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Original Research Article

Impact of the near-physiological temperature on the in vitro maturation of bovine oocytes: A comparative proteomic approach

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ABSTRACT

In vivo, the temperature inside preovulatory follicles of cows is approximately 1 °C lower than rectal temperature. However, standard bovine oocyte in vitro maturation (IVM) protocols use 38.5 °C based on rectal temperature. This study evaluated the effect of reducing IVM temperature to 37.5 °C on the proteomic profile of oocytes compared to the routine 38.5 °C. Nuclear maturation rate and cumulus cell (CC) expansion (30 COCs per group, 21 replicates) were assessed by observing the first polar body and using a subjective scoring method (0–4). Total nitrite concentrations in the culture medium were measured using the Griess method. Differential proteomics was performed using LC-MS/MS on pooled oocyte samples (500 matured oocytes per group, three replicates), followed by gene ontology enrichment, protein-protein interaction, and putative miRNA target analyses. No significant differences were observed between the groups in nuclear maturation, CC expansion, or nitrite concentration ($P > 0.05$). A total of 806 proteins were identified, with 7 up-regulated and 12 down-regulated in the treatment group compared to the control. Additionally, 12 proteins were unique to the control group, and 8 were unique to the treatment group. IVM at 37.5 °C resulted in the upregulation of proteins involved in protein folding and GTP binding, and the downregulation of enzymes with oxidoreductase activity and proteins involved in cytoskeletal fiber formation. Furthermore, 43 bovine miRNAs potentially regulating these genes (DES, HMOX2, KRT75, FARSA, IDH2, CARHSP1) were identified. We conclude that IVM of bovine oocytes at 37.5 °C induces significant proteomic changes without impacting nuclear maturation, cumulus cell expansion, or nitrite concentration in the IVM medium.

1. Introduction

In vitro production of bovine embryos (IVP) is widely used today in the agricultural market, accounting for 66 % of the embryos produced worldwide [1]. The in vitro maturation (IVM) stage involves the meiotic progression of the cumulus-oocyte complex (COC) to the metaphase II (M II) stage, at which point fertilization of the cell is possible [2,3]. However, oocytes matured in vitro are not as competent as those matured *in vivo* [4–6]. Therefore, IVM protocols have been adapted concerning the composition of the culture medium to closely mimic the natural *in vivo* process [7–9].

It is known that the deep rectal temperature in adult cattle is on average 38.5 °C [10], and this was considered for years to be the same temperature found in the abdominal organs [11]. Concurrently, acrosome reaction tests (important for the in vitro fertilization stage) showed satisfactory results at this temperature. These factors were taken into

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consideration to determine the average temperature of 38.5 °C for the adjustment of incubators in IVP protocols. However, more recent research has shown that the temperature of the reproductive tract organs is lower, around 37.5 °C [12–14].

Based on recent findings, in monovular cows, the temperature of ovulatory follicles is about 1.5 °C lower than the rectal temperature, while no temperature differences are detected in non-ovulatory cows [13]. Similarly, in bi-ovular cows under heat stress, ovulatory follicles have significantly lower temperatures (about 0.9 °C) than rectal temperatures, with no significant difference in the temperature of non-ovulatory follicles. This follicular cooling results in a 3.6-fold increase in pregnancy rates, indicating that lower follicular temperatures are necessary to trigger ovulation and positively correlate with pregnancy potential [14]. These findings suggest that adjusting incubator temperatures during IVP could be beneficial.

Previous studies using lower incubation temperatures in IVM protocols did not show significant differences in maturation, cleavage, blastocyst, and hatching rates [11,15–18]. However, *in vivo*, the pregnancy rate in cows where follicular cooling occurred was higher, suggesting that the "quality" of oocyte maturation increases [13]. Several factors may explain the temperature decrease in the pre-ovulatory follicle. Firstly, the dominant follicle is a fluid-filled structure, and the blood vessel supply, which aids in tissue heat exchange, only reaches the theca cell layer, contributing to the lower oocyte temperature. Endothermic reactions, possibly related to steroid hormone and protein synthesis, cumulus cell expansion, and proteoglycan hydration, might also generate localized cooling [19]. A deeper evaluation of the effect of IVM temperature on bovine oocyte using proteomics could shed a light on its influence on maturation process, improving maturation efficiency and embryo production [20–22]. Thus, this study aimed to compare the proteomic profile of bovine COCs matured in vitro at 37.5 °C vs. 38.5 °C.

2. Material and methods

All media and reagents used were from Sigma-Aldrich®, São Paulo, Brazil, unless otherwise specified.

2.1. Collection and selection of cumulus-oocyte complexes

The ovaries from predominantly crossbred Nelore cattle were brought from local slaughterhouses to the laboratory in an isothermal box containing sterile 0.9 % saline solution supplemented with 0.01 % antibiotic (Agrovet 5,000,000, Elanco®, São Paulo, Brazil) at 30 °C. In the laboratory, the ovaries were washed and transferred to a beaker containing sterile 0.9 % saline solution supplemented with 0.01 % antibiotic. Follicles measuring 3–8 mm were aspirated using a 10 mL syringe attached to an 18G needle. The COCs, along with the follicular fluid, were deposited into a 50 mL conical tube containing TCM-199 with HEPES supplemented with 5 % fetal bovine serum (FBS - Cultilab®, São Paulo, Brazil), 10 μL/mL antibiotic and antimycotic solution (AA), 1 mM glutamine, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit meiosis resumption during COC handling (VIANA et al., 2007). After sedimentation, grades I and II COCs were selected according to de LOOS et al. (1989) and then washed in drops of TCM-199 medium with HEPES supplemented with 5 % FBS, 10 μL/mL AA, and 1 mM glutamine to remove debris and IBMX. COCs were then washed in drops of IVM medium [TCM-199 supplemented with 10 % FBS, 0.7 μg/mL recombinant follicle-stimulating hormone (r-hFSH – Gonal-F, Merck® Serono S.p.A., Bari, Italy), 10 μL/mL AA, 0.2 mM sodium pyruvate, and 0.4 mM glutamine] and subjected to the IVM process.

2.2. IVM and cumulus expansion assessment

Groups of COCs were transferred to 4-well plates (NUNC® Rochester, New York, USA), containing 30 COCs per well with 500 μL [23] of IVM medium, and incubated for 22 h at the following temperatures: 38.5 °C (control group) or 37.5 °C (treated group). Thirty replicates were performed. Cumulus expansion was evaluated 22 h after the start of IVM using a subjective classification method: 0 – no response observed; 1 – minimal response; 2 – expansion of outer layers; 3 – expansion of all layers except the corona radiata; 4 – expansion of all layers [24,25]. Ten replicates were performed (n = 4410). After subjective classification of cumulus cell expansion, the COCs were washed in phosphate-buffered saline (PBS) solution with 0.1 % polyvinyl alcohol (PVA). The COCs were mechanically denuded by successive pipetting and washed again in drops of PBS with 0.1 % PVA, and analyzed under a stereomicroscope (200 ×, SMZ745, Nikon®, Tokyo, Japan). Oocytes were considered to be in MII when the presence of the first 'centrifuged at 3000×*g* for 5 min, the supernatant was removed, and the pellet was stored at −80 °C until proteomic analysis [26].

2.3. Measurement of total nitrite (NO_2^-) concentrations

Nitric oxide levels were measured indirectly by quantifying the concentration of total nitrite in the maturation media by the Griess method. *In vitro* maturation media from control (38.5 °C) and treatment (37.5 °C) groups were collected at the end of culture (22 h IVM) and stored at −20 °C until testing. Assays were performed using the Griess Reagent System kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Absorbance was read at 540 nm (Biotek, model Epoch, software Gen5, Santa Clara, USA). To determine the nitrite concentrations in samples, a standard curve (10–100 μM) was performed with the experimental samples calculated by the standard curve equation. The results are expressed in micromolar units.

2.4. Oocyte protein extraction and digestion for mass spectrometry analysis

The total protein extraction from oocyte samples was performed according to the protocol described elsewhere [27,28] with modifications. Samples (500 oocytes in PBS solution each, three biological replicates per treatment) were ground in liquid nitrogen containing 100 μL of extraction buffer consisting of 7M urea (GE Healthcare, NJ, USA), 2M thiourea (GE Healthcare), 2 % Triton X-100 (GE Healthcare), 1 % dithiothreitol (DTT, GE Healthcare), 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, MO, USA). The cells were then lysed in five cycles of 10 s of sonication followed by 1 min on ice, vortexed for 30 min, and centrifuged at 16,000×*g* for 20 min at 4 °C. The supernatants were collected, and protein concentration was determined using the 2-D Quant Kit® (GE Healthcare). Tryptic protein digestion (1:100 enzyme: protein, V5111, Promega, Madison, USA) was subsequently performed using the modified filter-aided sample preparation (FASP) method as described previously (REIS et al., 2021). The concentration of the peptides obtained from each biological replicate was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) at 205 nm.

2.5. Mass spectrometry analyses

Samples of digested oocyte protein extracts were injected into a nanoAcquity UPLC system connected to a Q-TOF SYNAPT G2-Si mass spectrometer (Waters®, Manchester, UK). Runs consisted of three biological replicates of 1.945 μg of peptide sample. Mass spectrometry was performed in positive and resolution mode (V mode), with ion mobility and in data-independent acquisition (DIA) mode. The transfer collision energy ramped from 19 V to 55 V in high energy mode, with cone and capillary voltages set at 30 V and 2750 V, respectively. This experiment was conducted with a source temperature of 70 °C. [Glu1]-fibrinopeptide B human at 100 fmol/μL was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectrum acquisition was performed using MassLynx v.4.0 software

(Waters®, Manchester, UK).

2.6. Proteomic data analysis

Spectra processing and database search conditions were carried out using ProteinLynx Global SERVER (PLGS) v.3.02 software (Waters®, Manchester, UK). The HDMSE analysis used the following parameters: Apex3D with 150 counts for low energy threshold, 50 counts for high energy threshold, and 750 counts for intensity threshold; one missed cleavage; minimum fragment ions per peptide equal to three; minimum fragment ions per protein equal to seven; minimum peptides per protein equal to two; fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphorylation (STY); false discovery rate (FDR) of 1 %. For protein identification, we used the *Bos indicus* x *Bos taurus* protein database (ID: UP000314981, January 2021), available in UniProtKB (www.uniprot.org). Label-free quantification analyses were performed using ISOQuant v.1.8 software (DISTLER et al., 2014). To ensure the quality of the results, only proteins present in all three runs were accepted for differential abundance analysis after data processing. Proteins were considered up-accumulated if the log2 fold change (FC) value was greater than 0.60 and down-accumulated if the log2 FC value was less than 0.60, according to Student's t-test (two-tailed, $P < 0.05$).

2.7. Bioinformatics

Functional enrichment analysis was performed using OmicsBox version 1.2.4 (https://www.biobam.com/omicsbox). Protein clustering and interactions were studied using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/, version 6.8) [29] and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (http://string-db.org/, version 11.0) with k-means clustering [30]. Clusters were manually curated to enhance visualization of protein-protein interactions. Genes associated with the bovine oocyte proteome were analyzed using the g:GOst module of the g: Profiler server [31] and the GENE2FUNC module of the Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA) platform [32]. Differentially expressed gene sets were pre-calculated in GENE2FUNC via a two-tailed *t*-test for each tissue against all others. Additionally, analyses included KEGG and Hallmark pathways as well as gene ontology based on the Molecular Signatures Database (MSigDB) [33]. Proteins with lower abundance in oocytes matured at 37.5 °C were analyzed for putative microRNA (miRNA) targets using *Bos indicus* x *Bos taurus* datasets from the TargetScan (http://targetscan.org) [34] and miRBase (http://mirbase.org) [35] servers. To avoid annotation errors, all identified miRNAs were verified for conservation among mammals using miRBase. To visualize the interaction between miRNAs and genes, data were submitted to the miRNet 2.0 server (https://www.mirnet.ca) [36].

2.8. Statistical analysis

All COCs were randomly distributed within each experimental group, and each experiment was repeated 21 times (nuclear maturation and cumulus expansion). Data consistency and descriptive statistics were analyzed (PROC UNIVARIATE, SAS, 2019). The effect of different treatments on the studied variables was assessed by analysis of variance (ANOVA) or Student's t-test at a 5 % probability level, without transformation.

3. Results

3.1. Nuclear maturation rate and cumulus cell expansion

No significant difference ($P > 0.05$) was observed in the nuclear maturation rate (78.7 ± 3.7 % vs. 78.5 ± 1.8 %) or in the cumulus cell expansion rate (74.0 ± 3.3 % vs. 72.5 ± 8.8 %) of oocytes cultured at 38.5 °C and 37.5 °C, respectively. The total nitrite concentration in the IVM medium of COCs cultured at 38.5 °C (17.7 ± 3.8 μM) was also not different from grouped matured at 37.5 °C (15.5 ± 1.1 μM) ($P \geq 0.05$, Table 1).

3.2. Proteomic analysis

A total of 806 proteins were identified (Supplementary Table S1) common to the groups of oocytes matured in vitro at 38.5 °C (control) and 37.5 °C (treatment). Additionally, 12 unique proteins were described in the control group and eight unique proteins in the treatment group (Fig. 1). Regarding differential protein accumulation, seven proteins were up-regulated and 12 were down-regulated. Table 2 presents the unique and differentially accumulated proteins identified in the 37.5 °C/38.5 °C contrast.

Gene enrichment analysis showed no difference between the functional profiles of the proteomes of oocytes matured at 38.5 °C and 37.5 °C (Fig. 2). The same was observed when evaluating the main hallmark and KEGG pathways (Fig. 3). The analysis of these metabolic pathways demonstrates that a significant portion of the proteome in the studied embryonic phase is related to metabolic activity, cytoskeleton regulation, protein synthesis, and protein quality control. When comparing only the differentially abundant and unique proteins between the experimental groups, a difference between these sets of proteins was observed. Bovine oocytes matured at 37.5 °C showed higher expression of proteins associated with the biological process of protein folding and molecular functions related to GTP metabolism. Conversely, there was a reduction in enzymes with oxidoreductase activity, as well as a decrease in proteins involved with the cytoskeleton (Fig. 4, Supplementary Table S2).

In silico analyzes were performed to identify the interaction of differently abundant proteins with other proteins and miRNAs. String analysis allowed the visualization of the protein-protein interaction network showing interactions among the unique proteins in oocytes matured at 37.5 °C group. Fig. 5 describes the interaction among proteins, together with a functional characterization based on gene ontology terms. The putative regulation of protein expression by miRNA was verified using mIRNet server (Fig. 6), which allowed the identification of six genes (DES, HMOX2, KRT75, FARSA, IDH2, CARHSP1) that are known to be regulated by 43 bovine miRNAs (Supplementary Table S3).

4. Discussion

The present study aimed to compare, for the first time, the proteomic profile of bovine oocytes matured in vitro at 38.5 °C vs. 37.5 °C, in order

Table 1
Effect of temperature reduction during IVM on nuclear maturation, cumulus cell expansion, and total nitrite concentration in the culture medium.

Group Treatment	$[NO_2^-]$ (μM)	M II (%)	*Cumulus* cells expansion (%)				
			G0	G1	G2	G3	G4
38.5 °C (control)	17.7 ± 3.8[a]	78.7 ± 3.7[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	26.0 ± 3.3[a]	74.0 ± 3.3[a]
37.5 °C	15.5 ± 1.1[a]	78.5 ± 1.8[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	27.5 ± 8.8[a]	72.5 ± 8.8[a]

Means in the same column followed by the same letter do not differ according to Student's t-test at a 5 % probability level (30 oocytes in each repetition with 21 replicates per group). $[NO_2^-]$: total nitrite concentration (n = 5 with duplicates), M II: oocytes that showed extrusion of the first polar body; G0: grade 0, no response observed; G1: grade 1, minimal response; G2: grade 2, expansion of the outer layers; G3: grade 3, expansion of all layers except the corona radiata; G4: grade 4, expansion of all layers.

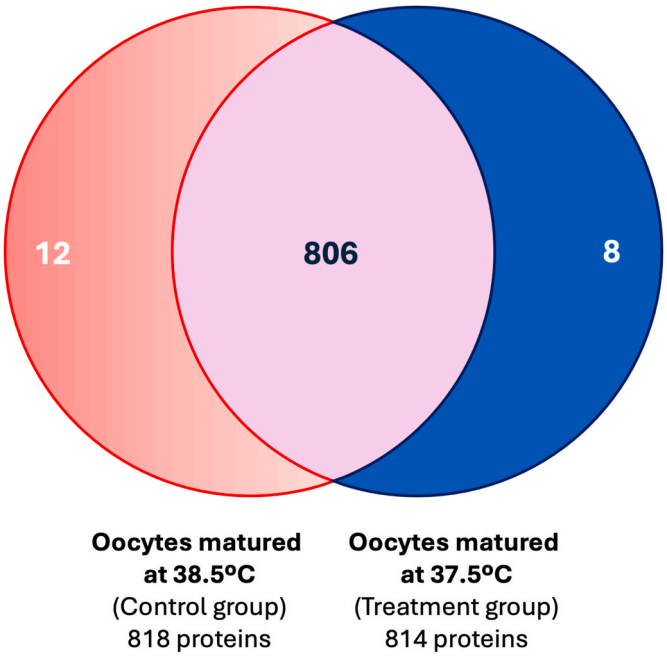

Fig. 1. Venn diagram demonstrating the distribution of identified proteins in bovine oocytes matured in vitro at 38.5 °C (control) and 37.5 °C (treatment).

to identify changes in metabolic pathways utilized by the oocyte when cultured at a temperature closer to physiological levels. As expected, there were no differences in nuclear maturation, cumulus cell expansion, or total nitrite concentration between the experimental groups. However, a 1 °C reduction in IVM temperature was sufficient to induce differences in the proteomes of oocytes cultured at 37.5 °C compared to the standard 38.5 °C. A total of 826 proteins were identified in both groups, with 20 proteins showing differential abundance. Additionally, 12 proteins were unique to the control group and 8 proteins were unique to the experimental group. In the section, the most relevant proteins will be discussed focusing in their putative roles in bovine oocyte maturation.

The absence of a significant difference in the nuclear maturation rate (78.7 % vs. 78.5 %) and cumulus cell expansion was expected, given the similar results found in previous studies evaluating lower temperatures in IVM [11,15]. These pioneering works suggested 39 °C as the optimal temperature for IVM and IVF in bovines due to (i) the core temperature of this species [10]; (ii) glycosaminoglycans production by COCs [37]; and (iii) better results in acrosome reaction [38,39] and sperm-ovum fusion and rates of post-fusion events [40,41]. However, the scientific and technological advancements in bovine IVM and IVF over the past decades have led to tighter control of these processes, significantly increasing the number of embryos transferred worldwide [42]. Despite these improvements, there is still room for enhancement. Challenges such as potentially reduced fertility from the use of sex-sorted sperm, decreased oocyte quality after in vitro maturation, and lower embryo cryotolerance contribute to reduced pregnancy rates compared to embryos produced *in vivo* (as reviewed by Ref. [43]).

The measurement of total nitrite did not differ between the experimental groups, indicating that a 1 °C reduction in the IVM procedure does not induce cellular damage from reactive oxygen species (ROS) nor alter the involvement of the NO/NOS system in controlling meiotic maturation of cumulus–oocyte complexes. ROS play a critical role in NO-based cell signaling, and significant changes in intracellular NO in bovine COCs can potentially harm oocyte viability. Our group has previously demonstrated that NO exhibits a dual effect on bovine oocytes, either inhibiting nuclear and cytoplasmic maturation or stimulating cytoplasmic maturation, depending on its concentration in the culture medium [23]. Additionally, NO derived from iNOS affects the in vitro maturation of bovine COCs by modulating the viability of cumulus cells and oocytes, the progression of meiosis after GVBD, the migration of cortical granules, and cleavage and blastocyst development [24].

Despite the absence of previously described alterations, bovine oocytes matured at 37.5 °C exhibited a distinct proteomic profile. A thorough analysis of the differential proteomics data provided a better understanding of how changes in the abundance of individual proteins, as well as groups of proteins revealed by protein-protein interaction

Table 2
Differentially abundant or unique proteins associated with temperature alteration in the in vitro maturation of bovine oocytes.

ID UniProtKB	Gene	Description	Log2 FC*
Up regulated proteins in oocytes incubated at 37.5°C			
A0A4W2F122	HSPA6	Heat shock protein family A (Hsp70) member 6	0.60
A0A4W2DK74	ZP3	Zona pellucida sperm-binding protein 3	0,74
A0A4W2BLY1	PRDX4	Peroxiredoxin 4	0.86
A0A4W2E9H1	TUBB1	Tubulin beta chain	0.91
A0A4W2EG40	IDH3A	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	1.10
A0A4W2EKU9	TUBAL3	Tubulin alpha chain	1,36
A0A4W2BX19	SLC25A31	Solute carrier family 25 member 31	2.36
Exclusive proteins in oocytes incubated at 37.5°C			
A0A4W2DE74	RAB8A	Ras-related protein Rab-8A	–
A0A4W2DYK7	GDI1	Rab GDP dissociation inhibitor	–
A0A4W2ETA0	PPIC	Peptidyl-prolyl *cis*-trans isomerase C	–
A0A4W2EPQ2	RAB2B	Ras-related protein	–
A0A4W2D705	CLU	Clusterin	–
A0A4W2DIF2	GFPT1	Glutamine–fructose-6-phosphate transaminase (isomerizing)	–
A0A4W2EXY1	PPIL3	Peptidyl-prolyl *cis*-trans isomerase	–
A0A4W2DBL6	TPM1	Tropomyosin alpha-3 chain	–
Down regulated proteins in oocytes incubated at 37.5°C			
A0A4W2C6J7	IDH2	Isocitrate dehydrogenase [NADP	−0.64
A0A4W2C1C8	NME1	Nucleoside diphosphate kinase A 1	−0.75
A0A4W2BJR7	CARHSP1	Calcium-regulated heat-stable protein 1	−0.81
A0A4W2HF60	HMOX2	Heme oxygenase (biliverdin-producing)	−0.85
A0A4W2D3J6	KRT75	Keratin 75	−0.94
A0A4W2E0S0	DYNLRB	Dynein light chain	−0.98
A0A4W2HHF4	MARS1	methionine–tRNA ligase, cytoplasmic isoform X1	−1.01
A0A4W2E576	HIST1H2BB	Histone H2B	−1.04
A0A4W2D795	DES	IF rod domain-containing protein	−1.07
A0A4W2EKX7	NOXRED1	NADP-dependent oxidoreductase domain containing 1	−1.22
A0A4W2CS91	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	−1.37
A0A4W2F2G1	FARSA	Peroxiredoxin-2	−3.51
Exclusive proteins in oocytes incubated at 38.5°C			
A0A4W2C6E9	HIST1H4I	Histone H4	–
A0A4W2BV44	GSTP1	GST class-pi; Glutathione S-transferase P	–
A0A4W2G7I1	PPA1	Inorganic diphosphatase	–
A0A4W2CEF3	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase	–
A0A4W2BQL0	LDHC	l-lactate dehydrogenase C	–
A0A4W2FPY3	XP_005901874.1	Aldo_ket_red domain-containing protein Dihydrodiol dehydrogenase 3-like	–
A0A4W2EP82	KRT75	Keratin, type II cytoskeletal 75	–
A0A4W2E5C2	NEFH	Neurofilament, heavy polypeptide;	–
A0A4W2EVJ9	BMP1	Bone morphogenetic protein 1	–
A0A4W2HLZ0	NME2	Histidine protein kinase NDKB	
A0A4W2DN76	TUBA1A	Tubulin alpha chain	–
A0A4W2D3G3	MESDC1	IF rod domain-containing protein	–

log with base 2 of the fold-change between experimental groups.

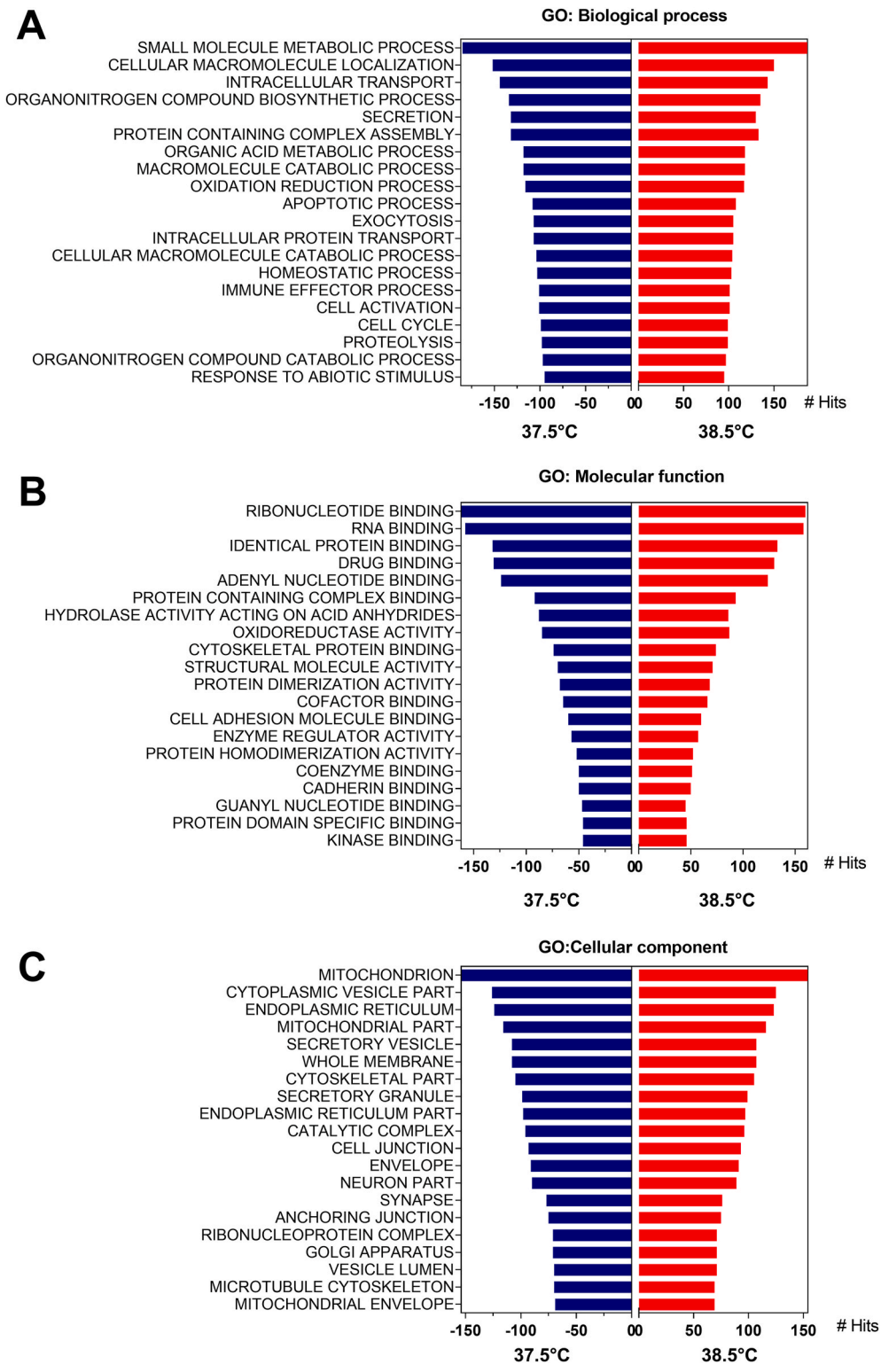

Fig. 2. Distribution of identified proteins in bovine oocytes matured in vitro at 38.5 °C (control) and 37.5 °C according to (A) biological process, (B) molecular function, and (C) cellular component. Gene ontology enrichment analysis by FUMA server.

analysis, influence the physiological processes of COCs. Twelve proteins were absent in the COCs from the experimental group. For instance, nucleoside diphosphate kinase (NDK), related to the NME2 gene, is known for its role in cell differentiation and proliferation (Boissan et al., 2009; Marino et al., 2012). However, its function has only been reported in *Xenopus* oocytes at the germinal vesicle (GV) stage (Ouatats et al., 1998). According to these authors, these proteins accumulate near the mitochondria and participate in transcription processes involving guanosine triphosphate (GTP). Histone H4 (H4) is associated with meiotic progression and chromosome alignment (Han et al., 2015; Lu et al., 2017), while alpha-tubulin is involved in the dynamics of meiotic spindle microtubules (Feng et al., 2016). L-lactate dehydrogenase (LDHC) has not yet been described in oocytes, but in other cells, this molecule is converted to pyruvate by lactate dehydrogenase (LDH)

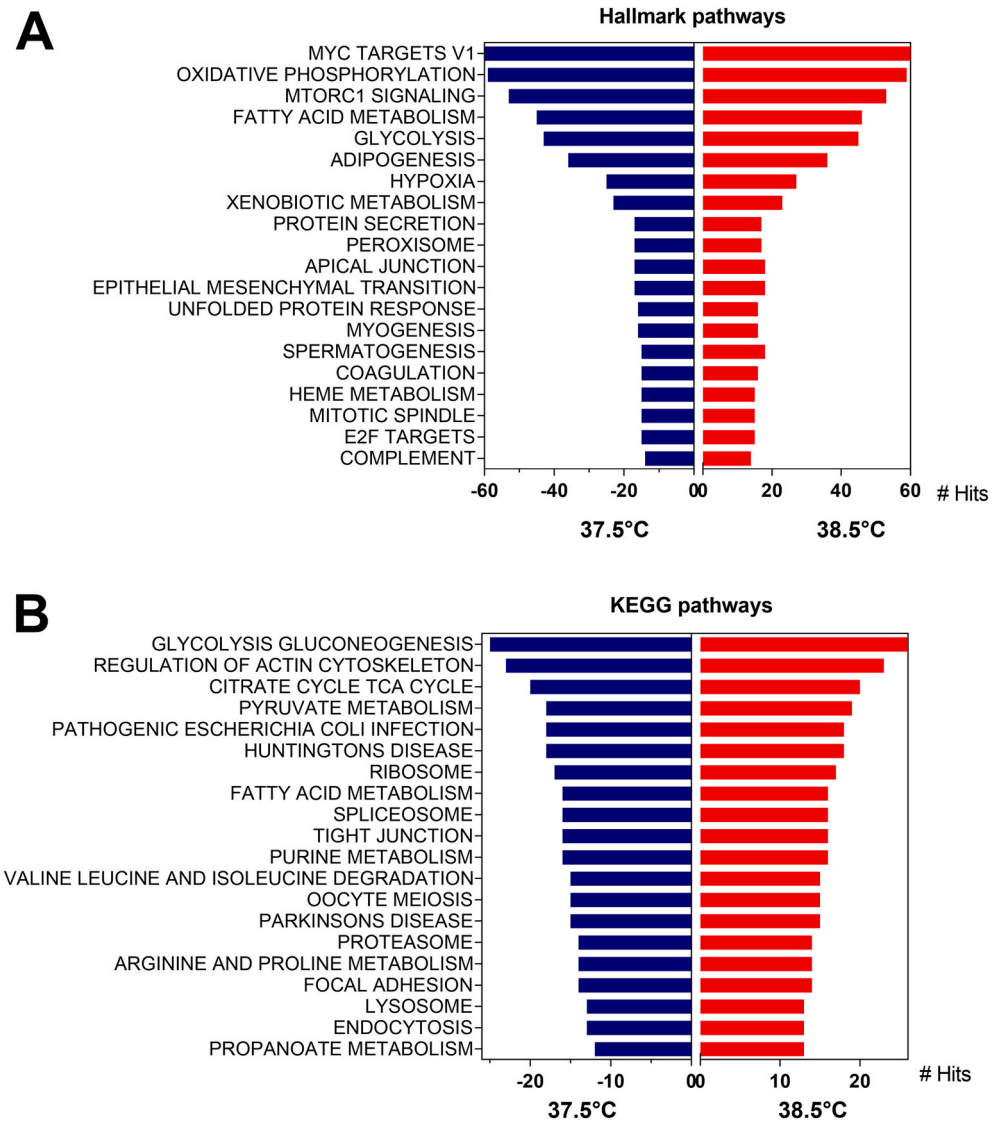

Fig. 3. Analysis of expressed genes in oocytes matured in vitro at 38.5 °C (control) and 37.5 °C by FUMA server. (A) Hallmark pathways indicating the most prevalent signaling pathways within the MSigDB gene set. (B) KEGG pathways indicating the most prevalent signaling pathways.

(Brown & Whiteley, 2009). LDH has been reported in oocytes as part of the glycolysis pathway (Brinster, 1968; Cetica et al., 1999; Kumar et al., 2013), as has glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bermejo-Álvarez et al., 2010).

Similarly, 8 proteins were exclusively found in oocytes matured at 37 °C, among them we highlight the peptidil-prolil *cis*-trans isomerase (PPIase) and clusterin. The synthesis of new proteins from maternal mRNA translation is crucial for oocyte maturation and embryo development [44]. During these stages, the endoplasmic reticulum (ER) is a primary site for the biosynthesis of proteins, lipids, and secretory proteins, playing a key role in meeting the increased demand for new proteins. Proper folding of these functional proteins in the ER is essential for maintaining appropriate oocyte maturation and embryo development. Therefore, regulation of ER stress and homeostasis is likely an important mechanism in these processes [45,46]. Thus, the presence of these proteins in oocytes matured at 37.5 °C indicates good oocyte quality.

Clusterin (CLU) functions as a chaperone and it's a well-known protein of the bull seminal plasma associated with fertility and freezability [47–49]. While it had not yet been described in oocytes, it was identified in the bovine follicular fluid [50]. Its role in inhibiting apoptosis in granulosa cells has been documented [51,52], and the mechanism might be associated with the ER stress and the UPR signaling pathway. ER stress is known impair mammalian oocyte maturation and preimplantation embryo development (reviewed by Ref. [44]). Further research is needed to determine if clusterin can also be used during the in vitro maturation of bovine oocytes to reduce ER stress and thereby improve oocyte quality, optimizing blastocyst production outcomes.

Among the 806 proteins identified in both groups, 19 proteins had differential abundance. In bovine oocytes matured in vitro at 37.5 °C, seven proteins were upregulated compared to the control group, with four of them having known functions in oocytes: peroxiredoxin 4 (PRDX4), zona pellucida sperm-binding protein 3 (ZP3), heat shock protein family A member 6 (HSP70), and NAD-dependent isocitrate dehydrogenase (NAD-IDH), mitochondrial subunit.

PRDX4 has been previously reported in oocytes [53], although its isolated activity has not been described. Proteins in the PRDX family are involved in chromosomal organization and meiotic spindle assembly and regulate signaling by modulating reactive oxygen species (ROS) concentration or protein interactions through redox signaling. In oocytes, this occurs at the meiotic spindle [54].

ZP3 is associated with fertilization, facilitating sperm binding to the zona pellucida and triggering the acrosome reaction [55–59]. It subsequently helps prevent polyspermy and plays a role in germinal vesicle

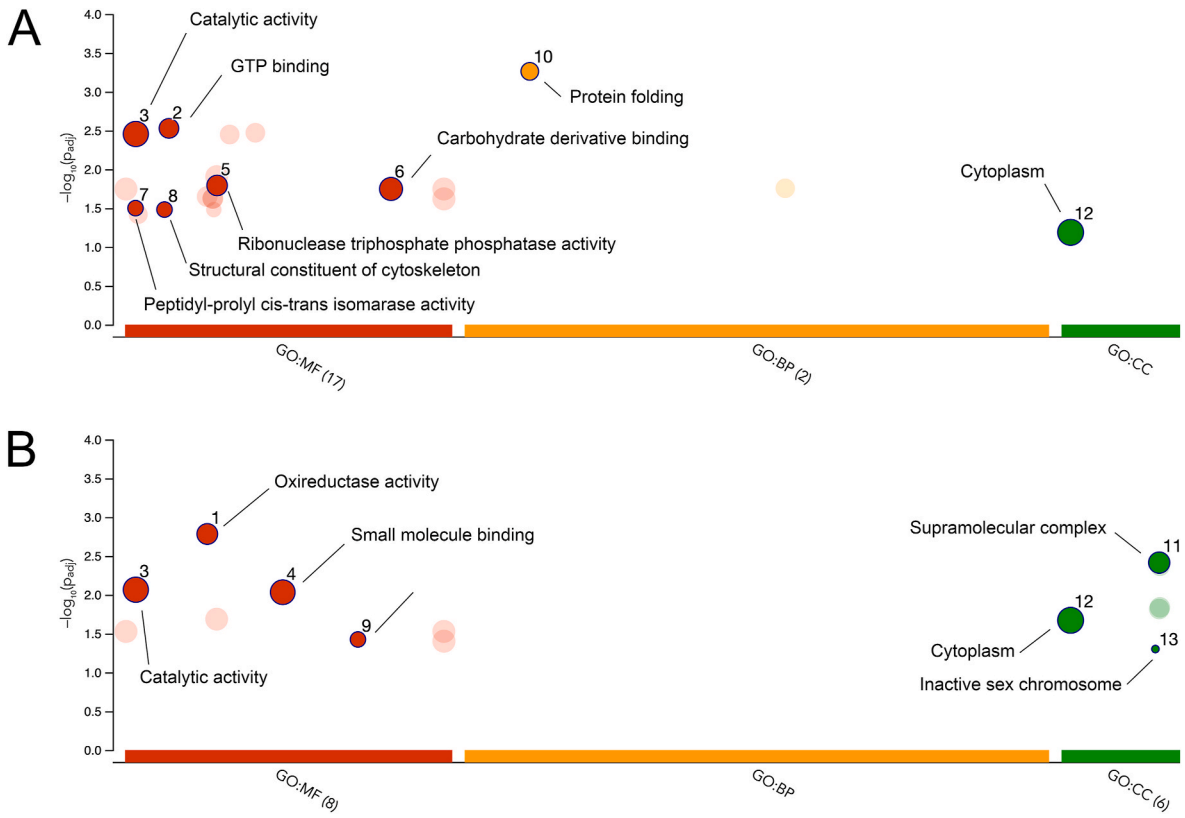

Fig. 4. Enrichment analysis of the differential proteome of bovine COCs in vitro maturated at 37.5 °C (A) and 38.5 °C (B). g:Profiler indicates the most representative gene ontology (GO) terms for differentially abundant proteins, revealing an up-regulation of proteins involved in protein folding and GTP metabolism, and down-regulation of proteins involved in cytoskeleton arrangement and enzymes with oxireductase activity.

breakdown by regulating cytoskeleton organization [60].

HSP70, is a multitask protein, acting in diverse processes, including assisting in nascent protein folding and regulating client protein location and translocation within the cell [61]. HSP70 (HSPA) is the first heat shock protein (HSP) detected during oogenesis due to its crucial role in cell division involving the PI3K/AKT pathway. Cytoplasmic HSP70 is the most conserved among HSP families [62], including the inducible HSP70, which is produced in small amounts under normal conditions but is highly responsive to heat shock and other environmental stresses [63]. Additionally, there is constitutively expressed HSP70, which is present under normal conditions and slightly upregulated under stress. HSP70 remains present until ovulation and plays a key role in regulating various aspects of folliculogenesis in animal models and human ovaries [64]. Its expression in ovarian cells is induced by hormonal stimulation [65,66], particularly controlling the functionality of estrogen, progesterone, and androgen [67]. In addition, HSP70 inhibits steroid effects and biosynthesis by downregulating estrogen and progesterone receptor expression [68,69]. Given its protective potential, Stamperna and colleagues evaluated the effects of supplementing HSP70 in in vitro matured bovine oocytes under thermoneutral and heat stress conditions. They found that exogenous HSP70 protected oocytes and cumulus cells, promoted blastocyst formation, inhibited apoptosis, facilitated signal transduction, and enhanced antioxidant protection. Additionally, HSP70 increased the expression of three HSP genes regardless of incubation temperature [70].

Additionally, there were twelve downregulated proteins in the experimental group, with five previously associated with oocytes: NDK, heme oxygenase (HO), dynein light chain (DYNLL1), thioredoxin (TRX), and NADP-dependent isocitrate dehydrogenase (NADP-IDH).

HO-1 may be considered a marker of oocyte competence, as its deficiency results in ovulation and fertilization failure [71]. DYNLL1 is part of the dynein protein complex involved in microtubule organization and cytoplasmic maturation [72,73]. TRX family member TXNDC9 participates in meiotic spindle conformation and redox balance during meiosis [74].

The IDH protein was identified twice: the NAD-IDH mitochondrial isoform was upregulated, while the NADP-IDH isoform was down-regulated. IDH plays a crucial role in the tricarboxylic acid cycle, with mitochondrial NAD-IDH using NAD and NADP as electron acceptors, while cytosolic NADP-IDH solely uses NADP [75]. NADP-IDH typically appears in higher concentrations in other mammalian cells and is involved in antioxidant defense [76]. Optimal ROS levels are essential for cellular signaling, while excessive ROS is detrimental [77,78]. Thus, reduced NADP-IDH accumulation could be due to NAD-IDH's action, negating the need for excessive antioxidant activity.

In silico protein-protein network analysis and functional enrichment of differentially abundant proteins revealed histone 4 (HIST1H4I, gene: H4C6) as the network's central node, linking over-expressed structural proteins and metabolic enzymes. However, no clear clustering was observed, suggesting that other factors, such as miRNAs, may be involved. To verify this, genes encoding proteins absent and down-regulated in oocytes matured at 37 °C were examined for potential miRNA regulation. This approach using bioinformatics have been used previously to identify putative miRNA targets from differential proteomic studies [21,22]. miRNAs' role in bovine oocyte maturation has been documented [79–82], showing that both dynamic and stable populations of miRNAs are present in bovine oocytes and zygotes, highlighting the critical role of small RNAs in oocyte maturation and early embryo development [83]. *In silico* screening identified 43 miRNAs that might be involved in the downregulation of six genes that encode bovine oocyte proteins. The miR-1343-5p isoform, associated with HO and CARHSP1, is linked to follicular development [84]. miR-339a, miR-423-3p, and miR-140 isoforms regulate oocyte maturation, with all miRNAs in this study connected to downregulated proteins in the

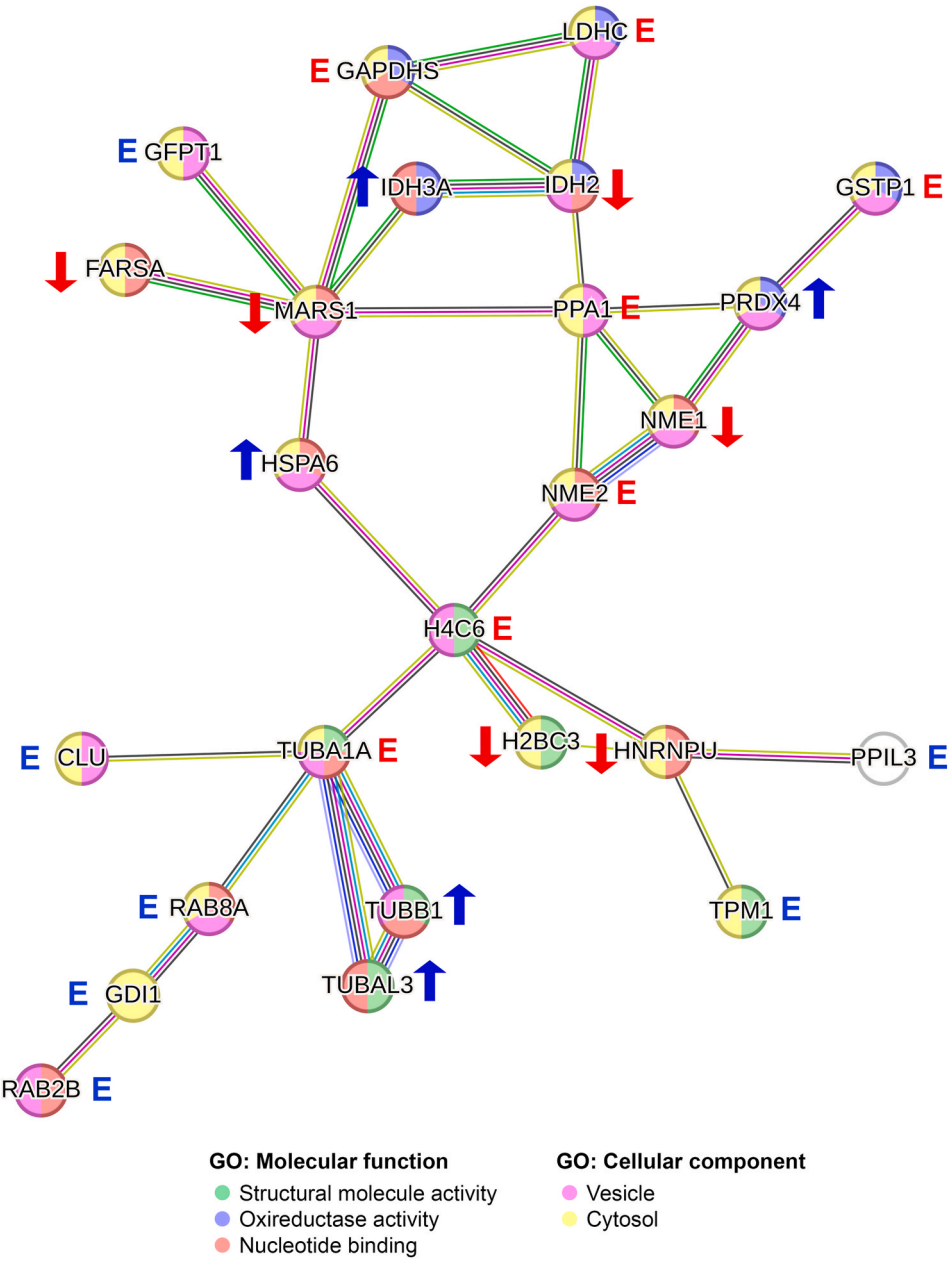

Fig. 5. Protein-Protein Interaction Network of Differentially Abundant and Exclusive Proteins in Bovine Oocytes Matured In Vitro at 38.5 °C and 37.5 °C. The network illustrates the protein-protein interactions among differentially abundant and exclusive proteins identified in bovine oocytes matured in vitro at 38.5 °C (control) and 37.5 °C (treatment). Blue arrows indicate up-regulated proteins, while red arrows indicate down-regulated proteins. Blue capital "E" denotes proteins exclusive to the treatment group, and red capital "E" denotes proteins exclusive to the control group. The network was generated using String v.12. Permalink: https://version-12-0.string-db.org/cgi/network?networkId=bTMYkBwqfB6l.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proteomic analysis [80]. Further research is necessary to determine if these proteins are absent or down-regulated in oocytes matured at 37.5 °C due to miRNA silencing or another mechanism.

5. Conclusion

In vitro maturation (IVM) of bovine oocytes at near-physiological temperatures does not affect nuclear maturation, cumulus cell expansion, or nitrite concentration in the IVM medium. However, it induces changes in the COCs proteome, including differential protein abundance and the presence of unique proteins at 38.5 °C (control) and 37.5 °C. At 38.5 °C, unique proteins related to cellular respiration and meiosis progression were identified, while at 37.5 °C, unique proteins associated with protein folding and apoptosis inhibition were found, suggesting a lower metabolic demand at this temperature. These proteins, which have not yet been functionally characterized in oocytes, are important candidates for future research on oocyte maturation. Further studies are needed to determine if 37.5 °C is the optimal temperature for IVM of bovine oocytes and to elucidate the roles of these unique and previously undescribed proteins.

CRediT authorship contribution statement

Winny Caldas Moreno Tavares: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Vinicius Maretto:** Writing – original draft,

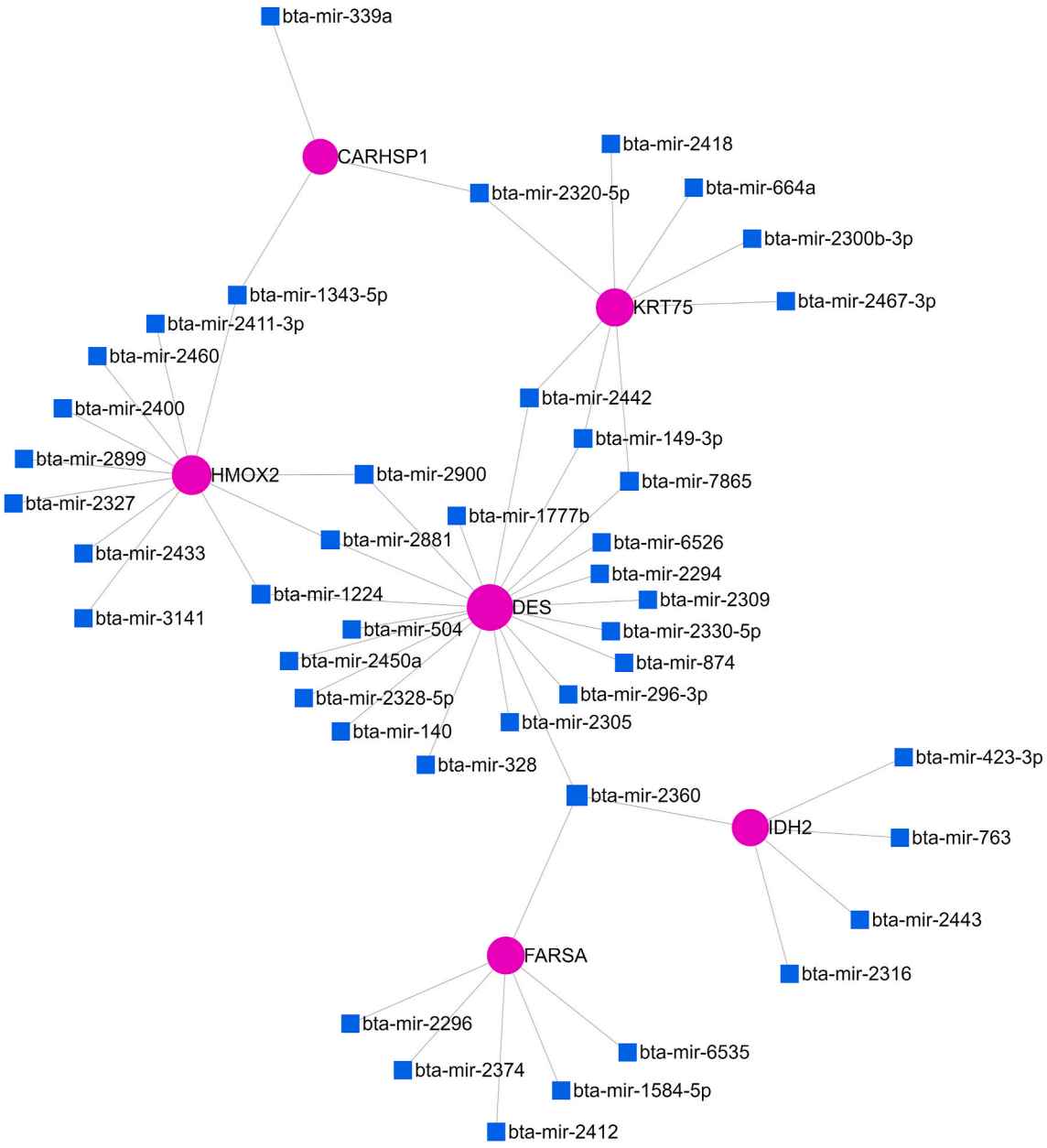

Fig. 6. Network analysis and gene set enrichment analysis of bovine miRNA (squares) associated with genes (circles) that enconde for proteins with significative lower abundance in bovine oocytes matured in vitro at 37.5 °C (treatment group). Scheme and analysis obtained by miRNet 2.0.

Visualization, Methodology, Investigation, Formal analysis. **Vanildo Silveira:** Writing – original draft, Validation, Supervision, Software, Resources, Methodology, Funding acquisition. **Vitor Batista Pinto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Ivan Cunha Bustamante-Filho:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis. **Celia Raquel Quirino:** Writing – review & editing, Formal analysis. **Wilder Hernando Ortiz Vega:** Writing – review & editing, Visualization, Software, Formal analysis. **Maria Clara Caldas-Bussiere:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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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.2024.07.026.

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