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Seasonal variations in the metabolomic profile of the ovarian follicle components in Italian Mediterranean Buffaloes

Michal Andrzej Kosior [a], Riccardo Esposito [a], Natascia Cocchia [a, *], Federica Piscopo [a], Valentina Longobardi [a], Nunzio Antonio Cacciola [a], Giorgio Antonio Presicce [b], Giuseppe Campanile [a], Hilde Aardema [c], Bianca Gasparrini [a]

[a] Department of Veterinary Medicine and Animal Production – Federico II University, Via F. Delpino 1, 80137, Naples, Italy
[b] ARSIAL, Centro Sperimentale per la Zootecnia, Via Lanciani 38, 00162, Rome, Italy
[c] Department of Farm Animal Health, Faculty of Veterinary Medicine - Utrecht University, Yalelaan 7, 3584 CL, Utrecht, the Netherlands

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ABSTRACT

The aim of this work was to evaluate the seasonal effect on the metabolomic profile of the ovarian follicle in Italian Mediterranean buffalo to unravel the causes of the reduced competence during the non-breeding season (NBS). Samples of follicular fluid, follicular cells, cumulus cells and oocytes were collected from abattoir-derived ovaries during breeding season (BS) and NBS and analyzed by 1H Nuclear Magnetic Resonance. The Orthogonal Projections to Latent Structures of the Discriminant Analysis showed clear separation into seasonal classes and Variable Importance in Projection method identified differentially abundant metabolites between seasons. Seasonal differences were recorded in metabolite content in all analyzed components suggesting that the decreased oocyte competence during NBS may be linked to alteration of several metabolic pathways. The pathway enrichment analysis revealed that differences in the metabolites between the seasons were linked to glutathione, energy generating and amino acid metabolism and phospholipid biosynthesis. The current work allows the identification of potential positive competence markers in the follicular fluid as glutathione, glutamate, lactate and choline, and negative markers like leucine, isoleucine and β-hydroxybutyrate. These results form a major basis to develop potential strategies to optimize the follicular environment and the IVM medium to improve the competence of oocytes during the NBS.

1. Introduction

In the last years there has been an increasing awareness of the pivotal role played by buffalo as a valuable protein source particularly for tropical and subtropical countries, leading to a remarkable worldwide increase in buffalo population, currently estimated to sit at 207 million heads [1]. The buffalo is a short-day breeder, exhibiting improved reproductive performance during months characterized by decreasing day length [2,3]. Reproductive seasonality is right now the main constraint of buffalo farming, leading to discontinuity of milk supply during the year. In Italy, as natural calving pattern is opposite to milk market demand, the out of breeding mating strategy (OBMS) is used [3]. The OBMS undoubtedly allows a more even calving distribution throughout the year but, a higher incidence of embryonic mortality is recorded during the non-breeding season (NBS) [2,4,5]. The higher incidence of embryonic mortality is partly due to reduced luteal function, and hence decreased progesterone secretion [6], which is related to delayed embryo growth and is associated to transcriptomic and proteomic changes [7,8], resulting in impeding embryo attachment. However, embryonic mortality during the NBS in Italian Mediterranean buffalo is also caused by decreased oocyte developmental competence, as clearly indicated by lower cleavage and blastocyst rates after in vitro fertilization [9,10]. In Murrah buffalo heifers the lowest recorded oocyte quality during the NBS was associated to reduced intrafollicular levels of estradiol and IGF-1 [11]. The oocyte acquires developmental competence, i.e. the capability to undergo fertilization and embryo development, during the last phase of growth that is tightly coordinated with that of the follicle [12]. The acquisition of oocyte developmental competence is a gradual

* Corresponding author. Via F. Delpino 1, 80137, Naples, Italy.
E-mail address: ncocchia@unina.it (N. Cocchia).

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process involving a precisely modulated spatio-temporal expression of various genes [12,13]. It is known that microRNAs (MiRNAs) play an important role in regulating gene expression [14]. Interestingly, seasonal variations in miRNAs content and transcriptomic profile in both buffalo oocytes and follicular cells were recently demonstrated [15]. The seasonal dependent changes in gene expression likely result in different metabolite abundance, influencing indeed the metabolic profile of the follicle. Therefore, the knowledge of metabolic profile of the follicle is fundamental to unravel the causes of reduced oocyte competence during the NBS. Metabolites are the most reliable indicators of phenotypic traits, as intermediate and/or end products of metabolic pathways. Metabolomics allows a broad identification of low molecular weight metabolites, that are the downstream products of genome, transcriptome and proteome expression [16,17] present in biological fluids, cells and tissues, and can provide a picture of the dynamic variations in response to environmental or genetic factors [18]. Currently several techniques are available for metabolomics, such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), Fourier transform infrared spectroscopy, Raman spectroscopy and nuclear magnetic resonance spectroscopy [17], which has been considered one of the strongest techniques for biological fluid research [19]. The follicular microenvironment where the oocyte grows certainly influences oocyte competence. It is known that follicular fluid composition, influenced by climate [20] and nutrition [21] affects the oocyte maturation process [22]. Metabolomic analysis of the follicular fluid has been carried out in cattle allowing the identification of some predictive markers for oocyte developmental competence [23]. Furthermore, variations in total fatty acid and amino acid profile were recorded in follicular fluid from cows compared with heifers, with low and high fertility, respectively [24]. To the best of our knowledge, metabolomics has so far not been applied to buffalo female reproduction. Therefore, the purpose of this experiment was to evaluate whether the season influences the metabolite content and metabolic pathways within the ovarian follicle in this species, to unravel the causes of the reduced competence during NBS and lay the basis for further studies to develop corrective strategies. To do so, we investigated the metabolomic profile of various components of the buffalo ovarian follicle in relation to season, such as follicular fluid, follicular cells, cumulus cells and oocytes by H1 Nuclear Magnetic Resonance (NMR) spectroscopy.

2. Material and methods

2.1. Experimental design

The samples were collected in October, i.e during the breeding season (BS) and in January, i.e., during the non-breeding season (NBS), over a total of 8 replicates (4/season). The ovaries were collected at a local slaughterhouse (Real Beef s.r.l., Flumeri (AV), Italy), under national food hygiene regulations from cyclic multiparous Italian Mediterranean buffalo cows (on average 40 per season), grown under controlled nutrition and housed inside barns in intensive farms located in the province of Caserta (Italy). The age and weight of the animals were respectively 4.8 ± 0.6 years and 547.2 ± 14.3 kg. Cyclic ovarian activity was assessed by two clinical examinations carried out 12 days apart before slaughter, to detect the presence of a follicle greater than 1 cm and/or corpus luteum on the ovary. The ovaries of healthy and cyclic animals were transported to the laboratory within 4 h after slaughter in physiological saline supplemented with 150 mg/L kanamycin at 30–35 °C. During each season four replicates were carried out to collect follicular fluid, follicular cells, cumulus cells and oocytes. Briefly, on the day of slaughter follicular fluid was aspirated, separated from follicular cells and half of the COCs found was denuded in order to collect both oocytes and cumulus cells; then these samples were stored at - 80 °C until analyses. The remaining COCs were in vitro matured, fertilized and cultured according to our standard protocol [10,25] briefly described below, to assess developmental competence.

2.2. Collection of follicular fluid and follicular cells

In the laboratory the ovaries were washed three times with physiological saline with 150 mg/L kanamycin to remove blood residues. Follicular fluid was aspirated from 3 to 5 mm diameter follicles using an 18 G needle under vacuum (40–50 mm Hg) and collected in Falcon tubes. Because of the limited volumes typically aspirated from small follicles, fluids from 20 follicles were pooled and poured into a petri dish for oocyte recovery. All the COCs found were allocated to a dish with HEPES-buffered TCM199 supplemented with 10% fetal calf serum (FCS; H199) and evaluated according to morphology and classified according to Di Francesco et al. (2011) [9]. The remaining fluid was transferred into 1.5 ml eppendorf tubes and centrifuged (300 g for 10 min). The supernatant was then stored as follicular fluid (FF). The remaining pellet was washed in PBS and subjected to two consecutive centrifugations (2000 g × 10 min), then the supernatant was removed and the pellet stored at −80 °C as follicular cells (FC). Grade A and B COCs, considered suitable for in vitro embryo production (IVEP), were quickly selected from the dish and washed thoroughly in medium H199, while the other oocyte categories were discarded.

2.3. Collection of cumulus cells and oocytes

For each replicate, A and B COCs were split into two groups, as previously described. In the first group the oocytes were pooled into groups of 10, then moved into vial tubes in PBS and vortexed for 3 min. Then the content of the tubes was moved to a petri dish, the oocytes were removed and transferred into eppendorf tubes in minimum volume and stored at −80 °C as oocytes (OO). The remaining liquid was centrifuged at 2500 g × 15 min and then, after removing the supernatant, the pellet was stored at - 80 °C as cumulus cells (CC).

2.4. In vitro embryo production

For each replicate, Grade A and B COCs recovered by follicular aspiration were rinsed in H199 medium and in vitro matured. The methods for in vitro maturation (IVM) described below have been reproduced in part from Gasparrini et al. (2000) [26]. Briefly, COCs were allocated to 50 μL drops (10 per drop) of IVM medium, i.e., in TCM199 buffered with 25 mM sodium bicarbonate and supplemented with 10% FCS, 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL 17 β-estradiol and 50 μg/mL kanamycin and incubated at 38.5 °C for 21 h in a controlled gas atmosphere of 5% CO2 in humidified air.

The methods for in vitro fertilization (IVF) and culture (IVC) described below have been reproduced from Di Francesco et al. (2012) [10]. Frozen straw from a bull previously tested for IVF were thawed at 37 °C for 40 s and sperm was selected by centrifugation (25 min at 300 g) on a Percoll discontinuous gradient (45% and 80%). The sperm pellet was re-suspended to a final concentration of 2×10^6 mL^{-1} in the IVF medium, consisting of Tyrode albumin lactate pyruvate supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine and 0.01 mM heparin. Insemination was performed in 50 μL drops of IVF medium under mineral oil (5 oocytes per drop) at 38.5 °C under humidified 5% CO2 in air. Twenty hours after IVF, presumed zygotes were removed from the IVF medium, stripped of cumulus cells by gentle pipetting and allocated to 20 μL drops of

IVC medium, i.e., synthetic oviduct fluid (SOF) including essential and non-essential amino acids and 8 mg/mL bovine serum albumin [27]. Culture was carried out under humidified air with 5% CO_2, 7% O_2 and 88% N_2 at 38.5 °C. On day 5 post-insemination (pi) the cleavage rate based on the number of oocytes was assessed, and the embryos were transferred into fresh medium until day 7 of IVC (end of culture). The embryos were scored for quality on the basis of morphological criteria [28], and the percentages of transferable embryos (Grade 1 and 2 tight morulae and blastocysts) and of Grade 1 and 2 blastocysts were recorded.

2.5. Extraction of the polar fraction from samples

The methods for extraction of the polar phase have been reproduced from Santonastaso et al. (2017) [29]. Briefly, each sample type was first re-suspended in 170 μL of H2O and 700 μL of methanol. For cell samples (FC, CC and OO) the pellet-containing solution was sonicated for 30 s to lyse the membranes, cell lysis was confirmed under optical microscope and lysates were further processed, while sonication was not carried out for cell-free samples (FF). The same fraction of each sample was processed, to avoid possible influence of cell concentration on the relative amount of metabolites. Then, 350 μL of chloroform was added, and the samples were mixed on an orbital shaker in ice for 10 min. After this, 350 μL of a 1:1 (v/V) H_2O/chloroform solution was added to each sample, that was vortexed for 5 s and centrifuged at 10000 rpm for 10 min at 4 °C. Following centrifugation three different phases were separated: an upper phase (containing polar metabolites), a middle phase (with cell debris, denatured proteins and RNA) and a lower phase (containing apolar metabolites). Therefore, both the aqueous (polar) and lipophilic (apolar) phases were collected and evaporated but for this work only the polar phase was analyzed.

2.6. 1H NMR metabolomic analysis

The methods for 1H NMR metabolomic analysis have been reproduced from Santonastaso et al. (2017) [29]. The polar fractions were dissolved in 630 μL of PBS-D_2O with the pH adjusted to 7.2, and 70 μL of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D_2O) was used as the internal standard. A 600 MHz Avance Bruker spectrometer with a TCI probe was used to acquire 1H NMR spectra at 300 K. An excitation sculpting pulse sequence was applied to suppress the water resonance. A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and gradient pulses of 1 ms each in duration adding 128 transients of 64k complex points, with an acquisition time of 4 s per transient. Time domain data were all zero filled to 256k complex points and an exponential amplification of 0.6 Hz prior to Fourier transformation was applied.

All of the 1H NMR spectra were automatically phased using the “apk” command in Topspin 4.0 (Bruker, Biospin, Germany) that performs an automatic phase correction using both a zero and first order correction. In few cases we optimized manually the spectra phasing. Then, we performed a baseline-correction using the “absn” command in Topspin 4.0 (Bruker, Biospin, Germany) that automatically fits the spectra baseline to a polynomial of degree given by the processing parameter absg (usually 5). We referenced the spectra to the CH_3 resonance of TSP at 0 ppm. The spectral 0.50–8.60 ppm region of 1H NMR spectra was integrated in buckets of 0.04 ppm by AMIX package (Bruker, Biospin, Germany). In details, we excluded, in the case of the polar spectra, the water resonance region (4.5–5.2 ppm) during the analysis. We normalized by MetaboAnalyst v5.0 tool [30] the bucketed region using a normalization procedure grouped into two categories: i) sample normalization that is for general-purpose adjustment for systematic differences among samples; and ii) data scaling that adjusts each variable/feature by a scaling factor computed based on the dispersion of the variable. In details, we normalized by sum (the total spectrum area) and used Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable).

2.7. Measurement of antioxidant markers in follicular fluid

The activities of total superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) were determined using commercial ELISA kits (SOD: E-BC-K020-M; CAT: E-BC-K031-M;

GSH-Px: E-BC-K096-M) from Elabscience Biotechnology Inc. (Houston, TX, USA), following the manufacturer's protocol. The total antioxidant capacity (TAC) was measured with a commercial kit (MAK187-1 KT, Sigma, Milan, Italy), following the manufacturer's instructions. The absorbance readings were recorded by using a microplate reader (BioTek™ Cytation™ 3, Winooski, VT, USA).

2.8. Statistical analysis

Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) and S-Plot were used by the Metabo Analyst tool to analyze differences between seasons in different types of samples because it can effectively deal with chemical shift variation in full-resolution 1H NMR datasets without the need of binning or alignment steps [31]. Furthermore, the Variable Importance in Projection (VIP) method was used to identify specific metabolites that show the greatest variations between seasons and their pattern.

The threshold of VIP score greater than 1 was used to select more relevant variables. Q2 was used as an estimate of the predictive ability of the model, and is calculated via cross-validation (CV). The metabolites were identified and quantified by Chenomx Profiler [32].

Finally, pathway analysis on polar metabolites was performed using the Metabo Analyst tool [31].

Differences in cleavage and blastocyst rates between seasons were analyzed by Chi square test.

Comparisons between samples of BS and NBS for SOD, GSH-Px, CAT activities and total antioxidative capacity (TAC) by ELISA were made by two-tailed unpaired Student's *t*-test, using GraphPad Prism 9.0 software (La Jolla, San Diego, CA, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. In vitro embryo production

The NBS cleavage rate was decreased compared to the BS (60.7 vs 76.8%; $P < 0.01$). Furthermore, during the NBS there was a decrease of the percentages of total transferable embryos (17.9 vs 28.6%; $P < 0.05$) and of grade 1 and 2 blastocysts (16.2 vs 26.2%; $P < 0.05$).

3.2. Metabolomic analysis of follicular fluid

The OPLS-DA plot evidenced that the FF samples from different seasons clustered separately, even though NMR spectra slightly overlapped. The distinct clusters per season suggest the presence of significant differences in proton signals (metabolites) between the seasons (Fig. 1-A). The VIP plot shows the 15 proton signals that correspond to specific metabolites with the largest seasonal variation in FF (Fig. 2-A). Interestingly, most of the metabolites (12/15) in FF were present in lower amounts during the NBS. More

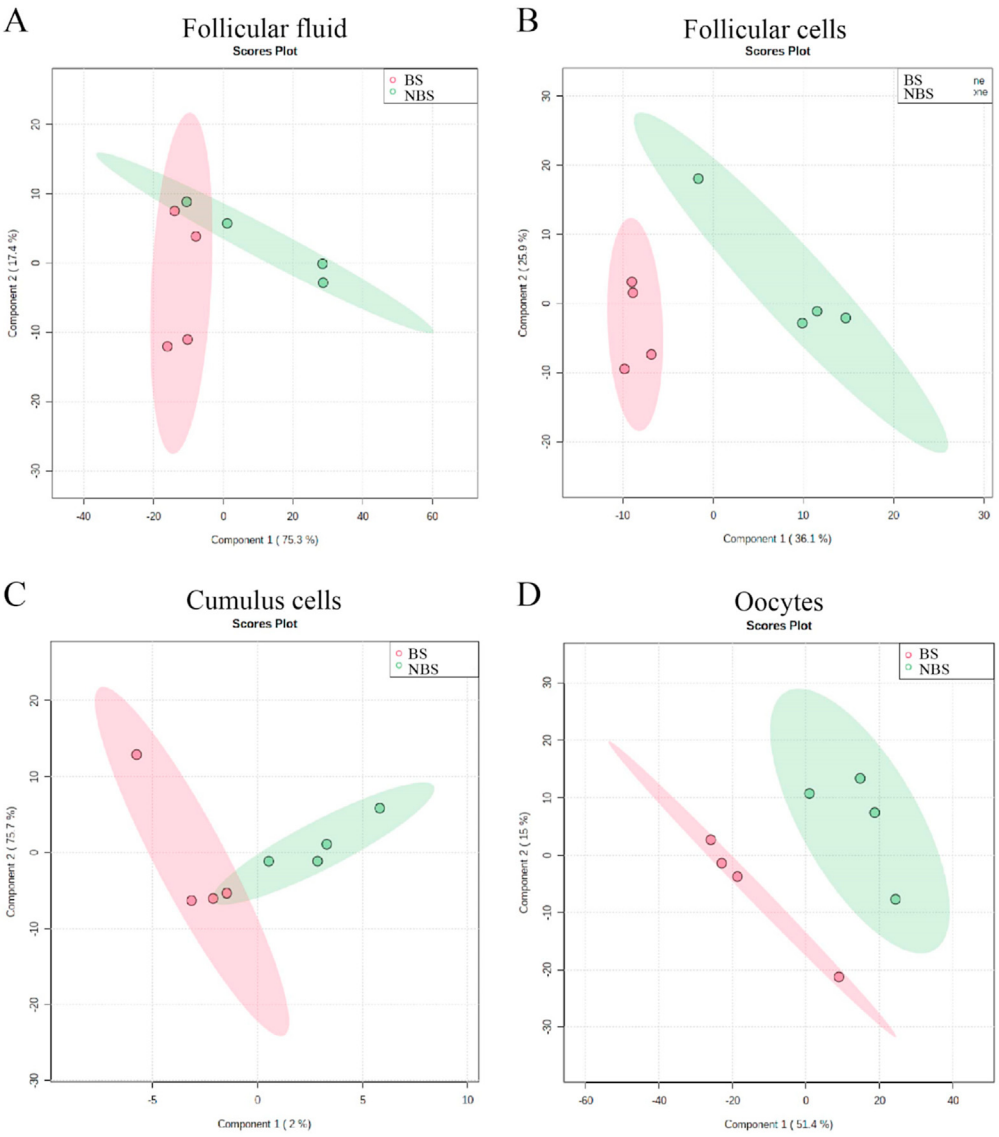

Fig. 1. Score plot of samples collected during the breeding (BS) and non-breeding (NBS) seasons. A) Follicular fluid; B) Follicular cells; C) Cumulus cells and D) Oocytes.

specifically, reduced levels of lactate, trimethylamine, lysine, serine, cysteine, glutamate, glutathione, glycerophosphocholine, proline, coline and phosphocoline were detected during the NBS. In contrast, β-hydroxybutyrate, leucine and isoleucine were more abundant during the NBS. The pathway analysis showed the main metabolic pathways in which the significant metabolites in FF are involved, such as glutathione metabolism, valine/leucine/isoleucine degradation and phospholipids biosynthesis (Supplementary files 1 and 2).

3.3. Metabolomic analysis of follicular cells

The OPLS-DA evidenced that samples of follicular cells collected in different seasons clustered into two distinct groups, suggesting identifiable differences in the metabolic profile in relation to season (Fig. 1-B). The VIP plot showed that the levels of tryptophan, glutathione, glucose, phosphocholine and glycerophosphocholine were reduced in the NBS, while those of lactate, 3-hydroxybutyrate, ATP, valine, leucine, isoleucine and lysine were increased (Fig. 2-B). The pathways enrichment analysis revealed that the metabolites showing seasonal variations in FC are mainly involved in glycidic metabolism, valine/leucine/isoleucine degradation, glutathione metabolism and phospholipids metabolism (Supplementary files 1 and 2).

3.4. Metabolomic analysis of cumulus cells

The OPLS-DA plot showed a clear separation of cumulus cells (CC) between the two different seasons, indicating differences in the metabolic profile of the NBS and BS (Fig. 1-C). The VIP plot showed that the content of hydroxyproline and glucose was lower whereas that of phosphocholine, glycerophosphocholine, threonine, valine, lactate, isoleucine and ATP was higher during the NBS compared to the BS (Fig. 2-C). The pathway analysis showed that the metabolites showing seasonal variations in CC were mainly involved in glycidic metabolism, valine/leucine/isoleucine degradation and phospholipid metabolism, as illustrated in Supplementary files 1 and 2.

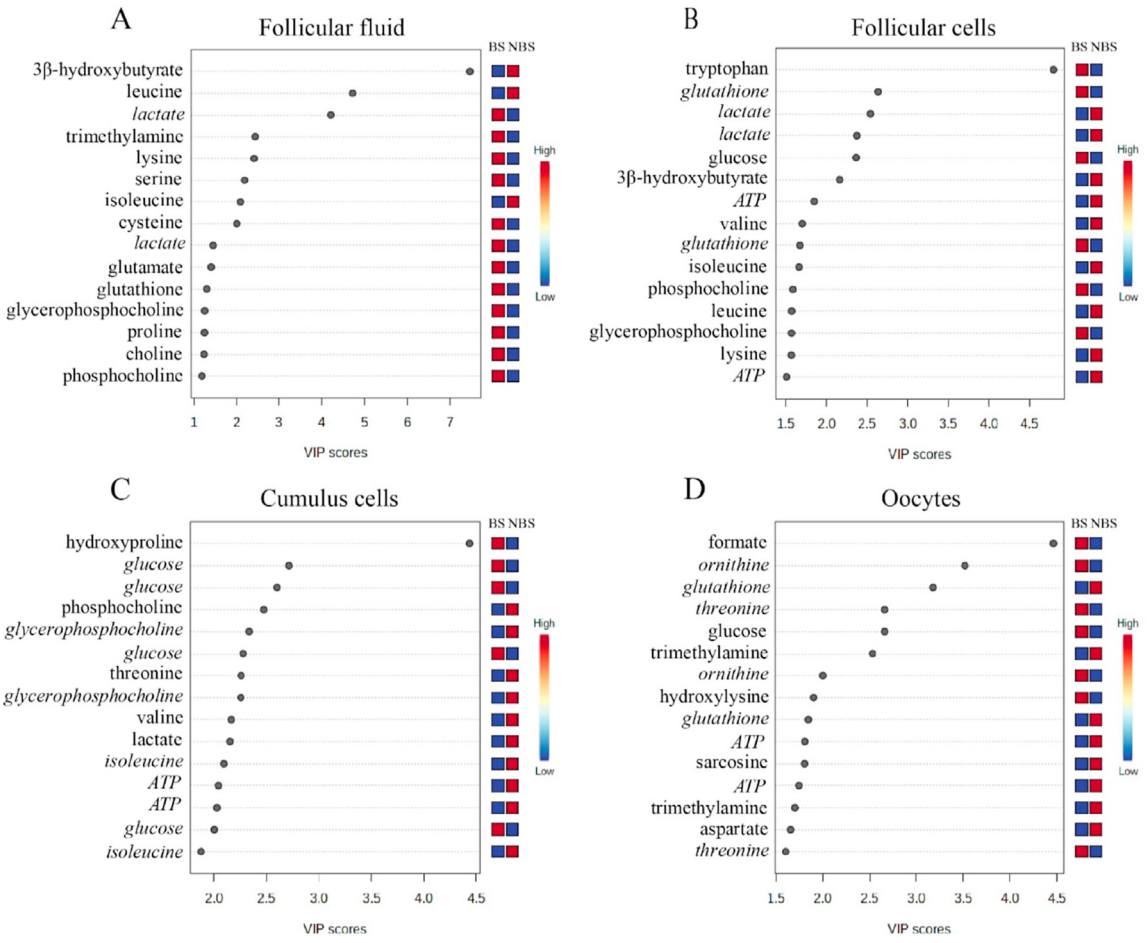

Fig. 2. Variable importance in projection (VIP) plot of samples collected during the breeding (BS) and non-breeding (NBS) seasons. A) Follicular fluid; B) Follicular cells; C) Cumulus cells and D) Oocytes. The metabolites for which more than 1H proton was present in the VIP plot are reported in italics.

3.5. Metabolomic analysis of oocytes

Oocyte samples collected in different seasons also clustered separately as shown in Fig. 1-D. Decreased levels of formate, ornithine, threonine, glucose and hydroxylysine and an increased content of glutathione, ATP, sarcosine, trimethylamine and aspartate were observed during the NBS compared to the BS (Fig. 2-D). The pathway analysis revealed that the main metabolic pathways influenced by season based on seasonal variations in metabolites in the OO are the urea cycle, ammonia recycling glycidic metabolism, glutathione metabolism, folate and aspartate metabolism in the OO (Supplementary files 1 and 2).

An overall representation of seasonal variations in metabolite content for the different types of samples is provided in Table 1.

3.6. Antioxidant markers in the follicular fluid (FF)

As shown in Fig. 3, no differences were found in superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the FF between seasons. Interestingly, there was a reduced catalase (CAT; $P \leq 0.05$) activity during the NBS. Furthermore, an increased total antioxidant activity (TAC; $P \leq 0.001$) was detected during the NBS (Fig. 3).

4. Discussion

To the best of our knowledge, this is the first study to use a metabolomic approach to characterize ovarian follicles in the

Table 1
Seasonal differences in metabolite content in follicular fluid (FF), follicular cells (FC), cumulus cells (CC) and oocytes (OO). ▲ (>1) and ▼(<1) indicate a respectively higher and lower concentration in the non-breeding season (NBS) vs breeding season (BS). The value in brackets is the ratio between normalized proton signals for each metabolite in the non-breeding season (NBS) vs breeding season (BS).

Metabolites	FF	FC	CC	OO
β-hydroxybutyrate	▲ (2.1)	▲ (1.8)		
Lysine	▼ (0.5)	▲ (1.6)		
Serine	▼ (0.3)			
Cysteine	▼ (0.3)			
Proline	▼ (0.2)			
Leucine	▲ (3.4)	▲ (2.1)		
Iso-Leucine	▲ (3.2)	▲ (2.5)	▲ (2.4)	
Sarcosine				▲ (1.9)
Valine		▲ (2.4)	▲ (2.5)	
Threonine			▲ (1.8)	▼ (0.4)
Ornithine				▼ (0.5)
Trimethylamine	▼ (0.1)			▲ (2.2)
Tryptophan		▼ (0.2)		
Aspartate				▲ (2.5)
Formate				▼ (0.4)
Glutamate	▼ (0.2)			
Glutathione	▼ (0.4)	▼ (0.5)		▲ (1.8)
Choline	▼ (0.5)			
Phosphocholine	▼ (0.1)	▼ (0.5)	▲ (0.5)	
Glycerophosphocholine	▼ (0.4)	▼ (0.7)	▲ (2.3)	
Glucose		▼ (0.4)	▼ (0.4)	▼ (0.4)
Lactate	▼ (0.8)	▲ (2.6)	▲ (3.2)	
ATP		▲ (2.1)	▲ (2.0)	▲ (3.5)

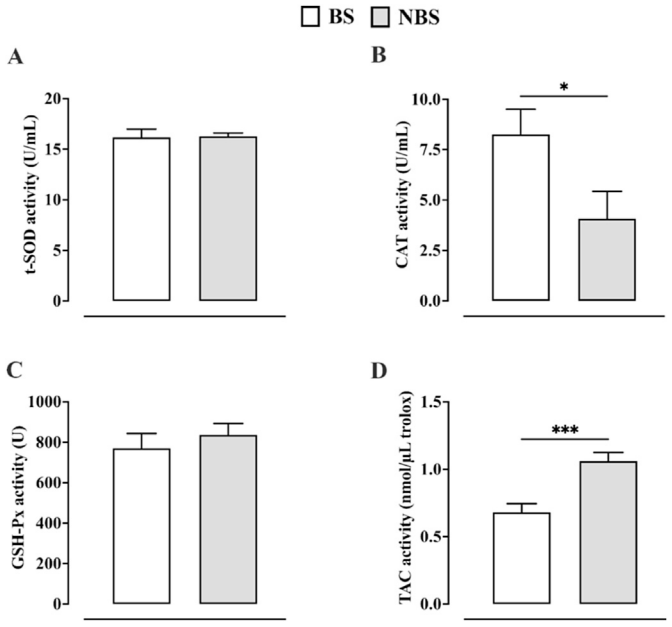

Fig. 3. Seasonal differences in follicular fluid for; A) superoxide dismutase (SOD), B) glutathione peroxidase (GSH-Px), C) catalase (CAT), and D) total antioxidant activity (TAC).*P ≤ 0.05; ***P ≤ 0.001.

Italian Mediterranean buffalo. Furthermore, this is the first report of seasonal variations in metabolite content in different components of the follicle, including the follicular fluid, follicular and cumulus cells, and oocytes. The rationale behind this work originates from the hypothesis that the environment in which buffalo oocytes develop is suboptimal during the NBS, leading to impaired oocyte competence. Indeed, seasonal variations in several amino acids, energy substrates, and cholines were observed at different levels of the follicle, which may be related to the decreased oocyte developmental competence observed in Italian Mediterranean buffaloes during the NBS.

Reduced oocyte competence, indicated by lower cleavage and blastocyst rates of the oocyte during the NBS, was also recorded in the current study, in agreement with previous reports [9,10]. Intriguingly, the majority of the metabolites exhibiting seasonal changes in the FF were reduced during the NBS, except for β-hydroxybutyrate (βHB), leucine, and isoleucine. Of particular interest was the higher content of βHB during the NBS in the FF and FC, which was the metabolite with the highest variation between seasons. This finding is in agreement with the higher follicular levels of total nonesterified fatty acids (NEFAs) and saturated palmitic and stearic acids previously reported during the NBS [33]. It is worth pointing out that at the Italian latitudes where the trial was performed, the NBS began in winter when the environmental temperatures were low, playing an additive role in the photoperiod. It is likely that, due to their tropical origin, buffaloes do not adapt well to cold temperatures and, especially during the transition from the mild to cold periods, inappropriate thermoregulation may lead to a negative energy balance (NEB). An NEB results in body fat and muscular protein mobilization to supply alternative energy for tissue and precursors to maintain the glucose levels within physiological ranges, resulting in increased levels of NEFA and βHB in the blood. In dairy cattle undergoing NEB, a correlation exists between the plasma and follicular levels of glucose, NEFA, βHB, urea, and total cholesterol [24,34]. It has been hypothesized that aberrant metabolic serum conditions that occur during NEB are reflected in the follicular environment and may negatively affect oocyte competence [35]. Indeed, low glucose levels, as well as high levels of urea and NEFA, particularly saturated palmitic and stearic acid, in follicular fluid are potentially toxic to bovine oocytes [36–41]. Furthermore, an additive toxic effect of βHB under moderate hypoglycemic conditions has been hypothesized [42]. The increased βHB content found in the current study and the elevated levels of total NEFAs, as well as palmitic and stearic acids (data reported in Ref. [33]) in FF samples suggest a condition of energy scarcity of buffaloes during the NBS, leading to NEB. The higher concentrations of leucine and isoleucine in the FF and FC during the NBS may also fit with an NEB condition and consequently protein mobilization, as these amino acids are markers of protein turnover [43]. In contrast, no seasonal changes were observed in the urea and glucose levels in the FF. However, a reduced amount of glucose was observed in the FC, CC, and OO during the NBS. The changes in the concentrations of glucose, lactate, and ATP are certainly suggestive of a seasonal impact on the energy metabolism of the follicle, which was also demonstrated by pathway analysis. Glycolysis represents the major energy pathway of the COC, with glucose as the main substrate for cumulus cells [44] and pyruvate, lactate, and ATP as the main end products. Indeed, cumulus cells are mainly glycolytic, whereas oocytes rely mainly on mitochondrial oxidation of pyruvate for ATP production [45,46]. Cumulus cells take up glucose from the follicular environment, metabolize it into pyruvate, and transfer it via gap junctions to the oocyte [47,48]. The reduced glucose levels in the FC, CC, and OO are in line with the hypothesized katabolic status of the animals during the NBS. However, the lower glucose levels and higher lactate levels detected in the FC and CC also suggest increased anaerobic glycolytic activity during the NBS; this hypothesis is not supported by the decreased lactate concentration in FF. Lactate, which is normally abundant in the follicular fluid and reproductive tract [49], modulates the cytosolic redox state. A higher lactate production has been associated with reduced oocyte competence in the mouse [50]. In contrast, decreased lactate levels, together with increased glucose levels, have been negatively associated with oocyte competence in humans [51]. The important role played by lactate in follicle metabolism and redox state suggests that the observed distinct concentrations of lactate between the seasons indicate disrupted energetic metabolism. The larger amount of ATP observed during the NBS in FC, CC and OO could be a consequence of the increased level of metabolites present in the follicular fluid (NEFA and ketone bodies), due to the presumed NEB condition, and uptake by the follicle. To satisfy energy requirements, an adequate amount of ATP in oocytes and embryos is fundamental for development, affecting nucleic acid and protein synthesis, and it has been suggested as a marker for the developmental potential of mouse [52] and bovine embryos [53,54]. Nevertheless, the association between ATP content and oocyte developmental competence is controversial, with contradictory findings in different species [55–57]. Interestingly, in a previous study, a higher triglyceride level was observed in the oocytes of the NBS [33]. Based on the previously described association between the developmental competence of oocytes in relation to the level of intracellular lipids and a link with high ATP levels [54], this finding requires further investigation. Overall, the findings in the current study certainly indicate a modification of the microenvironment of the developing oocyte, which may be derived from the serum changes linked to an NEB during the NBS that are reflected in the follicular fluid. The hypothesis that the observed decreased oocyte competence in buffaloes during the unfavorable season is associated with NEB and metabolic stress undoubtedly requires further investigation.

The results of this study also showed a seasonal difference between the profiles of amino acids and peptides at the follicular level. Among these, glutathione (GSH) and its precursors cysteine

and glutamate, as well as serine, which are involved in the synthetic pathway of cysteine, are of particular interest because of the known role of GSH in antioxidant cell defense and oocyte development. During the NBS, all of these metabolites were less abundant in FF, and the GSH levels were also reduced in FC. GSH contributes to sperm decondensation and male pronucleus formation after fertilization [58,59]. Maturing oocytes from several species, including buffalo [60,61], are capable of synthesizing GSH during maturation [59,62,63] to create a reservoir pool of GSH that protects embryos during early development until embryonic genome activation occurs, whereafter the embryo is capable of synthesizing GSH [64]. In our study, the reduced levels of serine, cysteine, and glutamate in the FF during the NBS were reflected in the reduced GSH content, suggesting that the oocyte grows in an environment deficient in antioxidant defense, which may account for the lower oocyte competence. The elevated intraoocyte concentration of GSH during the NBS observed in this study was unexpected, especially as GSH in the oocyte appears to be a marker of oocyte developmental competence and cytoplasmic maturation [65–67]. The higher GSH content in oocytes during the NBS may be related to the suggested higher nutrient supply at the level of the follicle due to increased levels of NEFAs [33] and β-HB, which is in line with the increased ATP content, suggesting a high energy status or decreased energy consumption by the oocyte. However, it cannot be ruled out that during the NBS, the oocytes growing in an environment lacking antioxidants increase the synthesis of GSH, resulting in a lower cysteine level in the FF to counteract oxidative stress. An analysis of the ROS levels, which was not performed in the current study, would certainly help understand the observed changes, as the intracytoplasmic GSH levels are related to the energy balance of the oocyte and ROS levels [68]. Nevertheless, the current dataset clearly demonstrates that during the NBS, the metabolism of GSH and its main precursors is affected at several levels within the follicle, as confirmed by pathway enrichment analysis, which may affect oocyte competence. To better characterize the oxidative status of the follicle, selected enzymatic markers (SOD, GSH-Px, and CAT) and TAC were analyzed in the FF. The reduced CAT activity found in the NBS is in line with the hypothesized reduced antioxidant defense in the follicular environment and with previous studies in which reduced levels of this enzyme were found in aged women known to produce less competent oocytes [69]. In another study, decreased GSH/catalase activity and increased SOD activity in human follicular fluid were demonstrated to be markers of oocyte senescence [70]. The lack of seasonal variations in the other enzymatic antioxidants (SOD and GSH-Px) observed in the current study is in contrast with previous studies in humans reporting a positive (GSH-Px) and negative (SOD) association with oocyte competence [70,71]. However, the pattern of SOD activity in our study agrees with a study conducted in Egyptian buffaloes, in which seasonal differences were also not found [72]. Furthermore, SOD activity in the buffalo follicular fluid did not vary in relation to oocyte competence, whereas the GSH levels were reduced in the follicular fluid corresponding to less competent oocytes [73]. The unexpectedly higher TAC level recorded during the NBS seems to be in contrast with the decreased CAT activity, along with the reduced GSH levels. However, a similar seasonal pattern of TAC activity was also reported in an earlier study on Egyptian buffaloes [72]. Interestingly, higher TAC levels in human follicular fluid associated with reduced oocyte competence were interpreted as the result of follicular antioxidant defense in response to persistent oxidative stress [74]. Therefore, taking into account all results, we speculate that other antioxidants may increase within the follicle during the NBS as a compensatory response to increased oxidative stress. Undoubtedly, a seasonal effect on the antioxidant balance within the follicle was observed, which may contribute to reduced oocyte competence during the NBS.

With regard to other amino acids, our results are in agreement with those of a previous study on the amino acid profiles of follicular fluid in cattle, in which the glutamate levels were higher and those of leucine and isoleucine were lower in the FF of competent versus noncompetent oocytes [75]. Interestingly, some of the FF amino acids that are considered predictive markers for oocyte competence in cattle, such as glutamate, leucine, and isoleucine, may also be candidate markers in buffalo based on the data from the current study. The lower follicular levels of proline, lysine, and serine found in our study during the NBS are in contrast with previous data from cattle, where decreased levels of lysine and proline in FF was related to increased oocyte competence and no relationship was found with the serine levels [75]. However, in humans, increased follicular proline levels were related to pregnancy success [51], which is in agreement with the higher proline levels and higher fertility recorded during the BS in the current study. Amino acids in bovine FF that were highly predictive of oocyte competence, such as alanine and glycine [23,51], were not different between the NBS and BS in our study, which demonstrates the importance of species-specific studies. Another interesting finding of the current study was the lower concentration of choline and its derivatives phosphocholine and glycerophosphocholine in in the FF and FC during the NBS. These data are in line with previous work in humans, where a reduction in the levels of choline, phosphocholine, and glycerophosphocholine in the FF was related to oocytes that did not cleaved after in vitro fertilization [51]. Seasonal changes in choline derivatives were also observed in somatic cells, showing opposite patterns in the FC and CC, likely related to the specific functions of the cell types. Cumulus cells are capable of rapidly phosphorylating choline into its derivatives, which can be taken up by the oocyte for energetic needs [76]. In any case, these changes are relevant as choline and phosphocholine are the precursors of phosphatidylcholine and are key components of biological membranes. The importance of these metabolites is confirmed by the compromised follicular development and oocyte maturation recorded in transgenic mice with an altered phosphatidylcholine production pathway [77].

Metabolic pathway analysis revealed that the metabolism of GSH and its precursors was affected by the season at most levels within the follicle (FF, FC, and OO). Seasonal effects were also observed in phosphatidylcholine biosynthesis and valine/leucine/isoleucine degradation in both FF and somatic cells. Furthermore, metabolic pathways involved in energy metabolism were mainly affected in somatic cells and oocytes, the latter also showing alterations in the metabolism of nitrogen, amino acids, and folate.

In conclusion, this study demonstrates a distinct composition of metabolites in the follicular components analyzed in buffalo between the NBS and BS. The seasonal differences in metabolites suggest that during the NBS, several metabolic pathways are altered, such as GSH metabolism and antioxidant capacity, energy-generating metabolism, phospholipid biosynthesis, and amino acid metabolism, which may be linked to decreased oocyte competence. Furthermore, potential markers of oocyte competence were identified in the FF; among these, the potential positive markers were GSH, glutamate, lactate, and cholines, whereas the negative markers were leucine, isoleucine, and βHB. The marked differences between the two seasons may suggest potential strategies to optimize the follicular environment or to adapt the IVM medium to improve the competence of oocytes collected during the NBS. However, future studies should investigate the mechanistic value of the observed differences, and absolute quantification of targeted metabolites is needed. In addition, evaluation of the ROS levels in the oocytes would help to understand the oocyte response to the

presumed suboptimal follicular conditions experienced during the NBS.

CRediT authorship contribution statement

Conceptualization and planning the experiments M.A.K, B.G. and N.C.; Samples collection R.E, V.L. and F.P; methodology for in vitro embryo production N.C, V.L and F.P; methodology for metabolites analysis M.A.K. and R.E.; analysis of antioxidant markers N.A.C; data analysis G.A.P and M.A.K.; writing-original draft preparation M.A.K., H.A. and B.G.; writing-review and editing M.A.K., B.G., H.A., N.A.C and G.C.; Funding acquisition B.G., G.A.P and G.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors have no conflict of interest to declare.

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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.2023.02.022.

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