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Original Research Article

Impact of heat stress on the miRNA cargo of pre-ovulatory follicular fluid extracellular vesicles and fertility in dairy cattle

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ABSTRACT

This study aimed to evaluate the impact of heat stress on bovine fertility and extracellular vesicles (EVs) distribution and cargo in the follicular fluid (FF) of pre-ovulatory follicles. Temperature and humidity data recorded on the farm were used to calculate the mean temperature humidity index (THI) during summer (S) and winter (W). Herd fertility was assessed as conception rates following artificial insemination during S and W. The miRNA cargo of the EVs contained in the pre-ovulatory follicle was studied in a representative group of synchronized animals during S and W (n = 10/season). The EVs were isolated by ultracentrifugation, and characterized by Nanosight, TEM, and Western Blot. EVs' miRNAs cargo was obtained by sequencing. The average THI index was 74 and 54 in S and W, respectively. The response to synchronization and conception rates decreased in S (81.5% and 24.4 %, respectively) compared to W (100% and 39.9 %, respectively). The EVs showed similar size distribution (mean size of 218 nm) and particle concentration (2.25e + 10 particles/ml) in both seasons. Seasonal variations of miRNA cargo were observed, with 83 differentially expressed (DE)-miRNAs, the most significant included 6 up-regulated (bta-miR-202, bta-miR-16a, bta-miR-186, bta-miR-532, bta-miR-154c, and bta-miR-19b), and 6 down-regulated (bta-miR-1306, bta-miR-130b, bta-miR-130a, bta-miR-320a, bta-miR-1246, and bta-miR-494) miRNAs during S compared to W. In conclusion, HS significantly alters the miRNA profile of pre-ovulatory FF-EVs in dairy cattle. Most of the DE-miRNA modulate ovarian function and follicular physiology, potentially explaining the reduced fertility observed in heat-stressed cows.

1. Introduction

Heat stress (HS) has become a major challenge in dairy farming, due to the combination of rising global temperatures and genetic selection for milk production. It occurs when an animal is unable to dissipate body heat, resulting in a disruption of thermoregulatory homeostasis. HS significantly impacts cattle breeding profitability by reducing productive [1] and reproductive performance [2,3]. HS is also associated with depression of the immune system [4], leading to an increased incidence of diseases such as mastitis [5], retention of fetal membranes [6], and metritis [7]. The effect of HS on reproduction occurs at both central and peripheral levels. Cows subjected to HS often show alterations of the hypothalamic-pituitary-gonad axis and cyclic ovarian activity [8]. Additionally, alterations in the follicular dominance, resulting in an increased incidence of multi-ovular cycles, have been reported [9]. At the ovarian level, HS can affect hormone secretion [8] and oocyte quality via multiple mechanisms [10], thereby reducing zygote formation.

HS negatively influences the oocyte developmental competence, i.e., its ability to be fertilized and subsequently develop into an embryo [11] in several ways. It is known that oocyte competence is affected by the follicular environment in which the oocyte grows [12]. The biochemical

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conditions during follicular growth before ovulation affect both the oocyte developmental competence and the follicle steroidogenic capacity in high-producing dairy cows [13]. The follicular fluid (FF) is a complex mixture of bioactive compounds that play a crucial role in determining oocyte quality [14,15]. Still, its composition varies in response to various environmental factors [16–20]. In high-producing dairy cows, HS has been shown to affect the follicular concentrations of key metabolites including glucose, cholesterol, Insulin-like Growth Factor 1 (IGF-1), urea, and Non-Esterified Fatty Acids (NEFAs), which are known to be related to oocyte competence [20]. Moreover, exposure to environmental thermal stress may damage granulosa and theca cells at early stages of proliferation and differentiation, potentially leading to long-term detrimental effects on follicular development and oocyte quality [21]. In vitro studies have shown that elevated culture temperatures directly impair the function and structure of granulosa and cumulus cells [22,23], reduce gap junction communication and cumulus support to the oocyte [23],and alter steroid production [24]. The FF contains extracellular vesicles (EVs), lipid bilayer-enclosed particles secreted by the somatic cells of the ovarian follicle, which are essential regulators of steroidogenesis, oocyte growth, and developmental competence, by mediating communication between somatic cells and the oocyte [25]. The EVs isolated from the FF contain miRNAs implicated in ovarian function and oocyte competence in bovine [26,27]. However, the EVs' composition varies in response to several factors, such as the metabolic status of dairy cows [28] and the photoperiod in seasonal species like buffalo [29], influencing oocyte competence. The hypothesis underlying this work was that HS may alter the miRNA profile of EVs within the FF, which may account for the reduced oocyte competence and fertility observed in animals exposed to HS. Therefore, this work aimed to compare the miRNA cargo of EVs contained in the FF collected from the pre-ovulatory follicles in dairy cows housed either in thermoneutrality conditions or naturally exposed to HS.

2. Materials and methods

2.1. Ethical approval

The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and all the animal treatments (PG/2024/0023589, February 27th, 2024).

2.2. Experimental design

The effect of HS was assessed on both the herd fertility and EVs distribution and cargo in the FF of pre-ovulatory follicles. For this purpose, herd fertility was monitored and evaluated as conception rates following artificial insemination (AI) during winter (W; n = 1951) and summer (S; n = 1089), i.e., periods characterized by low and high temperature humidity index (THI), respectively. To evaluate the miRNA cargo of the EVs contained in the pre-ovulatory follicle, a representative group of animals was selected after synchronization during W and S (n = 10/season) to collect the FF, from which the EVs were isolated and analyzed as reported below.

2.3. Temperature humidity index

Ambient temperature and humidity were recorded daily (always at 15:00 h) using a data logger (Sainlogic FT-083 – Sainlogic) placed in the feeding area. The device was positioned at the head height of the animals.

The temperature humidity index (THI) was calculated according to the following formula:

$$THI = (1.8 \times AT + 32) - (0.55 - 0.0055 \times RH) \times [(1.8 \times AT + 32) - 58]$$

where AT is the ambient temperature expressed in degrees Celsius, the term $(1.8 \times AT + 32)$ represents the conversion of temperature data to degrees Fahrenheit, and RH is the relative humidity as a fraction of unity [30].

2.4. Animals

The study was performed on Holstein dairy cows, kept under controlled nutrition and housed in a barn, on a farm located in the Caserta area (South of Italy), during winter and summer seasons. To assess herd fertility, all the animals were evaluated by a veterinarian, and only healthy and cyclic multiparous animals were inseminated (n = 1951 and 1089, respectively, during W and S). For FF collection, multiparous Holstein dairy cows (n = 21 and 27, respectively, in W and S) were enrolled in the trial. Then, 10 cows per season were selected to be similar in age (3.7 ± 0.2 years), parity (2.5 ± 0.76), and days in milk (43.0 ± 19.3) at the time of FF sampling.

2.5. Herd fertility assessment

The fertility of the herd was monitored, by recording the outcome of all AIs performed during W and S, regardless of the service number. Pregnancy diagnosis was carried out 35 days post-AI using transrectal ultrasonography. Conception rate (CR) was calculated as the ratio between the number of pregnancies and the number of inseminated animals.

2.6. Assessment of the body condition score (BCS)

The body condition score (BCS) was assessed during W and S on a 1 to 5 scale. Scores were assigned based on visual assessments of fat cover on key anatomical points such as the ribs, spine, hips, and tailhead, as previously described [31].

2.7. Stimulation protocol and follicular aspiration

All the animals were synchronized by using an Ovsynch protocol, consisting of administration of 0.012 mg of a GnRH agonist-buserelin acetate (Ingravida 0,0040 mg/ml, Fatro S.p.A., Italy) i.m. on Day 0 and Day 9, and by 0.524 mg of synthetic prostaglandin (Cloprostenol, Estrumate, Schering-Plough Animal Health, Italy) i.m. on Day 7.

Animals were subjected to a transrectal ultrasonographic examination approximately 16-18 h after the last GnRH administration, i.e., close to ovulation. The follicle size was calculated as the mean of two perpendicular diameters of each pre-ovulatory follicle. After the presence of a dominant follicle was confirmed, animals were subjected to anesthesia by intraepidural injection of procaine cloridrate (Procamidor, 20 mg/ml, VetViva Richter GmbH, Austria) and to transvaginal ultrasound-guided aspiration of the dominant follicle content. In brief, a 10 MHz micro-convex probe mounted on a transvaginal support device (Dramiński ® blue, Olsztyn, Poland), was used to scan ovaries, and the dominant follicle was aspirated using a 16G needle.

The FF was collected into 15 mL conical tubes (Falcon, Corning Science, Reynosa, México) and transferred to the laboratory. After searching and removal of the oocyte, the FF was subjected to two consecutive centrifugations (7000 g × 10 min and 2000 g × 10 min at 4 °C) and stored at −80 °C until further analyses. Among all the samples collected, 10 FF samples per season were selected for further analysis.

2.8. Extracellular vesicles (EVs) isolation from the follicular fluid

Follicular fluid was collected from multiparous Holstein dairy cows (n = 10 and 10, in S and W, respectively) and used for EVs isolation by ultracentrifugation at 100,000g at 4 °C for 1 h (Beckman Coulter OptimaX, Milan, Italy). The pellet was resuspended in 200 μl of phosphate buffer solution (PBS) and stored at −20 °C for EV characterization and RNA extraction.

2.9. Nanoparticle tracking analysis (NTA)

Follicular fluid extracellular vesicles size and concentration were determined by Nanoparticle tracking analysis (NTA) with a NanoSight NS300 system (Malvern Technologies, Malvern, UK) configured with a 532 nm laser. All samples were diluted in filtered PBS (1:200 vol/vol) in order to obtain an ideal particle per frame value (20–100 particles/frame). Three videos of 60 s were captured for each preparation and analyzed with NTA software version 3.2. From each video, the mean, mode, and median EV size were used to calculate sample concentration expressed in nanoparticles/mL.

2.10. Dot Blot

Isolated FF-EVs were characterized by Dot Blot analysis as previously reported [32,33]. Briefly ten samples of FF (W) and ten samples of FF (S) were pooled in separate vials. Then, 0.8 μL of EVs pooled preparation was dropped on nitrocellulose membrane (PROTAN BA 85, 0.45 mm; Whatman, Germany); drops were dried at room temperature for 15 min and non-specific sites were saturated with a blocking step, performed by a solution of BSA 4% in TBS-T (0.05% of Tween20). Membranes were incubated for 2 h at room temperature on orbital shaker with a primary antibody mouse IgG, diluted 1:500 in a solution containing 2% BSA in TBS-T, respectively: mAb anti-ALIX, clone C11 (Santa Cruz Biotechnology Inc.), mAb anti-TSG101, clone C-2 (Santa Cruz Biotechnology Inc.), mAb anti-CD63, clone H5C6 (BD Pharmigen), mAb anti-CD81, clone B-11 (Santa Cruz Biotechnology Inc.) and Calnexin, clone TO-5 (Sigma). Strips were washed for 5 min, 3 times with TBS-T, then membranes were incubated with horseradish peroxidase-conjugated anti--mouse secondary antibody (Jackson Immunoresearch), diluted 1:2500 in 2% BSA in TBS-T, for 1 h under orbital shaking. Final washes were performed 3 times, 5 min each, in TBS-T, and the signal was detected using Bio-Rad Clarity Western ECL Substrate (Bio-Rad) and imaged using a Chemidoc XRS (Bio-Rad).

2.11. Transmission electron microscopy

After ultracentrifugation, the isolated EVs were fixed for 1 h at room temperature with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffered solution at pH 7.4. Samples were postfixed on ice using 1% OsO_4, 1.5% potassium ferrocyanide in 0.1 M cacodylate solution, for 1 h in dark conditions. After washing, samples were stained with 0.5% uranyl acetate solution and dehydrated using a series of ethanol/water mixtures. Finally, samples were infiltrated for 2 h with ethanol solution containing resin (Araldite-Epon) (1:1 vol) and incubated twice with 100% Epon resin for 1h. Then, the samples were polymerized at 60 °C for 48 h. Sections of 70 nm were collected on 300-mesh uncoated copper grids using an Ultracut E microtome (Reichert, Austria) and observed with a Zeiss LEO 912 ab Energy Filtering TEM operating at 120 kV. Digital images were acquired using a CCD-BM/1 K system operating with the iTEM (Olympus Soft Imaging Solutions).

2.12. RNA extraction

RNA was extracted from isolated EVs (n = 10 and 10, in S and W, respectively). EVs were solubilized in Trizol (Invitrogen), supplemented with chloroform, and centrifuged following the manufacturer's instructions. The upper aqueous solution containing RNA was purified using the NucleoSpin miRNA kit (Macherey–Nagel, Germany) protocol. The Agilent Bioanalyzers 2100 instrument (Santa Clara, CA, USA) was used to determine the quality and quantity of isolates' RNAs, which were stored at −80 °C until use.

2.13. Library preparation

Small RNA libraries were obtained with the QIAseq miRNA Library Kit (QIAGEN Venlo, Hulsterweg, NL), according to the manufacturer's instructions with QIAseq miRNA 96 Index IL and 14 cycles of amplification. Libraries were purified with pippin-prep instrument (Sage Science, MA, USA). The quality checks of libraries and concentration were assessed by Agilent 2100 bioanalyzer. Libraries were sequenced on Illumina NovaSeq X, 150 cycles paired-end. Data are available at the Sequence Reads Archive (SRA), BioProject accession number, PRJNA1344866.

2.14. Data analysis

After quality checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming with Trimmomatic (version 0.32)58, Illumina raw sequences were analyzed with miRDeep2 (miRDeep2 (version 2.0.0.5)59 for miRNA detection and discovery. *Bos taurus* miRNAs were input to support known miRNA detection. The miRDeep2 quantifier module was used to quantify the identified miRNAs. The EdgeR Bioconductor package version 3.6 (Bioconductor, https://bioconductor.org/packages/release/bioc/html/edgeR.html) was used to evaluate the differentially expressed miRNAs (DE-miRNAs) between groups [34].

Differences in CR and in the response to synchronization between S and W were analyzed by Chi square test. Differences if the follicular diameter and BCS were evaluated by Student's *t*-test. The level of significance was set at $P < 0.05$.

3. Results

3.1. THI

The mean temperatures were 15.6 ± 0.3 and 34.3 ± 0.5 °C, respectively, in W and S. The average humidity was 70.0 ± 1.9 and 44.6 ± 1.9% respectively, in W and S. The mean THI was 59.0 ± 0.3 and 74.0, respectively, in W and S.

3.2. Herd fertility

Reduced herd fertility was recorded in response to HS, as shown by the decrease in CR observed during S compared to W (24.4 and 39.9 %, respectively; $P < 0.01$).

3.3. Response to stimulation protocol

The response to the synchronization protocol was 100% (21/21) and 81.5 % (22/27), respectively, in W and S. No differences were observed in the mean diameter of the dominant follicle between W and S (15.6 ± 1.6 mm vs 15.4 ± 2.6 mm, respectively).

3.4. Body condition score

The BCS was lower ($P < 0.05$) in the S than in the W group (2.6 ± 0.3 and 3.4 ± 0.4, respectively).

3.5. Extracellular vesicles characterization

The EVs isolated from the FF of the pre-ovulatory follicle did not show differences in size distribution (mean size of 202.56 ± 31.65, 232.55 ± 34.91 nm) and particle concentration (1.73E+10 ± 5.12E+09, 2.77E+10 ± 1.95E+10 particles/ml) between the W and S groups, as shown in Supplementary file S1 (an example of NTA profile was reported in Fig. 1A). Dot Blot showed the presence of specific EVs markers such as CD63, CD81, Alix, and TSG101, and a negligible contamination of Calnexin (Fig. 1B). Observation by electron microscope revealed that preparations contained EVs (Fig. 1C).

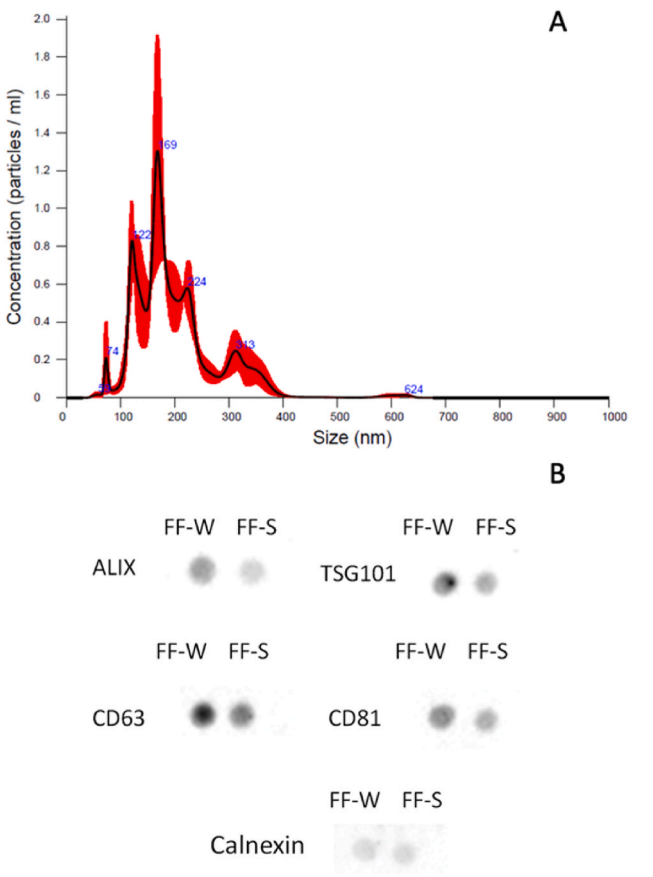

Fig. 1. EVs isolated from Follicular Fluid (FF) collected in winter (W) and summer (S) season by: A) Nanoparticle Tracking Analysis (NTA), B) Dot Blot for EVs internal markers (TSG101 and Alix), membrane markers (CD63 and CD81); and Calnexin as a marker of cell contamination in EVs preparation. C) Transmission Electron Microscopy that revealed typical morphologies characteristic of vesicles.

3.6. Small RNA sequencing

Ten samples of FF-EVs per season were characterized for their miRNA content. About 42.6 million reads were sequenced for FF-W and FF-S samples; about 5.9% of them were assigned to miRNAs (see Supplementary File S2 for statistics). Sample FF-S1 had very low coverage and, therefore, was not included in the subsequent data analysis.

In total, 585 *Bos taurus* bta-miRNAs were identified (at least 1 count in three samples). Principal Component Analysis (PCA) and hierarchical clustering showed a clear separation in terms of miRNA cargo of the FF-EVs samples collected in the two seasons (Fig. 2A and B).

The FF isolated from the pre-ovulatory follicle contained EVs that showed a specific miRNA cargo with 83 differentially expressed miRNAs (DE-miRNAs) (False Discovery Rate FDR <0.05) between S and W (Supplementary file S3). Within them, 28 were under-expressed and 55 over-expressed during S. Considering the most significant DE-bta-miRNA (FDR <10exp-3, LogFC >|2|, n = 12), 6 miRNAs were over-expressed, and 6 miRNAs were under-expressed during S (Table 1).

3.7. Analysis of target genes

Gene Ontology (GO) analysis was performed on DE-miRNAs target genes (Supplementary file S4). As each miRNA potentially targets hundreds of genes, we limited the GO analysis considering only the first 20 genes targeted by the total DE-miRNAs (55 over and 28 under) and 100 genes targeted by the most significant DE-miRNAs (6 over and 6 under), sorted by 3′UTR binding energy and longest consecutive pairing.

The GO analysis did not reveal any pathway enriched for under-expressed miRNAs in the S season. On the contrary, FF-EVs from heat-stressed cows were enriched with miRNAs targeting genes related to cell migration and differentiation, tyrosine kinase signaling, and positive regulation of biological processes, as shown in Fig. 3.

4. Discussion

The rationale for the present study stems from the need to elucidate the causes of reduced oocyte competence observed in dairy cattle naturally exposed to HS, as improved knowledge of the molecular factors involved may allow the development of corrective strategies. Specifically, this work aimed to investigate whether season-induced HS might influence the miRNA profile of the EVs contained in the FF of pre-ovulatory follicles in Holstein dairy cows. To our knowledge, this is the first report of HS-associated variations in miRNAs content in the FF EVs collected from pre-ovulatory follicles in dairy cattle, which may account for reduced fertility.

Heat Stress has been shown to have a detrimental impact on dairy cows when THI exceeds 72 [35]. In this study, the average THI in S was 74. HS led to a decrease in herd fertility, as evidenced by reduced CR during periods characterized by elevated THI, in agreement with previous reports [2]. Among cows enrolled for FF characterization, the response to synchronization was also lower in S compared to W. On the other hand, the average diameter of the dominant follicle was unaffected, in contrast with previous studies in which a reduction of the diameter of the follicle was associated with HS [21,36].

The reduced BCS observed in S was expected and is in line with a previous study reporting a reduction in feed intake during hot periods, leading to negative energy balance [37]. High environmental temperatures may prolong the post-partum negative energy balance period, due to increased body fat mobilization to compensate for the inadequate nutrient intake [38]. This results in the release of substances such as non-esterified fatty acids, β-hydroxybutirate, urea, and bilirubin into the bloodstream – and consequently into the FF – which may negatively affect the acquisition of oocyte developmental competence [39–41]. The loss of body condition and decreased fertility were associated with alterations in the cargo of the EVs present in the FF of preovulatory follicles.

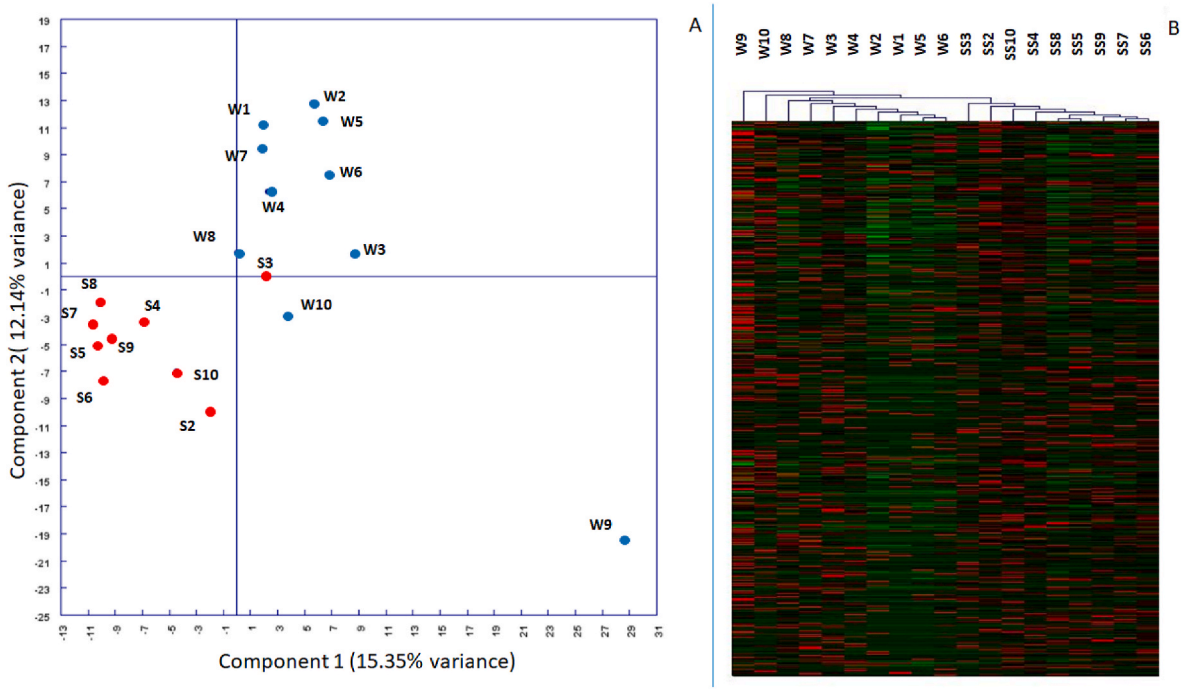

Fig. 2. A) Principal Component Analysis and B) hierarchical clustering of samples analyzed (extracellular vesicles from follicular fluid FF-EVs) in the summer (S) and winter (W) season based on their miRNA cargo.

Table 1
Most significant differentially expressed miRNAs (FDR <10exp-3, LogFC >|2|) between summer (S) and winter (W) season. In the table the logarithmic Fold Change (logFC), the logarithmic Count Per Milion reads (logCPM), and the False Discovery Rate (FDR) are reported.

miRNA	logFC	logCPM	FDR
bta-miR-202	3.86174029	15.92578	5.91E-08
bta-miR-16a	2.287449228	15.41692	5.91E-08
bta-miR-1306	−6.10975503	9.664463	9.66E-08
bta-miR-186	3.882917732	10.02469	1.51E-07
bta-miR-130b	−2.44341128	13.35702	1.51E-07
bta-miR-130a	−2.60119969	15.37776	2.17E-06
bta-miR-532	3.257829705	11.95393	6.82E-06
bta-miR-154c	7.925011641	2.905925	5.79E-05
bta-miR-320a	−2.14858066	12.66888	9.59E-05
bta-miR-19b	2.023581056	12.48217	0.000556
bta-miR-1246	−2.88510937	9.942072	0.00083
bta-miR-494	−3.27398063	6.945757	0.000959

In this work, the EVs isolated from the pre-ovulatory follicle FF had a similar size distribution and concentration between seasons. This finding aligns with a recent study, reporting no differences in EVs' average size and concentration in antral follicles collected from cross-breed beef cattle following ovarian stimulation with FSH, subjected or not to seasonal HS [42]. The mean size and concentration of the EVs in our study were consistent with those previously reported in bovine and buffalo FF [43,44]. Metabolic changes associated with HS may alter the follicular environment and compromise the oocyte competence during summer. It was demonstrated that exposure to HS during the peri-ovulatory period modifies the follicular proteome and enhances the intrafollicular levels of pro-inflammatory cytokines in bovine [45]. Moreover, there is indirect evidence of alterations of FF-EVs in response to HS in cattle. It has been shown that supplementation of IVM medium with EVs isolated from the FF of HS-subjected animals alters the expression of genes related to oocyte competence [46]. When granulosa cells are cultured in vitro under HS-induced conditions, the number of secreted EVs and the associated miRNA cargo change [47]. Despite this, a characterization of the miRNA cargo of FF-EVs of vivo-derived pre-ovulatory follicles in animals subjected to HS has not yet been performed.

In this study, the reduced fertility in S was associated with changes in the miRNA profile of pre-ovulatory FF-EVs. Indeed, seasonal variations in the expression of large numbers of miRNAs were recorded in the FF-EVs isolated from the pre-ovulatory follicles. Considering only the most significant DEmiRNAs, 6 miRNAs were over-expressed, and 6 miRNAs were under-expressed during S compared to W. More specifically, bta-miR-202, bta-miR-16a, bta-miR-186, bta-miR-532, bta-miR-154c, and bta-miR-19b were over-expressed, while bta-miR-1306, bta-miR-130b, bta-miR-130a, bta-miR-320a, bta-miR-1246, and bta-miR-494 were under-expressed in S compared to W. It is interesting to note that many DE-miRNAs regulate follicular physiology, ovarian function, and stress response. Among these, bta-miR-19b and bta-miR-1246 were also differentially expressed in EVs obtained from FF samples of antral follicles during the hot and cold seasons in crossbreed cattle, with bta-miR-19b being over-expressed and bta-miR-1246 under-expressed in S [42], consistent with our findings. However, bta-miR-19b and bta-miR-1246, involved in the stress response, oxidative stress, and immune response, were both over-expressed in the serum of bovines exposed to HS [48]. Moreover, serum bta-miR-19b was over-expressed in both pregnant and non-pregnant cows exposed to HS [49]. Interestingly, an in vitro study revealed that when oocytes are exposed to HS during IVM, a reduction of blastocyst rate is associated with an increase in bta-miR-19b expression in the embryos [50]. An intriguing but challenging finding of this work is the over-expression of bta-miR-202 during Scontrasting with earlier studies suggesting a modulatory role of this miRNA on follicular function. In particular, bta-miR-202 was associated with follicle development and steroidogenic capacity in cattle [51]. In addition, this miRNA, which is only expressed in gonads, was up-regulated in large healthy follicles compared to atretic ones [52]. Another DE-miRNA identified in the present study in FF-EVs was Bta-miR-16a, which was also differentially expressed in the serum of *Bos Indicus* cattle subjected to HS, although with an opposite pattern [53]. Furthermore, in the FF-EVs of animals exposed to HS we observed a reduced expression of bta-miR-130b, known to promote bovine granulosa and cumulus cells viability and proliferation, oocyte maturation, and embryo development

Fig. 3. Gene Ontology (GO) Analysis of: A) the first 20 genes targeted by 55 overexpressed miRNAs and B) the first 100 genes targeted by 6 most significant overexpressed miRNAs (FDR>0.01, LogFC>|2|) present in the extracellular vesicles isolated from follicular fluid (FF) in the S season.

in vitro [54]. Another miRNA that was down-regulated in S in our study was miR-130a, involved in the control of steroidogenesis in goat granulosa cells and in the regulation of fertility in sow [55,56]. Another interesting finding concerns the under-regulation during S of miR-320, previously identified as a competence marker in humans, as high levels of its expression in the FF are associated with embryo quality [57]. Finally, the GO analysis revealed that FF-EVs collected in heat-stressed cows were enriched with miRNAs targeting genes related to tyrosine kinase signaling. This pathway plays a key role in meiotic resumption, oocyte quality, and developmental potential [58,59]. The miRNA changes observed in the present study may reflect, at least in part, an adaptive follicular response to HS aimed at protecting the oocyte from thermal insult, as suggested by in vitro studies. Indeed, in bovine models, FF-derived EVs have been shown to modulate gene expression in cumulus cells and oocytes, influencing pathways related to cellular stress response and developmental competence [60–62].

In conclusion, HS significantly alters the miRNA profile of FF-derived EVs of pre-ovulatory follicles in dairy cattle. Most of the DE-miRNA are involved in the regulation of ovarian function and follicular physiology, suggesting that alterations of the miRNA cargo of FF-EVs may account for the reduced fertility observed in cows exposed to HS. An improved knowledge of the molecular changes at the follicular level associated with HS is fundamental to developing potential strategies to counteract deleterious effects of HS on oocyte developmental competence and fertility in dairy cows.

CRediT authorship contribution statement

Michal Andrzej Kosior: Writing – original draft, Data curation. **Luca Masiello:** Writing – original draft, Investigation, Data curation. **Federica Piscopo:** Investigation. **Riccardo Esposito:** Investigation. **Annalisa Rizzo:** Writing – review & editing, Supervision, Conceptualization. **Valentina Longobardi:** Writing – review & editing, Investigation, Data curation. **Anna Lange-Consiglio:** Writing – review & editing, Validation, Formal analysis. **Emanuele Capra:** Writing – review & editing, Validation, Formal analysis. **Barbara Lazzari:** Investigation. **Paola Gagni:** Investigation. **Bianca Gasparrini:** Writing – review & editing, Supervision, Resources, Conceptualization.

Data availability statement

The datasets are available from the corresponding author on reasonable request.

Conflict of interest

The Authors declare they do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2026.117910.

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