[19] Sabés-Alsina M, Johannisson A, Lundeheim N, Lopez-Bejar M, Morrell JM. Effects of season on bull sperm quality in thawed samples in northern Spain. Vet Rec 2017;180:251. https://doi.org/10.1136/vr.103897.
[20] Rahman MB, Vandaele L, Rijsselaere T, Maes D, Hoogewijs M, Frijters A, et al. Scrotal insulation and its relationship to abnormal morphology, chromatin protamination and nuclear shape of spermatozoa in Holstein-Friesian and Belgian Blue bulls. Theriogenology 2011;76:1246–57. https://doi.org/10.1016/j.theriogenology.2011.05.031.
[21] Nichi M, Bols PEJ, Züge RM, Barnabe VH, Goovaerts IGF, Barnabe RC, Cortada CNM. Seasonal variation in semen quality in Bos indicus and Bos taurus bulls raised under tropical conditions. Theriogenology 2006;66:822–8. https://doi.org/10.1016/j.theriogenology.2006.01.056.
[22] Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab 2008;93:3199–207. https://doi.org/10.1210/jc.2007-2616.
[23] Holland MK, Storey BT. Oxygen metabolism of mammalian spermatozoa. Generation of hydrogen peroxide by rabbit epididymal spermatozoa. Biochem J 1981;198:273–80. https://doi.org/10.1042/bj1980273.
[24] Wathes DC, Abayasekara DRE, Aitken RJ. Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 2007;77:190–201. https://doi.org/10.1095/biolreprod.107.060558.
[25] Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 2001;56:275–86. https://doi.org/10.1016/S0093-691X(01)00562-3.
[26] Guimarães ACG, Leivas FG, Santos FW, Schwengber EB, Giotto AB, Machado ClU, Gonçalves CGM, Folchini NP, Brum DS. Reduction of centrifugation force in discontinuous percoll gradients increases in vitro fertilization rates without reducing bovine sperm recovery. Anim Reprod Sci 2014;146:103–10. https://doi.org/10.1016/j.anireprosci.2014.02.016.
[27] Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 1979;31:531–7. https://doi.org/10.1016/S0015-0282(16)43999-3.
[28] Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 1989;41:183–97. https://doi.org/10.1095/biolreprod41.1.183.
[29] Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. J Reprod Fertil 1993;98:257–65. https://doi.org/10.1530/jrf.0.0980257.
[30] Aitken RJ, Harkiss D, Buckingham DW. Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol Reprod Dev 1993;35:302–15. https://doi.org/10.1002/mrd.1080350313.
[31] Silva PFN, Gadella BM, Colenbrander B, Roelen BAJ. Exposure of bovine sperm to pro-oxidants impairs the developmental competence of the embryo after the first cleavage. Theriogenology 2007;67:609–19. https://doi.org/10.1016/j.theriogenology.2006.09.032.
[32] Schiller J, Fuchs B, Arnhold J, Arnold K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. Curr Med Chem 2003;10:2123–45. https://doi.org/10.2174/0929867033456828.
[33] Tunc O, Tremellen K. Oxidative DNA damage impairs global sperm DNA methylation in infertile men. J Assist Reprod Genet 2009;26:537–44. https://doi.org/10.1007/s10815-009-9346-2.
[34] Senner CE. The role of DNA methylation in mammalian development. Reprod Biomed Online 2011;22:529–35. https://doi.org/10.1016/j.rbmo.2011.02.016.
[35] Matsuda Y, Tobari I. Repair capacity of fertilized mouse eggs for X-ray damage induced in sperm and mature oocytes. Mutat Res 1989;210:35–47. https://doi.org/10.1016/0027-5107(89)90042-0.
[36] Fatehi AN, Bevers MM, Schoevers E, Roelen BAJ, Colenbrander B, Gadella BM. DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl 2006;27:176–88. https://doi.org/10.2164/jandrol.04152.
[37] Rahman MB, Vandaele L, Rijsselaere T, Zhandi M, Maes D, Shamsuddin M, et al. Oocyte quality determines bovine embryo development after fertilisation with hydrogen peroxide-stressed spermatozoa. Reprod Fertil Dev 2012;24:608–18. https://doi.org/10.1071/RD11237.
[38] Khadem N, Poorhoseyni A, Jalali M, Akbary A, Heydari ST. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. Andrologia 2014;46:126–30. https://doi.org/10.1111/and.12056.
[39] Paranjpe SS, Veenstra GJC. Establishing pluripotency in early development. Biochim Biophys Acta Gene Regul Mech 2015;1849:626–36. https://doi.org/10.1016/j.bbagrm.2015.03.006.
[40] Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 2007;447:425. https://doi.org/10.1038/nature05918.
[41] Piedrahita JA. The role of imprinted genes in fetal growth abnormalities. Birth Defects Res A Clin Mol Teratol 2011;91:682–92. https://doi.org/10.1002/bdra.20795.
[42] Roberts RM. Interferon-τ and pregnancy. J Interferon Cytokine Res 1996;16:271–3. https://doi.org/10.1089/jir.1996.16.271.
[43] Li Z, Srivastava P. Heat-shock proteins. Curr Protoc Im 2004;58. A.1T.-A.T.6, https://doi.org/10.1002/0471142735.ima01ts58.
[44] Beere HM. ‘The stress of dying’: the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 2004;117:2641–51. https://doi.org/10.1242/jcs.01284.
[45] Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35:495–516. https://doi.org/10.1080/01926230701320337.
[46] Koninklijk Nederlands Meteorologisch Instituut (KNMI). Ministerie van Infrastructuur en Waterstaat. The Royal Netherlands Meteorological Institute; 2019. https://www.knmi.nl/nederland-nu/klimatologie. [Accessed 1 August 2018].
[47] Wydooghe E, Heras S, Dewulf J, Piepers S, Van den Abbeel E, De Sutter P, et al. Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. Reprod Fertil Dev 2014;26:717–24. https://doi.org/10.1071/RD13043.
[48] Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. Int J Androl 2011;34:2–13. https://doi.org/10.1111/j.1365-2605.2009.01042.x.

[49] Wydooghe E, Vandaele L, Beek J, Favoreel H, Heindryckx B, De Sutter P, et al. Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. Anal Biochem 2011;416:228–30. https://doi.org/10.1016/j.ab.2011.05.033.
[50] Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A, Peelman LJ. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev Biol 2005;5:27. https://doi.org/10.1186/1471-213X-5-27.
[51] Van Poucke M, Peelman L. Flexible, multi-use, PCR-based nucleic acid integrity assays based on the ubiquitin C gene. BioRxiv 2017:168195. https://doi.org/10.1101/168195.
[52] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406–15. https://doi.org/10.1093/nar/gkg595.
[53] Ishii T, Matsuki S, Iuchi Y, Okada F, Toyosaki S, Tomita Y, et al. Accelerated impairment of spermatogenic cells in sod1-knockout mice under heat stress. Free Radic Res 2005;39:697–705. https://doi.org/10.1080/10715760500130517.
[54] Brewer LR, Corzett M, Balhorn R. Protamine-induced condensation and decondensation of the same DNA molecule. Science 1999;286:120–3. https://doi.org/10.1126/science.286.5437.120.
[55] Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H, Wolf E. Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments. Anim Reprod Sci 2014;149:46–58. https://doi.org/10.1016/j.anireprosci.2014.05.016.
[56] Generoso WM, Cain KT, Krishna M, Huff SW. Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. Proc Natl Acad Sci U S A 1979;76:435–7. https://doi.org/10.1073/pnas.76.1.435.
[57] Brandriff B, Pedersen RA. Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. Science 1981;211:1431–3. https://doi.org/10.1126/science.7466400.
[58] Karoui S, Díaz C, González-Marín C, Amenabar ME, Serrano M, Ugarte E, et al. Is sperm DNA fragmentation a good marker for field AI bull fertility? J Anim Sci 2012;90:2437–49. https://doi.org/10.2527/jas.2011-4492.
[59] Jia Y, Castellanos J, Wang C, Sinha-Hikim I, Lue Y, Swerdloff RS, et al. Mitogen-activated protein kinase signaling in male germ cell apoptosis in the rat. Biol Reprod 2009;80:771–80. https://doi.org/10.1095/biolreprod.108.072843.
[60] Sutovsky P, Schatten G. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. Int Rev Cytol 2000;195:1–65. https://doi.org/10.1016/s0074-7696(08)62703-5.
[61] Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Delivering spermatozoan RNA to the oocyte. Nature 2004;429:154. https://doi.org/10.1038/429154a.
[62] Miller D, Brinkworth M, Iles D. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. Reproduction 2010;139:287–301. https://doi.org/10.1530/REP-09-0281.
[63] Steger K, Cavalcanti MCO, Schuppe HC. Prognostic markers for competent human spermatozoa: fertilizing capacity and contribution to the embryo. Int J Androl 2011;34:513–27. https://doi.org/10.1111/j.1365-2605.2010.01129.x.
[64] Rahman MB, Kamal MM, Rijsselaere T, Vandaele L, Shamsuddin M, Van Soom A. Altered chromatin condensation of heat-stressed spermatozoa perturbs the dynamics of DNA methylation reprogramming in the paternal genome after in vitro fertilisation in cattle. Reprod Fertil Dev 2014;26:1107–16. https://doi.org/10.1071/RD13218.
[65] Feil R, Fraga MF. Epigenetics and the environment: Emerging patterns and implications. Nat Rev Genet 2012;13:97–109. https://doi.org/10.1038/nrg3142.
[66] Jin B, Li Y, Robertson KD. DNA methylation: Superior or subordinate in the epigenetic hierarchy? Genes Cancer 2011;2:607–17. https://doi.org/10.1177/1947601910393957.
[67] Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell Res 2011;21:381–95. https://doi.org/10.1038/cr.2011.22.
[68] Vasilatou D, Papageorgiou SG, Dimitriadis G, Pappa V. Epigenetic alterations and microRNAs: new players in the pathogenesis of myelodysplastic syndromes. Epigenetics 2013;8:561–70. https://doi.org/10.4161/epi.24897.
[69] Trombetta JJ, Gennert D, Lu D, Satija R, Shalek AK, Regev A. Preparation of single-cell RNA-seq libraries for next generation sequencing. Curr Protoc Mol Biol 2014;107. 4.22.1-4.22.17, https://doi.org/10.1002/0471142727.mb0422s107.