UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

Doctoral Thesis

"Seasonal factors affecting oocyte competence in Italian Mediterranean Buffalo"

Coordinator
Prof. Giuseppe Cringoli

Candidate
Michal Andrzej Kosior

Tutor
Prof. Marco Russo

Co-Tutor
Prof. Bianca Gasparrini

Index

List of Abreviations

AA Arachidonic Acid
AI Artificial Insemination
An Antral (follicles)
ATP Adenosine Triphosphate
BS Breeding Season
CC Cumulus Cells
CC-IO Cumulus Cells from Immature Oocytes
CC-MO Cumulus Cells from Mature Oocytes
COC Cumulus-Oocyte Complex
DEG Differentially Expressed Gene
DEmiRNA Differentially Expressed miRNA
EV Extracellular Vesicles
FC Follicular Cells
FCS Fetal Calf Serum
FDR False Discovery Rate
FF Follicular Fluid
FSH Follicle Stimulating Hormone
GC-MS Gas Chromatography–Mass Spectrometry
GnRH Gonadotropin Releasing Hormone
GSH Glutathione
HB Hydroxybutyrate
HDL High-Density Lipoproteins
ICSI IntraCytoplasmatic Sperm Injection
IGF-1 Insuline-like Growing Factor 1
IL8 interleukin-8
IO Immature Oocytes
IVEP In Vitro Embryo Production
IVF In Vitro Fertilization
IVM In Vitro Maturation
IVS In Vitro Culture
LA Linoleic Acid
LC-MS Liquid Chromatography-Mass Spectrometry
LEM Late Embryonic Mortality
LH Luteinizing Hormone
miRNA MicroRNA
MO Mature Oocytes

List of Abreviations

MV Microvesicles
NBS Non-Breeding Season
NEB Negative Energy Balance
NEFA Non-Esterificated Fatty Acid
NMR Nuclear Magnetic Resonance
OBMS Out of Breeding Mating Strategy
OPLS-DA Orthogonal Projections to Latent Structures Discriminant Analysis
OPU Ovum Pick-Up
PBS Phosphate Buffer Solution
PCA Principal Component Analysis
pO Pre-Ovulatory (follicles)
PVA Polyvinyl Alcohol
ROS Reactive Oxygen Species
SM Spent Medium
T4 Thyroxine
TAG Triglycerides
VIP Variable Importance in Projection
β-HB Beta-Hydroxybutyrate

List of Figures

List of Figures

List of Figures

Lista delle tabelle

corresponding cumulus cells (CC-MO) and spent medium (SM). ▲ higher concentration in the NBS vs BS; ▼ lower concentration in the NBS vs BS

Abstract

The purpose of this thesis was to investigate the causes and the molecular mechanisms underlying the phenomenon of reproductive seasonality in the Italian Mediterranean Buffalo, with specific reference to oocyte developmental competence. The focus of this thesis was paid on the oocyte and the follicular environment, to unravel the causes of reduced oocyte competence during the non breading season, using novel approaches. Therefore, in Experiment 1 the miRNA and transcriptomic profiles of buffalo oocytes and corresponding follicular cells were compared between seasons. Then a further characterization of the follicular MiRNA content was carried out in Experiment 2. Specifically, the miRNA cargo of extracellular vescicles isolated from the follicular fluid was characterized both in relation to the stage of development (antral vs preovulatory follicles) and to the season. Finally, in Experiment 3 it was evaluated whether season influences the metabolome of the follicle. In particular, to further unveil molecular differences related to season, the metabolomic profile of various components of the ovarian follicle, such as follicular fluid, follicular cells, cumulus cells and immature oocytes was investigated. In addition, the metabolome of cumulus cells and oocytes after in vitro maturation, as well as that of the spent medium, was characterized. The results showed a seasonal variation in both oocytes and follicular cells in the expression of miRNA, known to be involved in follicular maturation and development regulation. Furthemore specific miRNAs contained in extracellular vescicles isolated from follicular fluid are involved in regulating follicular development and modulating seasonal effects on oocyte competence. In particular, a differential expression of miRNAs involved in lipid and steroid metabolism, response to glucocorticoid and estradiol stimulus was observed in relation to follicular growth. Interestingly, differences in miRNAs cargo were also shown between seasons in both antral and pre-ovulatory follicles. The results of Experiment 3 demonstrated substantial seasonal differences in metabolites content in all the follicular components, suggesting that the reduced oocyte competence during the non breading season is due to alterations of several metabolic pathways, such as glutathione metabolism, energy generating metabolism, phospholipid biosynthesis and amino acid metabolism. The lipid composition of the follicular environment also varied significantly in relation to season. The findings suggest that during non breading season metabolic changes take place, likely related to a condition of negative energetic balance, influencing the follicular environment and are in part reflected at the somatic cells and the oocyte in the attempt to counteract

suboptimal conditions. The third experiment also permitted to identify potential positive markers of oocyte competence as glutathione, glutamate, lactate and cholines, whereas a negative association with competence was found for leucine, isoleucine and β-hydroxybutyrate. Taken together, these results pay the way to develop corrective strategies to modulate the follicular environment or, more easily the IVM medium to improve the competence of oocytes retrieved during the NBS. However, the amount of data produced confirm the complexity of the phenomenon of seasonality and undoubtedly suggest to carry out further studies to better interpret some of the changes observed.

I. Seasonality and reproduction

The effect of season on mammal reproduction is a complex phenomenon. It is the consequence of numerous and intricate interactions between events related to the adaptation of the species to the environment. In fact, the finality of seasonal breeding strategies in many wildlife animals is always to give birth to their progeny in the most favorable period of the year and consequently give them the highest possibilities to survive. Despite this, in many species, such as some farm animals and pets, in which the correlation between season and success in the offspring growth is less obvious due to human intervention regarding feeding and shelter, a more or less marked reproductive seasonality often persists. This shows that at the base of this phenomenon there are not only environmental factors, but also endogenous ones, deriving from hundreds of thousands of years of evolution of a species. Also in the human species (Homo sapiens) there is a slight preference for reproduction/conception in certain seasons; for example some studies suggest that in hot summers in sub-equatorial areas the deterioration of sperm may result in lower conception rates and reduction in the birth rate in spring [1]. On the contrary, in the regions of the far north of Europe and America, where a long period of darkness exists during the winter, a depression of the anterior pituitary-ovarian axis leads to a decrease in the probability to conception. In these areas, there is a peak in the number of conceptions in the late spring - summer period with a consequent increase in number of deliveries in the spring. In the past studies have also been conducted on the effects of the variation of the light / dark ratio on bovine performances, a species known to be poorly photosensitive. It was observed that the gradual transition to a housing regime with 16 hours of light and 8 hours of darkness could increase dry matter ingestion and milk production [2], but the long-term effects of this management are not known. Similarly, it seems that a prolongation of daylight hours can favor the growth rate of calves and therefore accelerate the onset of puberty [3,4].

II. Biological mechanisms underlying seasonality in mammals

As mentioned above, we can divide the factors underlying seasonality into exogenous and endogenous factors. The first ones include the cyclic alteration of seasons, the possibility to access food and water, temperature, humidity etc. Among the factors reported here, it is important to underline the pivotal role of the photoperiod that influences both other exogenous and

endogenous factors. The effect of the photoperiod on the availability of food and water in nature appears obvious if we think about the normal changing of the seasons through the solar year. In fact, in the equatorial regions, where the length of day and night remains roughly constant throughout the year, the availability of food / water is the main factor underlying the reproductive seasonality of animals. It was demonstrated that a negative energetic balance, resulting from a difference between the calories introduced through foraging and those consumed by the animal, can result in a depression of the activity of the Gonadotropin Releasing Hormone (GnRH) pulse generator and consequently impact reproductive efficiency [5]. On the other hand, as we approach the poles, photoperiod variations are certainly associated with plant growth cycles and consequently with food availability, but they also have a direct effect on the animals. The main mechanism underlying this phenomenon is related to the secretion of melatonin by the anterior pituitary gland. This is evident in the intensive and semi-intensive breeding farms of species such as buffalo, sheep, some cattle breeds and horses in which, despite the feeding is kept roughly constant throughout the year, a more or less marked seasonality persists and it is strongly correlated to solar cycles. Melatonin regulates the reproductive cyclical activities in many species both positively and negatively regarding the amount of light hours; we can divide the livestock species in short-day breeders such as goats, sheep and buffaloes or long- day breeders such as horses. Melatonin is a hormone primarily released by the pineal gland and it is involved in synchronizing circadian rhythms, including sleep-wake timing, blood pressure regulation, and in control of seasonal rhythmicity including reproduction, fattening, moulting and hibernation [6]. Melatonin has an essential role in the synchronization of reproductive functionality with the cyclical alternations of seasons. The presence of melatonin receptors has been demonstrated in the male and female reproductive tracts of many mammalian species, including humans, cattle, buffalo, rats and mice [7,8,9,10]. In buffalo (Bubalus bubalis), as in other ruminant species characterized by a marked female reproductive seasonality, the seasonal changes are reflected in the circadian secretion of melatonin by the brain and consequently in the secretion by the hypothalamic neurons of GnRH. The variation in the function of GnRH neurons greatly influences the secretion of gonadotropins (Follicle stimulating hormone - FSH and Luteinizing hormone - LH) by the pituitary gland and consequently the reproductive functionality of the species. [11,12]. Melatonin also has a peripheral role, both because a part of this neurotransmitter can be secreted

in some way as a hormone into the bloodstream and therefore can directly affect peripheral organs, and because it can be synthesized directly by tissues and cells. The effect of melatonin on peripheral tissues / organs is also evidenced by the finding of its receptors on numerous tissues, including those associated with the reproductive system as testicles, ovaries, corpora lutea etc. [13,14,15]. Melatonin is also synthesized in the mitochondria, and acts as an antioxidant but also as a paracrine or autocrine agent [16]. In buffalo, the presence of melatonin receptors was demonstrated in ovarian tissue and melatonin was isolated in the follicle [17]. The essential role of melatonin in buffalo reproduction can be partially explained by its powerful role as an antioxidant mentioned above. Numerous in vitro studies have shown how the administration of exogenous melatonin can improve the conditions of oocyte and sperm development under conditions characterized by a higher production of reactive oxygen species. Likewise, it has been shown that supplementation of exogenous melatonin can influence reproductive performances even in vivo [18]. In particular, the role of melatonin as a strategy for the resumption of ovarian activity in acyclic buffaloes has been studied. An experiment was carried out in Argentina, during the low breading season to evaluate the use of melatonin on water buffaloes to increase pregnancy rates in a fixed-time insemination program. In this study, the administration of 18 mg/50 kg of melatonin ten days before the beginning of the synchronization protocol has no effect on follicular development, formation of the corpus luteum, as well as on pregnancy rates. [19]. In another trial conducted on buffalo heifers, melatonin, given prior to an ovsynch synchronization protocol during the non-breeding season (NBS) for 28 days prior to AI, improved luteal profile (size and progesterone secretion) and conception rate (50% vs 20%)[20]. Similary a melatonin implant 24 days before a sincronization protocol was effective in resuming cyclicity and inducing ovulation in anaestrus buffalo heifers [21]. This difference could probably be due to the different exposure time of the follicle to melatonin and be somehow attributable to an effect during the pre-antral growth phases of the follicle. Improving of the luteal function, after protocols based on implant of melatonine is also described during NBS, both in heifers and lactating buffaloes [22,23,24]. Melatonine subcutaneous implants were also effective in post partum animals, inducing an increase in superoxide dismutase two weeks after the treatment and an early onset on estrus and ovulation compared to control [25]. Despite the strong evidence, the exact mechanisms by which melatonin acts at the follicle and oocyte levels still remain to be elucidated. In fact, the new researches are focusing

on the investigation of molecules such as lipids, metabolites, ectosomes, exosomes, proteins etc. involved in intra and inter cellular communication within the follicular environment which may account for the decreased oocyte competence recorded in the NBS of seasonal species like buffalo and perhaps are influenced by melatonin.

III. Influence of seasonality on buffalo farming

It is well known that buffalo is a short-day breeder, i.e. an animal which tends to increase its reproductive activity when day light hours decrease. As already mentioned, this phenomenon is, at least in European latitudes, a heritage of the tropical origin of the species. The Water buffalo currently present in Europe is native of tropical and subtropical areas corresponding to the great valley of the Indus river (corresponding to the territories between Pakistan and northwest India). There is evidence of the presence and somehow of the cult of the water buffalo in the Harappa civilization, about 3000 years before Christ in the Indus river valley. Throughout South India villages the water-buffalo sacrifices were related to the worshipping of goddess and there are also evidences of the existence of a male divinity called the “buffalo king” [26] In addition, remains of buffalo carcasses dating back to 3000/1600 years before Christ have been found in that area [27]. A proof of the bronze age society's close connection with the water buffalo is the finding of a seal from Mohenjo-daro showing a half- human and half-buffalo hybrid surrounded by various animals and attacked by a tiger [28]. In any case because of its origin the species exhibits a pattern of reproductive efficiency closely related to environmental and climatic conditions of breeding. The buffaloes become increasingly influenced by the photoperiod at increasing distance from the equator [29,30]. The reproductive seasonality of the species affects the profitability of buffalo farming, resulting in cycles of calving and milk production. In fact, in Italy, the maximum reproductive efficiency occurs in the autumn/winter period, in which daily light hours are lower [30]. Zicarelli analyzed the calving distribution of buffalo in free mating conditions as a function of latitude; the author observed how, approaching south, the calving distribution tends towards the first months of the year [31] In Italy (between the 47th and the 37th parallel) the distribution of the births tends to be concentrated between the end of July and December. A similar pattern was also observed in India, Pakistan, Venezuela and Egypt [32].Interestingly, all these countries, despite differences in environmental temperature and precipitation amount,

share a comparable pattern of daylight hours, confirming the importance of photoperiod as one of the main factors affecting buffalo reproductive performances. Moreover, in Italy buffalo is bred mainly under intensive systems, indicating that the differences observed in the reproductive efficiency are mostly due to the photoperiod rather than to the diet which remains roughly constant throughout the year, at least in terms of energetic input [33]. In Italy, in order to satisfy market demand for buffalo milk and mozzarella cheese, out of season mating strategy is commonly utilized in buffalo farming. This approach improves the temporal distribution of calving throughout the year but it may lead to reduced fertility [34]. Longer post-partum anestrous periods and a higher incidence of embryonic mortality are often observed during the months starting from winter until mid-summer i.e. months characterized by increasing daylight length [35]. In addition, during the mid-winter / early spring i.e. the period of transition from breeding to non (low) breading season, a decreased ovulation rate after Ovsynch was observed compared to breeding season [36].

One of the causes of the reduced reproductive efficiency in buffalo during the NBS is the increased incidence of embryonic losses. These are termed, according to the time of occurrence, as early embryonic mortality (within 25 days after conception), late embryonic mortality (between 25 and 45 days) and fetal mortality (after 45 days). Each of these phenomena has its own specific etiopathogenesis, but on the whole they are multifactorial phenomena. Unlike the cow, a higher incidence of late embryonic mortality compared to the early mortality is observed in buffalo. Overall, the incidence of late embryonic mortality ranges between 7.3% and 23%, reported respectively during the breeding season in Italian Mediterranean Buffalo [37]. Analyzing the literature as a whole, although different percentages are reported, all authors agree on the greater incidence of this phenomenon during the season characterized by an increase in light hours, which suggests the photoperiod as the main culprit [38]. In an earlier trial conducted on Italian Mediterranean buffaloes, the incidence of late embryonic mortality (LEM) was associated with the presence of infectious agents responsible for abortion only in 8% of the animals [39]. About half of the buffaloes undergoing non-infectious LEM had lower progesterone concentrations as early as at 10 and 20 days after AI compared to buffaloes that maintained pregnancy after 40 days. This suggests that the phenomenon of mortality is partly due to a decline in the functionality of the corpus luteum during the NBS, that may also result from a poor quality of the embryo, unable to prevent luteolysis, presumably due to insufficient secretion of interferon

tau). A season-dependent decline in corpus luteum functionality results in lower progesterone levels, known to influence the growth of the embryo [40,41]. In fact, buffaloes that encounter LEM showed delayed embryo development on day 25 [42]. In another trial the higher incidence of LEM during the NBS was associated to impaired luteal function resulting in reduced progesterone levels [37]. Interestingly, seasonal differences in progesterone secretion were also recorded in buffaloes maintaining pregnancies. It has been shown that the amount of progesterone is correlated with the extent of the vascularization of the corpus luteum in buffalo [43]. This may result from a lower vascularization of the ovulatory follicle during the NBS. Although this parameter has not been measured in buffalo, a correlation between the vascularity of the follicle and the quality of the corpus luteum is known in cattle [44]. In horses a seasonal variation in the extent of follicle vascularization is also correlated with the quality of the corpus luteum and the levels of circulating progesterone [45].

Several studies have confirmed that reduced luteal function is in part responsible for embryonic loss in buffalo during the NBS in buffalo [46,47,48]. However, the early finding that half of the animals undergoing LEM showed levels of progesterone comparable to those of pregnant animals suggests that other factors may play a role. An additional factor determining reproductive failure in buffalo during NBS is a reduction of the oocyte developmental competence, as indicated by reduced cleavage and blastocyst yields following in vitro fertilization also reported in small ruminants [49, 50, 51]. The influence of season on follicular growth and oocyte competence in buffalo has been evaluated by different authors. Despite few differences related to latitudes, breed, management and donors' age, a noticeable effect of season was demonstrated. In Indian buffaloes the decreased efficiency of Ovum pick-up (OPU) and in vitro embryo production (IVEP) during the NBS was mainly attributed to reduced follicular population [52]. In addition to reduced follicular development, Egyptian authors also reported decreased oocyte competence, as well as altered expression of key genes in cumulus-oocyte complexes (COCs) during the NBS [53].

A retrospective study of data collected for 3 years from slaughterhouse ovaries of Italian Mediterranean buffaloes revealed that oocyte developmental competence, i.e. the oocyte capability to undergo fertilization and subsequent embryo development, was reduced during the NBS, despite similar numbers of oocytes [49]. Furthermore, in a later work it was confirmed that oocyte competence is severely affected by season, as

shown by significantly reduced blastocyst production during the NBS compared to the BS in buffaloes undergoing OPU, despite similar follicular and oocyte populations [54]. A reduced oocyte competence during the unfavorable season was also reported in sheep, another short-day breeder, as indicated by the worse response to multiple ovulation [55], lower cleavage after IVF of ovulated oocytes [56] and reduced blastocyst rates from abattoir-derived oocytes [57].

The influence of season on oocyte quality was also investigated in Murrah buffalo heifers in Brazil [58]. In particular, the reduced follicular population and oocyte quality recorded during the NBS were associated to lower levels of estradiol in plasma and lower levels of both estradiol and IGF-1 in the follicular fluid. This is intriguing due to the known role played by IGF-1 in regulating follicular development, by increasing the proliferation of granulosa cells and estradiol secretion in synergy with FSH [59,60], as well as in influencing oocyte viability [61] and maturation [62]. Furthermore, given the promoting role of IGF-1 on follicular production of vascular endothelial growth factor [63], it was hypothesized that during the breeding season (BS) an improved angiogenesis of the follicle may result in increased oocyte quality, as well as in improved development of a functional corpus luteum. It is, in fact, known that environmental factors that interfere with follicular growth may have carryover effects on oocyte quality and corpus luteum function. Recently, seasonal differences in microRNA (miRNA) profile were observed in sheep ovaries during estrus [64]. This may account for the reduced oocyte competence during the NBS, as the pathway analysis of target genes revealed that the identified differentially expressed miRNAs are correlated with ovarian activity, regulation of hormone secretion, follicular development and angiogenesis.

Another interesting observation is that the season does not seem to affect the receptivity of the uterus in buffalo. In fact, [65] despite the decreased embryo production per donor recorded during the NBS, no differences in pregnancy rates were observed when embryo transfer was carried out in the BS vs NBS (38 vs 33%, respectively). However, embryos produced in the BS had a higher capacity to establish and maintain pregnancy than those produced in NBS, as shown by higher pregnancy rate, regardless of the moment of implantation (46.5% vs. 22.4%). Interestingly, in Italian Mediterranean buffalo the poor oocyte quality observed during NBS seems to be reflected in reduced embryo quality and cryotolerance [66,67]. This may suggest that embryo quality in buffalo would also depend on oocyte

competence, unlike cattle in which it is primarily influenced by culture conditions [68,69].
Given the similar response of recipients throughout the year, it is possible to optimize off-season breeding strategies through the use of embryos produced at favorable times of the year. The decrease in the efficiency of artificial insemination during NBS could be partially overcome by optimizing the management of embryo production. In the current situation, oocyte retrieval for IVEP should be limited to the BS to produce and cryopreserve higher quality embryos in adequate numbers to satisfy the farm needs that can be transferred throughout the year. This is a valid approach to save resources and reduce costs, but further exacerbates the main limitation of advanced reproductive technologies in this species, i.e. the poor number of oocytes that can be recovered per donor [70, 71], resulting in high embryo production costs, deriving from the low number of primordial and antral follicles [72,73,74], together with a high rate of atresia [75,76].
Despite the strong evidence of seasonal influences in buffalo reproduction, the reasons underlying the phenomenon of reduced developmental competence at a molecular level are still poorly understood. A better characterization of the follicular environment may undoubtedly pay the way for the development of potential corrective strategies.

IV. New technologies for the identification of fertility markers

In all mammals, reproductive function is fundamental for the perpetration of the species; various causes may determine reproductive failure whose consequences are often dire. Although the factors vary depending on the species examined, female fertility is mainly orchestrated by array of molecular messengers that can be induced or switched off in a time- and tissue-dependent manner [77].
Female fertility is a major factor affecting livestock breeding. The cost of days open is a commonly used index to understand the impact of subfertility and reproductive diseases on the profitability of the farm. It basically indicates the amount of money (such as expenses + lost income) for each extra day a cow is not pregnant. Generally, this cost ranges between 3.00 and 6.00 euros per animal per day. To address the constant growth linked to the demand for animal proteins it is crucial to increase the reproductive efficiency of the main livestock species. Optimizing reproductive efficiency means producing more food per unit of space and time, as well as optimizing

the use of limited resources such as water and soil and reducing the release of harmful substances.
An interesting stochastic simulation study of 10,000 herds of 200 cows demonstrated that effective reproductive management of dairy cows achieved through the use of hormonal therapy can lead to economic and environmental benefits. Indeed, it was predicted that in average size farms replacing visual detection of estrus with a hormonal treatment (ovsynch) before first AI service would allow 3.6 % reduction of methane emission per year, which is equivalent to the average environmental impact of two cars or a medium family home [78]. It follows the importance of optimizing fertility management of livestock in the current scenario for a proper development of sustainable breeding.
In the last years, the research on the identification of both male and female fertility markers in various species has constantly led to new and interesting discoveries, in order to optimize the reproductive performances of livestock, as well as to safeguard endangered species and manage the problem of human infertility. More recently the so-called " omics " sciences have become very popular because they are suitable for the complete study of the complex interactions among molecules in the different layers of complex biological systems. Major omics technologies include genomics, transcriptomics, proteomics, and metabolomics. The validation of biomarkers related to this type of technology, such as genes, transcripts, proteins, metabolites, associated with fertility phenotypes has aroused great interest in scientists due to their great potential to improve the reproductive efficiency of livestock. The “omics” sciences are various disciplines in biology whose names end with the suffix –omics. The term derives from the Englishization in "ics" of the Greek suffix "ikos", which means "pertinent" added to the suffix "oma", probably deriving from sanskrit OM that indicates completeness. Therefore, it can be translated simply as the science relevant to a particular branch of biology. Some examples of omics sciences are genomics, epigenomics, microbiomics, lipidomics, proteomics, glycomics, foodomics, transcriptomics, metabolomics, culturomics, ethomics, etc. Among these, the most useful for understanding the narrative of this thesis will concisely be described. Genomics is an interdisciplinary branch of biology focused on the structure, function, evolution, mapping and editing of genomes, where genome means not the single gene and its role but the complete DNA of an organism. Therefore, genomics studies the complete DNA and its structural configuration, it takes into consideration all the genes of an organism, their interrelationships and the way in which

they influence the organism. According to the World Health Organization, genomics can be defined as the study of the total or part of the genetic or epigenetic sequence information of organisms, and attempts to understand the structure and function of these sequences and of downstream biological products [79] Similarly, the transcriptomics is the biotechnology that analyzes the transcriptome, i.e the entire profile of the messenger RNAs (mRNAs) transcribed by an organism or a particular organ, tissue or cell. The comparison of transcriptomes can allow the identification of genes that are differentially expressed in differentiated cells, organisms or conditions, as well as to study their response to different treatments or stimuli. The metabolic or metabolomic profile is the analysis of various polar and non-polar molecular metabolites in cells and biological fluids using various forms of spectral and analytical approaches, allowing to determine the metabolic changes associated with physiological, para-physiological and pathological states [80].The advantage of this type of approach is significant, since variations in the metabolome are the result of the interaction of many factors and metabolomics offers a more ample vision of a particular phenomenon. . In other words, a small variation in gene expression can cause a large change in the metabolomic profile and furthermore its interaction with other factors can further amplify its effect. Furthermore, it can be useful in detecting subtle cellular events, variations in the expression of single metabolic pathways etc. Studies have highlighted the potential of the study of the follicular metabolome in assessing the quality and competence of the embryo and oocyte development [81,82,83]. MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules (containing +- 22 nucleotides) that direct post-transcriptional repression of mRNA targets in various eukaryotic lineages affecting the expression of many mRNAs [84]. MiRNAs are abundant in many mammalian cell types including those associated with reproductive tract, but they can also be found in biological fluids as extracellular circulating miRNAs [85]. In extracellular fluids these could be coupled with extracellular vesicles, high density lipoproteins or proteins as Argonaute proteins. Extracellular circulating miRNAs are involved in cellular communication [85]; indeed, many researchers are focusing on the possibility of using these molecules as early markers of specific diseases [86,87,88]. It is known that a fine synchronized interaction between genes and their regulatory mRNA and MiRNAs is essential for a proper development of the oocyte [89]. MiRNAs have been demonstrated to be involved in the activation and selection of primordial follicles of mouse

[90,91]. MiRNAs are also involved in the phenomenon of follicular selection and dominance; in fact, in cattle an up-regulation of some MiRNAs has been observed associated with the size and health of the follicle. In a study three miRNA (MiR-144, miR-202 and miR-873) were up-regulated in large healthy follicles compared to large atretic ones [92]. In another study, in support of this hypothesis, it was shown that smaller follicles have a more heterogeneous "MiRNA population" than dominant follicles and that there is a clear separation in the MiRNA expression profiles of granulosa cells collected from dominant and subordinate follicles [93,94,95,96]. Many studies have also highlighted an important role of these molecules in the proliferation, differentiation, survival and ability to produce hormones by granulosa and cumulus cells grown in vitro [97,98]. Consequently it seems that the MiRNAs may have a key role in the creation of an appropriate follicular microenvironment fundamental for a correct growth, oocyte maturation and acquisition of developmental competence.

Regarding seasonal variability in MiRNA expression associated with reproductive function, changes in transcriptome and miRNA expression were investigated in sheep, another short-day breeder as buffalo, where oocyte competence was observed to decline during the NBS, as indicated by impaired in vitro embryo development [51]. Transcriptome variations potentially associated with off-season reproduction were reported in sheep ovaries [99] and differences in miRNA profiling in ovaries of Tan sheep and Small Tail Han sheep were related to seasonal anoestrus and BS [100]. Similarly, seasonal differences were observed in relation to hormone production, follicular growth and angiogenesis in Kazakh sheep [101]. According to our knowledge, there are currently no works that have studied, from this point of view, the decline of competence in bubaline oocytes during the NBS.

An increasing attention has been recently paid to extracellular vesicles (EVs) that are lipid bound vesicles secreted by cells into the extracellular space [102], normally classified according to their size or role played. The main subtypes of EVs are microvesicles (MVs), exosomes, and apoptotic bodies [103]. Exosomes are vesicles with a monolayer membrane, secreted by practically all cytotypes and universally present in biological fluids. For years their role has been considered only in the elimination of waste substances from the cell, whereas more recently it has been discovered they are involved in inter-cellular communication, as well as in the immune defense, being able to present antigens [104]. It appears that among other roles, exosomes are also involved in tissue repair and regeneration [105].

Like exosomes, microvesicles are intracellular messengers secreted and absorbed by almost all cell types; similarly to exosomes, microvesicles are involved in cellular communication, both locally and non-locally. They are also able to transport substances with regulatory function such as miRNA, proteins, growth factors etc. from a donor to a recipient cell [106]. Apoptotic bodies basically have a different origin as they derive from dying cells, they are larger than the other two categories reaching dimensions of 5000 nm [107]. Unlike the two categories just mentioned, they contain organelles, chromatin and proteins associated with the nucleus. Extracellular vesicles have been found practically in all cytotypes, including those of the reproductive system, so they certainly have an active role in reproduction. Regarding reproductive biological fluid, EVs have been found in prostatic secretion [108] and are involved in sperm capacitation, acrosome reaction and fertilization. It was demonstrated that also the epididymal epithelium is able to release EVs into sperm in many species including human, hamster, ram, mouse and bovine [109,110,111,112,113].

The vesicles system inside the follicle certainly contributes to the complex bidirectional communication between the granulosa cells and the oocyte. The presence of EVs in the follicular fluid has been demonstrated in numerous species, including humans, pigs, horses and cattle [114,115,116,117]. They carry miRNAs that are predicted to target key elements in pathways involved in the regulation of many follicular and oocyte functions in mammals, such as wingless signaling pathway (WNT), transforming growth factor beta (TGFb), mitogen-activated protein kinase (MAPK), eurotrophin, epidermal growth factor receptor (ErbB) pathways, ubiquitin-mediated pathways etc [114,115,116,118]. Therefore, they are linked to follicular formation, growth and ovulation, to oocyte maturation, to steroidogenesis, luteogenesis, and to the proliferation of cumulus and granulosa cells. Furthermore, the presence of locally secreted EVs in the oviductal fluid, called oviductosomes, has been demonstrated, suggesting the involvement of EVs also on fertilization and early embryonic development [119, 120].

The content of the extracellular vesicles found in the follicular fluid in relationship to follicular development has been characterized both in bovine and horses [121,122,123], but, according to our knowledge, this was never investigated in buffalo. It was demonstrated that equine follicular fluid contains different miRNAs which expression changes between ovulatory and anovulatory follicles, in part reflecting changes observed in granulosa cells [124]. Moreover, in equines it was shown that granulosa cells are able

to absorb the microvesicles present in the follicular fluid [125]. In the study cited above it was also demonstrated an interesting difference in the miRNA content between the follicular fluid of young and old mares, suggesting a role for EVs in age-associated reduced fertility and loss of oocyte competence in this specie. The presence of EVs was also demonstrated in bovine [126]. Likewise, a difference in the content of EVs as a function of the degree of follicular development was also demonstrated in cattle [126]. In cattle EVs extrapolated from follicular fluid of preovulatory oocytes contain a higher number of upregulated miRNAs, suggesting greater transcriptional activity during oocyte growth [115,127]. Furthermore, the addition of bovine follicular EVs during in vitro maturation was beneficial for COCs, by promoting cumulus expansion and regulating gene expression [128], as well as improving oocyte maturation and embryo development [129].

V. Aim of the thesis

The main purpose of this thesis was to investigate the causes and the molecular mechanisms underlying the phenomenon of reproductive seasonality in the Italian Mediterranean Buffalo, with specific reference to oocyte developmental competence. Despite the evidence of reduced oocyte competence during the NBS in buffalo [37,49], severely affecting farm profitability as well as the success of reproductive technologies, the follicular environment, where the oocyte grows and acquires the developmental competence, has not yet been characterized at molecular levels. Therefore, the focus of this thesis was paid on the oocyte and the follicular environment, to unravel the causes of reduced oocyte competence during the NBS, using novel approaches. It is known that the oocyte quality plays a fundamental role for successful reproduction, both in vivo and in vitro, and a decline in developmental competence is a major limiting factor. The developmental competence, i.e. the ability of the oocyte to properly undergo fertilization and embryogenesis, is acquired during the last growth phase of the follicle and hence is closely linked to the follicular environment and influenced by a series of metabolic and hormonal changes, as well as a fine regulated expression of various genes. The development of a competent oocyte at the appropriate time involves complex and bidirectional interactions between the various parts of the follicle, i.e. oocyte, cumulus cells and granulosa cells, as well as extrafollicular factors. The finely regulated expression of key genes is pivotal for the correct development of

the oocyte. Gene expression is known to be modulated by miRNAs, that contribute to the overall cargo of extracellular vesicles (EVs). Extracellular vesicles are particles delimited by a lipid bilayer, released and taken up by practically all cells, and are considered important mediators of cell-to- cell communication. Within the follicle they provide a pathway for autocrine and paracrine communication between theca, granulosa, cumulus cells and the maturing oocyte. Therefore, in Experiment 1 the miRNA and transcriptomic profiles of buffalo oocytes and corresponding follicular cells were compared between BS and NBS. Then a further characterization of the follicular MiRNA content was carried out in Experiment 2. Specifically, the miRNA cargo of EVs isolated from the follicular fluid was characterized both in relation to the stage of development (antral vs preovulatory follicles) and to the season (NBS vs BS).

Finally, in Experiment 3 it was evaluated whether season influences the metabolome of the follicle. This approach was chosen because the variations in gene expression and the interactions with the environment are reflected downstream in metabolic changes. In particular, to further unveil molecular differences related to season, the metabolomic profile of various components of the ovarian follicle, such as follicular fluid (FF), follicular cells (FC), cumulus cells (CC-IO) and immature oocytes (IO) was investigated. In addition, the metabolome of cumulus cells and oocytes after in vitro maturation (CC-MO and MO, respectively), as well as that of the spent medium (SM), was characterized. The purpose of this experiment was to study the difference of metabolic pathways between seasons, to better understand the processes associated with reproductive dysfunction observed during NBS and to identify new potential fertility markers. This work lays the ground for future studies to develop corrective strategies based on the manipulation of the follicular environment and/or the in vitro maturation system during NBS.

References

[1]Rojansky N, Brzezinski J, Schenker G, 1992. Seasonality in human reproduction: an update, Human Reproduction, Volume 7, Issue 6, Pages 735–745

[2]Peters RR, Chapin LT, Leining KB and Tucker HA, 1978. Supplemental lighting stimulates growth and lactation in cattle. Science, 199: 911-912.

[3]Dahl GE, Buchanan BA and Tucker HA, 2000. Photoperiodic effects on dairy cattle: A review. Journal of Dairy Science, 83: 885-893

[4]Dahl GE, 2005. Let there be light: Photoperiod Management of Cows for Production and Health. Proceedings 42nd Florida Dairy Production Conference, Gainesville, 3: 41.

[5]Bronson FH, 2009. Climate change and seasonal reproduction in mammals Phil. Trans. R. Soc. B 364, 3331–3340;

[6]Doghramji K, 2007. Melatonin and its receptors: a new class of sleep-promoting agents. Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine, 3(5 Suppl), S17–S23.

[7]Wang SJ, Liu WJ, Wu CJ, Ma FH, Ahmad S, Liu BR,and Yang LG, 2012. Melatonin suppresses apoptosis and stimulates progesterone production by bovine granulosa cells via its receptors (MT1 and MT2). Theriogenology, 78, 1517–1526.

[8]Machado EB, Souza BB, Guimarães RC, Azevedo JSN, Gonçalves EC, Ribeiro HFL, Filho ES, 2016. Polymorphism in the melatonin receptor gene in buffalo populations of the Brazilian Amazon. Genet. Mol. Res, 15, 1-6.

[9]Lee CJ, Do BR, Lee YH, Park JH, Kim SJ, Kim JK and Yoon HS, 2001. Ovarian expression of melatonin Mel1a receptor mRNA during mouse development. Molecular Reproduction and Development, 59, 126–132.

[10] He C, Wang J, Li Y, Zhu K, Xu Z, Song Y, and Liu G, 2015. Melatonin- related genes expressed in the mouse uterus during early gestation promote embryo implantation. Journal of Pineal Research, 58, 300– 309.

[11] Parmeggiani A, Seren E, Esposito L, Borghese A, 1993. Plasma levels of melatonin in buffalo cows. Egyptian Society of Animal Production, Cairo
(Egypt).

[12] Weems PW, Goodman RL, Lehman MN, 2015. Neural mechanisms controlling seasonal reproduction: principles derived from the sheep model and its comparison with hamsters. Front Neuroendocrinol;37:43e51.

References

[13] Frungieri MB, Calandra RS, Rossi S, 2017. Local actions of melatonin in somatic cells of the testis. Int J Mol Sci; 18:1170.

[14] Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan DX, Sugino N, 2009. Melatonin and the ovary: physiological and pathophysiological implications.Fertil Steril;92:328e43

[15] Xiao L, Hu J, Zhao X, Song L, Zhang Y, Dong W, 2018. Expression of melatonin and its related synthase and membrane receptors in the oestrous corpus luteum and corpus luteum verum of sheep. Reprod Domest Anim;53:

[16] Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, 2003. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res;34:75–8.

[17] Soni N, Pandey AK, Kumar A, Verma A, Kumar S, Gunwant P, 2019. Expression of MTNR1A, steroid (ERa, ERb, and PR) receptor gene transcripts, and the concentration of melatonin and steroid hormones in the ovarian follicles of buffalo. Domest Anim Endocrinol:106371.

[18] Al-Hamedawi TM, 2020. Effect of Melatonin and in Combination with CIDR on Reproductive Performance in anestrus lactating Iraqi buffaloes (Bubalus bubalis). The Iraqi Journal of Veterinary Medicine, 44(2), 99-102.

[19] Berdugo JA, Konrad JL, Yuponi R, Bandeo A, Camelino R, Cardona-Maya WD, Crudeli GA, 2021. Effect of melatonin on follicular development parameters in a fixed-timed artificial insemination programme in water buffalo. Veterinarska stanica, 52(6), 0-0.

[20] Phogat JB, Pandey AK, Balhara AK, Ghuman SS, Gunwant P, 2018. Effects of melatonin supplementation prior to Ovsynch protocol on ovarian activity and conception rates in anestrous Murrah buffalo heifers during out of breeding season. Reproductive biology, 18(2), 161-168.

[21] Ghuman S, Singh J, Honparkhe M, Dadarwal D, Dhaliwal GS, Jain AK, 2010. Induction of ovulation of ovulatory size nonovulatory follicles and initiation of ovarian cyclicity in summer anoestrous buffalo heifers (Bubalus bubalis) using melatonin implants. Reprod. Domest. Anim. 45, 600–607.

[22] Singh J, Ghuman S, Dadarwal D, Honparkhe M, Dhaliwal GS, Jain AK, 2009.Estimations of blood plasma metabolites following melatonin implants treatment for initiation of ovarian cyclicity in true anestrus buffalo heifers. Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab 141 004 India

References

[23] Ramadan TA, Sharma RK, Phulia S, Balhara AK, Ghuman SS, Singh I, 2014. Effectiveness of melatonin and controlled internal drug release device treatment on reproductive performance of buffalo heifers during out-of-breeding season under tropical conditions. Theriogenology 82, 1296–1302. doi:10.1016/J.THERIOGENOLOGY.2014.08.014

[24] Ramadan TA, Sharma RK, Phulia S, Balhara AK, Ghuman SS, Singh I, 2016. Manipulation of reproductive performance of lactating buffaloes using melatonin and controlled internal drug release device treatment during out-of-breeding season under tropical conditions. Theriogenology 86, 1048–1053. doi:10.1016/J.THERIOGENOLOGY.2016.03.034

[25] Lochan S, Honparkhe M, Cheema RS, Kumar A, Sghuman SP, Brar PS, 2020. Ameliorating postpartum reproductive cyclicity using exogenous melatonin implant in water buffalo (Bubalus bubalis (). Indian Journal of Animal Sciences, 90(2), 181-184.

[26] Asko Parpola, 2015. The Roots of Hinduism: The Early Aryans and the Indus Civilization. Oxford University Press Inc.

[27] Suryanarayan S, Cubas M, Heron CO, Shinde VS, Singh RN, O'Connell TC, Petrie CA, 2021. Lipid residues in pottery from the Indus Civilisation in northwest India, Journal of Archaeological Science, Volume 125,105291, ISSN 0305-4403, https://doi.org/10.1016/j.jas.2020.105291.

[28] Srinivasan D, 1997. Many Heads, Arms, and Eyes: Origin, Meaning, and Form of Multiplicity in Indian Art. Brill. ISBN 9004107584.

[29] Zicarelli L, Esposito L, Campanile G, Di Palo R, Armstrong DT, 1997. Effects of using vasectomized bulls in artificial insemination practice on the reproductive efficiency of Italian buffalo cows. Animal Reproduction Science, 47(3), 171-180.

[30] Campanile G, Baruselli PS, Neglia G, Vecchio D, Gasparrini B, Gimenes LU, 2010. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. Animal Reproduction Science, 121(1-2), 1-11.

[31] Zicarelli L, 2013. Influence of seasonality on buffalo production. World, 1961, 1961.

[32] Zicarelli L, 2016. Influence of seasonality on buffalo production. In 'The Buffaloes (Bubalus bubalis) – Production and Research'. (Ed. G. A. Presicce.) pp. 196–224. (Bentham Science Publishers: Sharjah, United Arab Emirates.

[33] Zicarelli L, 1994. Management under different environmental condition. Buffalo J. 17–38.

[34] Gasparrini B, 2018. Effects of reproductive season on embryo development in the buffalo. Reproduction, Fertility and Development 31, 68-81.https://doi.org/10.1071/RD18315

[35] Zicarelli L, 2007. Can we consider buffalo a non precocious and hypofertile species? Ital. J. Anim. Sci. 6, 143–154. doi:10.4081/IJAS.S2.143

[36] Rossi P, Vecchio D, Neglia G, Di Palo R, Gasparrini B, Michael, JD, Campanile G, 2014. Seasonal fluctuations in the response of Italian Mediterranean buffaloes to synchronization of ovulation and timed artificial insemination. Theriogenology, 82(1), 132-137.

[37] Di Francesco S, Neglia G, Vecchio D, Rossi P, Russo M, Zicarelli L, Campanile G, 2012. Influence of season on corpus luteum structure and function and AI outcome in the Italian Mediterranean buffalo (Bubalus bubalis). Theriogenology, 78(8), 1839-1845.

[38] Campanile G, Neglia G, Michael JD, 2016. Embryonic and fetal mortality in river buffalo (Bubalus bubalis). Theriogenology, 86(1), 207-213.

[39] Campanile G, Neglia G, Gasparrini B, Galiero G, Prandi A, Di Palo R, Zicarelli L, 2005. Embryonic mortality in buffaloes synchronized and mated by AI during the seasonal decline in reproductive function. Theriogenology, 63(8), 2334-2340.

[40] Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans A, Kenny DA, Roche JF, Lonergan, P, 2008. Effect of increasing progesterone concentration from Day 3 of pregnancy onsubsequent embryo survival and development in beef heifers. Reprod.Fertil. Dev. 20, 368–375. doi:10.1071/RD07204

[41] Neglia G, Restucci B, Russo M, Vecchio D, Gasparrini B, Prandi A, Di Palo R, D'Occhio MJ, Campanile G, 2015. Early development and function of the corpus luteum and relationship to pregnancy in the buffalo. Theriogenology 83, 959–967. doi:10.1016/J.THERIOGENOLOGY.2014.11.035

[42] Balestrieri, M. L., Gasparrini, B., Neglia, G., Vecchio, D., Strazzullo, M., Giovane, A., Campanile, G. (2013). Proteomic profiles of the embryonic chorioamnion and uterine caruncles in buffaloes (Bubalus bubalis) with normal and retarded embryonic development. Biology of reproduction, 88(5), 119-1.

[43] Neglia, G., Restucci, B., Russo, M., Vecchio, D., Gasparrini, B., Prandi, A., Campanile, G. (2015). Early development and function of the corpus luteum and relationship to pregnancy in the buffalo. Theriogenology, 83(6), 959-967.

[44] De Tarso, S. G. S., Gastal, G. D. A., Bashir, S. T., Gastal, M. O., Apgar, G. A., & Gastal, E. L. (2017). Follicle vascularity coordinates corpus luteum blood flow and progesterone production. Reproduction, Fertility and Development, 29(3), 448-457.

[45] Ishak, G. M., Bashir, S. T., Gastal, M. O., & Gastal, E. L. (2017). Pre-ovulatory follicle affects corpus luteum diameter, blood flow, and progesterone production in mares. Animal reproduction science, 187, 1-12.

[46] Russo, M., Vecchio, D., Neglia, G., Pacelli, C., Prandi, A., Gasparrini, B., Zicarelli, L., D'Occhio, M. and Campanile, G. (2010), Corpus luteum Function and Pregnancy Outcome in Buffaloes during the Transition Period from Breeding to Non-Breeding Season. Reproduction in Domestic Animals, 45: 988-991. https://doi.org/10.1111/j.1439-0531.2009.01472.x

[47] Esposito L, Salzano A, Russo M, de Nicola D, Prandi A, Gasparrini B, Campanile G, Neglia G. Corpus Luteum Color Doppler Ultrasound and Pregnancy Outcome in Buffalo during the Transitional Period. Animals. 2020; 10(7):1181. https://doi.org/10.3390/ani10071181

[48] Campanile, G., Neglia, G., Gasparrini, B., Galiero, G., Prandi, A., Di Palo, R., Zicarelli, L. (2005). Embryonic mortality in buffaloes synchronized and mated by AI during the seasonal decline in reproductive function. Theriogenology, 63(8), 2334-2340.

[49] Di Francesco, S., Boccia, L., Campanile, G., Di Palo, R., Vecchio, D., Neglia, G., ... & Gasparrini, B. (2011). The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (Bubalus bubalis). Animal reproduction science, 123(1-2), 48-53.

[50] Souza-Fabjan, J. M., Correia, L. F., Batista, R. I., Locatelli, Y., Freitas, V. J., & Mermillod, P. (2021). Reproductive Seasonality Affects In Vitro Embryo Production Outcomes in Adult Goats. Animals, 11(3), 873.

[51] Mara, L., Sanna, D., Casu, S., Dattena, M., & Muñoz, I. M. (2014). Blastocyst rate of in vitro embryo production in sheep is affected by season. Zygote, 22(3), 366-371.

[52] Manjunatha, B. M., Ravindra, J. P., Gupta, P. S. P., Devaraj, M., & Nandi, S. (2009). Effect of breeding season on in vivo oocyte recovery and embryo production in non-descriptive Indian river buffaloes (Bubalus bubalis). Animal reproduction science, 111(2-4), 376-383.

[53] Abdoon, A. S., Gabler, C., Holder, C., Kandil, O. M., & Einspanier, R. (2014). Seasonal variations in developmental competence and relative abundance of gene transcripts in buffalo (Bubalus bubalis) oocytes. Theriogenology, 82(8), 1055-1067.

[54] Di Francesco, S., Novoa, M. V. S., Vecchio, D., Neglia, G., Boccia, L., Campanile, G., ... & Gasparrini, B. (2012). Ovum pick-up and in vitro embryo production (OPU-IVEP) in Mediterranean Italian buffalo performed in different seasons. Theriogenology, 77(1), 148-154.

[55] Mitchell, L. M., Dingwall, W. S., Mylne, M. J. A., Hunton, J., Matthews, K., Gebbie, F. E., ... & McEvoy, T. G. (2002). Season affects characteristics of the pre-ovulatory LH surge and embryo viability in superovulated ewes. Animal reproduction science, 74(3-4), 163-174.

[56] Stenbak, T. K., Redmer, D. A., Berginski, H. R., Erickson, A. S., Navanukraw, C., Toutges, M. J., ... & Grazul-Bilska, A. T. (2001). Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology, 56(1), 51-64.

[57] Mara, L., Sanna, D., Casu, S., Dattena, M., & Muñoz, I. M. (2014). Blastocyst rate of in vitro embryo production in sheep is affected by season. Zygote, 22(3), 366-371.

[58] Salzano, A., Gasparrini, B., Vecchio, D., Longobardi, V., Baruselli, P. S., Balestrieri, A., ... & Neglia, G. (2019). Effect of photoperiod on follicular IGF-1 and oocyte quality independently of metabolic status in buffalo heifers. Italian Journal of Animal Science.

[59] Glister, C., Tannetta, D. S., Groome, N. P., & Knight, P. G. (2001). Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. Biology of Reproduction, 65(4), 1020-1028.

[60] Gong, J. G., McBride, D., Bramley, T. A., & Webb, R. (1994). Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on bovine granulosa cell steroidogenesis in vitro. Journal of Endocrinology, 143(1), 157-164.

[61] Walters, K. A., Binnie, J. P., Campbell, B. K., Armstrong, D. G., & Telfer, E. E. (2006). The effects of IGF-I on bovine follicle development

and IGFBP-2 expression are dose and stage dependent. Reproduction, 131(3), 515-523.

[62] Izadyar, F., Colenbrander, B., & Bevers, M. M. (1997). Stimulatory effect of growth hormone on in vitro maturation of bovine oocytes is exerted through the cyclic adenosine 3′, 5′-monophosphate signaling pathway. Biology of reproduction, 57(6), 1484-1489.

[63] Schams, D., Kosmann, M., Berisha, B., Amselgruber, W. M., & Miyamoto, A. (2001). Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. Experimental and clinical endocrinology & diabetes, 109(03), 155-162.

[64] Zhai, M., Xie, Y., Liang, H., Lei, X., & Zhao, Z. (2018). Comparative profiling of differentially expressed microRNAs in estrous ovaries of Kazakh sheep in different seasons. Gene, 664, 181-191.

[65] da Silva, J. C. B., Alves, M. B. R., Bridi, A., Bohrer, R. C., Escobar, G. S. L., de Carvalho, J. A. B. A., ... & Perecin, F. (2021). Reproductive seasonality influences oocyte retrieval and embryonic competence but not uterine receptivity in buffaloes. Theriogenology, 170, 77-84.

[66] Gasparrini, B. (2019). Effects of reproductive season on embryo development in the buffalo. Reproduction, Fertility and Development, 31(1), 68-81.

[67] Kosior, M. A., Parente, E., Salerno, F., Annes, K., Annunziata, R., Albero, G., ... & Gasparrini, B. (2019). 26 Season affects cryotolerance of in vitro-produced buffalo embryos. Reproduction, Fertility and Development, 31(1), 139-139.

[68] Rizos, D., Ward, F., Duffy, P. A. T., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Molecular reproduction and development, 61(2), 234-248.

[69] Lonergan, P., Rizos, D., Gutierrez-Adan, A., Fair, T., & Boland, M. P. (2003). Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. Reproduction in Domestic Animals, 38(4), 259-267.

[70] Gasparrini, B. (2002). In vitro embryo production in buffalo species: state of the art. Theriogenology, 57(1), 237-256.

[71] Gasparrini, B. (2019). Effects of reproductive season on embryo development in the buffalo. Reproduction, Fertility and Development, 31(1), 68-81.
[72] Samad, H. A., & Nasseri, A. A. (1979). A quantitative study of primordial follicles in buffalo heifer ovaries. Compendium, 13.
[73] Danell, B. (1987). Oestrous behaviour, ovarian morphology and cyclical variation in follicular system and endocrine pattern in water buffalo heifers. Sveriges Lantbruksuniv..
[74] Kumar, A., Solanki, V. S., Jindal, S. K., Tripathi, V. N., & Jain, G. C. (1997). Oocyte retrieval and histological studies of follicular population in buffalo ovaries. Animal reproduction science, 47(3), 189-195.
[75] Ocampo, M. B., De Asis, A. T., Ocampo, L. C., & Kanagawa, H. (1994). Histological observation of follicular atresia in swamp buffalo. Buffalo Bull, 13(3).
[76] Palta, P. (1998). Endocrinological observation of atresia in individual buffalo ovarian follicles. Indian J Anim Sci, 68, 444-447.
[77] Tesfaye, D., Salilew-Wondim, D., Gebremedhn, S., Sohel, M. M. H., Pandey, H. O., Hoelker, M., & Schellander, K. (2017). Potential role of microRNAs in mammalian female fertility. Reproduction, Fertility and Development, 29(1), 8-23.
[78] Archer, S. C., Hudson, C. D., & Green, M. J. (2015). Use of stochastic simulation to evaluate the reduction in methane emissions and improvement in reproductive efficiency from routine hormonal interventions in dairy herds. Plos one, 10(6), e0127846.
[79] https://www.who.int/news-room/questions-and-answers/item/genomics
[80] Brison, D. R., Hollywood, K., Arnesen, R., & Goodacre, R. (2007). Predicting human embryo viability: the road to non-invasive analysis of the secretome using metabolic footprinting. Reproductive biomedicine online, 15(3), 296-302.
[81] Singh, R., & Sinclair, K. D. (2007). Metabolomics: approaches to assessing oocyte and embryo quality. Theriogenology, 68, S56-S62.
[82] Sinclair, K. D., Lunn, L. A., Kwong, W. Y., Wonnacott, K., Linforth, R. S. T., & Craigon, J. (2008). Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. Reproductive biomedicine online, 16(6), 859-868.
[83] Revelli, A., Delle Piane, L., Casano, S., Molinari, E., Massobrio, M., & Rinaudo, P. (2009). Follicular fluid content and oocyte quality: from

single biochemical markers to metabolomics. Reproductive biology and endocrinology, 7(1), 1-13.
[84] Bartel, D. P. (2018). Metazoan micrornas. Cell, 173(1), 20-51.
[85] Turchinovich, A., Samatov, T. R., Tonevitsky, A. G., & Burwinkel, B. (2013). Circulating miRNAs: cell–cell communication function?. Frontiers in genetics, 4, 119.
[86] Backes, C., Meese, E., & Keller, A. (2016). Specific miRNA disease biomarkers in blood, serum and plasma: challenges and prospects. Molecular diagnosis & therapy, 20(6), 509-518.
[87] Kim, Y. K. (2015). Extracellular microRNAs as biomarkers in human disease. Chonnam medical journal, 51(2), 51-57.
[88] Farrell, D., Shaughnessy, R. G., Britton, L., MacHugh, D. E., Markey, B., & Gordon, S. V. (2015). The identification of circulating MiRNA in bovine serum and their potential as novel biomarkers of early Mycobacterium avium subsp paratuberculosis infection. PloS one, 10(7), e0134310.
[89] Tesfaye, D., Gebremedhn, S., Salilew-Wondim, D., Hailay, T., Hoelker, M., Grosse-Brinkhaus, C., & Schellander, K. (2018). MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development. Reproduction, 155(3), R121-R135.
[90] Zhang, H., Jiang, X., Zhang, Y., Xu, B., Hua, J., Ma, T., ... & Shi, Q. (2014). microRNA 376a regulates follicle assembly by targeting Pcna in fetal and neonatal mouse ovaries. Reproduction, 148(1), 43-54.
[91] Zhang, Q., Sun, H., Jiang, Y., Ding, L., Wu, S., Fang, T., ... & Hu, Y. (2013). MicroRNA-181a suppresses mouse granulosa cell proliferation by targeting activin receptor IIA. PloS one, 8(3), e59667.
[92] Donadeu, F. X., Sontakke, S. D., & Ioannidis, J. (2017). MicroRNA indicators of follicular steroidogenesis. Reproduction, Fertility and Development, 29(5), 906-912.
[93] Hatzirodos, N., Irving-Rodgers, H. F., Hummitzsch, K., Harland, M. L., Morris, S. E., & Rodgers, R. J. (2014). Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. BMC genomics, 15(1), 1-19.
[94] Hatzirodos, N., Hummitzsch, K., Irving-Rodgers, H. F., Harland, M. L., Morris, S. E., & Rodgers, R. J. (2014). Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. BMC genomics, 15(1), 1-26.
[95] Salilew-Wondim, D., Ahmad, I., Gebremedhn, S., Sahadevan, S., Hossain, M. M., Rings, F., ... & Tesfaye, D. (2014). The expression

pattern of microRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. PloS one, 9(9), e106795.

[96] Gebremedhn, S., Salilew-Wondim, D., Ahmad, I., Sahadevan, S., Hossain, M. M., Hoelker, M., ... & Tesfaye, D. (2015). MicroRNA expression profile in bovine granulosa cells of preovulatory dominant and subordinate follicles during the late follicular phase of the estrous cycle. PloS one, 10(5), e0125912.

[97] Pan, B., Toms, D., Shen, W., & Li, J. (2015). MicroRNA-378 regulates oocyte maturation via the suppression of aromatase in porcine cumulus cells. American Journal of Physiology-Endocrinology and Metabolism, 308(6), E525-E534.

[98] Maalouf, S. W., Liu, W. S., & Pate, J. L. (2016). MicroRNA in ovarian function. Cell and tissue research, 363(1), 7-18.

[99] Chen, L., Liu, K., Zhao, Z., Blair, H. T., Zhang, P., Li, D., & Ma, R. Z. (2012). Identification of sheep ovary genes potentially associated with off-season reproduction. Journal of Genetics and Genomics, 39(4), 181-190.

[100] Di, R., He, J., Song, S., Tian, D., Liu, Q., Liang, X., ... & Chu, M. (2014). Characterization and comparative profiling of ovarian microRNAs during ovine anestrus and the breeding season. BMC genomics, 15(1), 1-15.

[101] Zhai, M., Xie, Y., Liang, H., Lei, X., & Zhao, Z. (2018). Comparative profiling of differentially expressed microRNAs in estrous ovaries of Kazakh sheep in different seasons. Gene, 664, 181-191.

[102] Maas, S. L., Breakefield, X. O., & Weaver, A. M. (2017). Extracellular vesicles: unique intercellular delivery vehicles. Trends in cell biology, 27(3), 172-188.

[103] Doyle, L. M., & Wang, M. Z. (2019). Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells, 8(7), 727.

[104] Bobrie, A., Colombo, M., Raposo, G., & Théry, C. (2011). Exosome secretion: molecular mechanisms and roles in immune responses. Traffic, 12(12), 1659-1668.

[105] Chaput, N., & Théry, C. (2011, September). Exosomes: immune properties and potential clinical implementations. In Seminars in immunopathology (Vol. 33, No. 5, pp. 419-440). Springer-Verlag.

[106] Pant, S., Hilton, H., & Burczynski, M. E. (2012). The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and

frontiers for pharmacological and biomarker opportunities. Biochemical pharmacology, 83(11), 1484-1494.

[107] Crescitelli, R., Lässer, C., Szabó, T. G., Kittel, A., Eldh, M., Dianzani, I., ... & Lötvall, J. (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. Journal of extracellular vesicles, 2(1), 20677.

[108] Ronquist, K. G., Ronquist, G., Carlsson, L., & Larsson, A. (2009). Human prostasomes contain chromosomal DNA. The Prostate, 69(7), 737-743.

[109] Yanagimachi, R., Kamiguchi, Y., Mikamo, K., Suzuki, F., & Yanagimachi, H. (1985). Maturation of spermatozoa in the epididymis of the Chinese hamster. American Journal of Anatomy, 172(4), 317-330.

[110] Sullivan, R., & Saez, F. (2013). Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. Reproduction, 146(1), R21-R35.

[111] Sullivan, R. (2016). Epididymosomes: role of extracellular microvesicles in sperm maturation. Front Biosci (Schol Ed), 8, 106-114.

[112] Frenette, G., & Sullivan, R. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Molecular Reproduction and Development: Incorporating Gamete Research, 59(1), 115-121.

[113] Fornes, M. W., Barbieri, A., & Cavicchia, J. C. (1995). Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia, 27(1), 1-5.

[114] da Silveira, J. C., Veeramachaneni, D. R., Winger, Q. A., Carnevale, E. M., & Bouma, G. J. (2012). Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. Biology of reproduction, 86(3), 71-1.

[115] Sohel, M. M. H., Hoelker, M., Noferesti, S. S., Salilew-Wondim, D., Tholen, E., Looft, C., ... & Tesfaye, D. (2013). Exosomal and non-exosomal transport of extra-cellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. PloS one, 8(11), e78505.

[116] Santonocito, M., Vento, M., Guglielmino, M. R., Battaglia, R., Wahlgren, J., Ragusa, M., ... & Di Pietro, C. (2014). Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs

control pathways involved in follicular maturation. Fertility and sterility, 102(6), 1751-1761.

[117] Matsuno, Y., Kanke, T., Maruyama, N., Fujii, W., Naito, K., & Sugiura, K. (2019). Characterization of mRNA profiles of the exosome-like vesicles in porcine follicular fluid. PLoS One, 14(6), e0217760.

[118] Machtinger, R., Laurent, L. C., & Baccarelli, A. A. (2016). Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Human reproduction update, 22(2), 182-193.

[119] Banliat, C., Le Bourhis, D., Bernardi, O., Tomas, D., Labas, V., Salvetti, P., ... & Saint-Dizier, M. (2020). Oviduct fluid extracellular vesicles change the phospholipid composition of bovine embryos developed in vitro. International journal of molecular sciences, 21(15), 5326.

[120] Harris, E. A., Stephens, K. K., & Winuthayanon, W. (2020). Extracellular vesicles and the oviduct function. International Journal of Molecular Sciences, 21(21), 8280.

[121] Hung, W. T., Hong, X., Christenson, L. K., & McGinnis, L. K. (2015). Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biology of reproduction, 93(5), 117-1.

[122] Navakanitworakul, R., Hung, W. T., Gunewardena, S., Davis, J. S., Chotigeat, W., & Christenson, L. K. (2016). Characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. Scientific reports, 6(1), 1-14.

[123] Uzbekova, S., Almiñana-Brines, C., Labas, V., Teixeira-Gomes, A. P., Combes-Soia, L., Tsikis, G., ... & Singina, G. (2020). Protein Cargo of Extracellular Vesicles From Bovine Follicular Fluid and Analysis of Their Origin From Different Ovarian Cells. Frontiers in veterinary science, 7, 821.

[124] Donadeu, F. X., & Schauer, S. N. (2013). Differential miRNA expression between equine ovulatory and anovulatory follicles. Domestic animal endocrinology, 45(3), 122-125.

[125] da Silveira, J. C., Winger, Q. A., Bouma, G. J., & Carnevale, E. M. (2015). Effects of age on follicular fluid exosomal microRNAs and granulosa cell transforming growth factor-β signalling during follicle development in the mare. Reproduction, Fertility and Development, 27(6), 897-905.

[126] Gebremedhn, S., Salilew-Wondim, D., Ahmad, I., Sahadevan, S., Hossain, M. M., Hoelker, M., ... & Tesfaye, D. (2015). MicroRNA expression profile in bovine granulosa cells of preovulatory dominant

and subordinate follicles during the late follicular phase of the estrous cycle. PloS one, 10(5), e0125912.

[127] Noferesti, S. S., Sohel, M. M. H., Hoelker, M., Salilew-Wondim, D., Tholen, E., Looft, C., ... & Tesfaye, D. (2015). Controlled ovarian hyperstimulation induced changes in the expression of circulatory miRNA in bovine follicular fluid and blood plasma. Journal of ovarian research, 8(1), 1-16.

[128] Hung, W. T., Hong, X., Christenson, L. K., & McGinnis, L. K. (2015). Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biology of reproduction, 93(5), 117-1.

[129] da Silveira, J. C., Andrade, G. M., Del Collado, M., Sampaio, R. V., Sangalli, J. R., Silva, L. A., ... & Meirelles, F. V. (2017). Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One, 12(6), e0179451.

Chapter 1

Experiment 1: Seasonal effects on miRNA and transcriptomic profile of oocytes and follicular cells in buffalo (Bubalus bubalis)

Seasonal effects on miRNA and transcriptomic profile of oocytes and follicular cells in buffalo (Bubalus bubalis).
Emanuele Capra, Barbara Lazzari, Marco Russo, Michal Andrzej Kosior, Giovanni Della Valle, Valentina Longobardi, Alessandra Stella, Anna Lange Consiglio & Bianca Gasparrini
Seasonal effects on miRNA and transcriptomic profile of oocytes and follicular cells in buffalo (Bubalus bubalis). Sci Rep 10, 13557 (2020). https://doi.org/10.1038/s41598-020-70546-5

1.1 Introduction

Water buffalo (*Bubalus bubalis*) is an important livestock resource for both developing and developed countries. The major factor affecting buffalo farming profitability is reproductive seasonality, resulting in cycles of calving and milk production. Buffalo is a short-day breeder, with increased fertility in response to decreasing day length [1,2]. This photoperiod dependent seasonality pattern is more pronounced as distance from the equator, together with variations in the light/dark ratio, increases. In Italy, in order to satisfy market demand, out of breeding mating strategy (OBMS), consisting in interrupting sexual promiscuity or the use of artificial insemination (AI) during the breeding season (BS), is commonly utilized [2]. The OBMS improves the distribution of calving throughout the year, but it reduces fertility [3]. Longer post-partum anoestrus periods as well as higher incidence of embryonic mortality are observed in months with increasing daylight length and particularly in mid-winter, which coincides with the transition to seasonal anoestrus at Italian latitudes [1,4]. The embryonic mortality is due to inadequate luteal growth and function, resulting in reduced progesterone secretion [5]. This has a negative impact on embryo growth, associated with alterations in transcriptomic and proteomic profiles of the embryos and chorioamnios/caruncles [6,7], which ultimately impair embryo attachment to the uterine endometrium. An additional factor determining reproductive failure in the non-breeding season (NBS) is the oocyte developmental competence. Indian authors reported decreased efficiency of ovum pick-up (OPU) during the NBS, mainly due to the reduced follicular population [8]. A seasonal effect on the number of follicles and oocytes, as well as on oocyte competence, has also been reported in Egyptian buffaloes [9]. In Italian Mediterranean buffaloes, season clearly influences oocyte competence, as indicated by improved blastocyst yields recorded during months with decreasing daylight [10,11]. In Murrah buffalo heifers, the decreased oocyte quality recorded during long day months was associated to reduced concentration of oestradiol both in plasma and follicular fluid, as well as of intrafollicular IGF-1 [12]. Despite the evidence of a seasonal influence in buffalo, the molecular mechanisms affect- ing oocyte competence in the NBS are poorly understood.

A fine-tuned spatio-temporal expression of multiple genes is known to be essential for follicular development and oocyte maturation, and requires a strict interaction between mRNAs and regulatory miRNAs [13]. In addition, in many tissues a time-controlled gene expression is mediated by miRNAs, which regulate core clock genes coordinating daily rhythms in physiology and behaviour [14]. A relationship between the variation of mRNAs

abundance for specific genes related to folliculogenesis in ovaries and changes in photoperiods was previously reported in other non-ruminant species such as the Siberian hamster [15]. Changes in transcriptome and miRNA expression in relation to season were further investigated in sheep, another short-day breeder, where oocyte competence was observed to decline during the NBS, as indicated by impaired in vitro embryo development [16]. Furthermore, transcriptome variations potentially associated with off-season reproduction were reported in sheep ovaries [17]. Again, differences in miRNA profiling in ovaries of Tan sheep and Small Tail Han (STH) sheep were related to ovine anoestrus and BS [18]. Seasonal differences in the expression of miRNAs involved in hormone regulation, follicular growth and angiogenesis were also observed in Kazakh sheep ovaries during oestrus [19]. Recently, an integrated analysis of mRNA and miRNA expression in European mouflon (Ovis musimon) and sheep (Ovis aries) depicted a miRNA-mRNA regulatory network associated with reproductive traits in Ovis species [20].

MicroRNAs play a significant role during follicle development in bovine [21,22]. In buffalo, heat stress was observed to alter the blood miRNA and mRNA content [23]. The role of miRNAs is also demonstrated in the regulation of lactating physiology in the buffalo mammary gland [24], and miRNA expression changes were observed in buffalo corpus luteum during pregnancy [25].

Therefore, in this study we investigated, for the first time, if reproductive failure in the NBS is associated to changes in gene expression affecting oocyte developmental competence in buffalo. To evaluate seasonal effects on oocyte competence, the miRNA and transcriptomic profiles of oocytes and corresponding follicular cells were characterized from abattoir-derived ovaries collected in the BS and NBS.

1.2 Materials and methods

1.2.1 Collection of oocytes and granulosa cells

The study was carried out in Southern Italy (latitude 40.5°– 41.5° N and longitude 13.5–15.5) in October, i.e. autumn (BS) and January, i.e. mid-winter (NBS). Buffalo ovaries were collected at a local slaughterhouse (Real Beef s.r.l., Flumeri (AV), Italy under national food hygiene regulations, and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30–35 °C within 4 h after slaughter. In order to reduce

variability, the ovaries were collected from a homogeneous population of buffaloes, i.e. 134 cyclic multiparous Italian Mediterranean Buffalo cows with a mean weight and age of 552.6. ± 12.1 kg and 5.3 ± 0.4 years, over a total of 10 replicates (5/season). 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. For each day of collection (n = 10), 2–8 mm follicles were aspirated under controlled pressure to collect both OOs and FCs for molecular analyses, while a group of cumulus oocyte complexes (COCs) were in vitro matured, fertilized and cultured up to the blastocyst stage (n = 238 and 234, respectively in the BS and NBS).

Follicular fluid was aspirated using an 18 G needle under vacuum (40–50 mm Hg) in Falcon tubes and poured into a petri dish for COC recovery. The COCs were evaluated according to morphology and classified according to Di Francesco et al. [10]. 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. For each replicate, COCs were denuded of their cumulus cells by gentle pipetting and denuded oocytes were washed in phosphate buffer solution (PBS) + 0.1% polyvinyl alcohol (PVA), pooled (20/pool), snap frozen in liquid nitrogen and stored at − 80 °C until RNA isolation. The follicular fluid was centrifuged at 300×g for 10 min at 4 °C to separate the follicular fluid and the FCs. After centrifugation, the supernatant was centrifuged again at 2000g for 10 min and the pellet containing FCs was snap frozen in liquid nitrogen and stored at − 80 °C until RNA isolation.

In vitro embryo production. Unless otherwise stated, reagents were purchased from Sigma Chemical Company (Milano, Italy). The methods for in vitro maturation (IVM) described below have been reproduced in part from Gasparrini et al. [26]. For each replicate, Grade A and B COCs recovered by follicular aspiration were rinsed in HEPES-buffered TCM199 supplemented with 10% fetal calf serum (FCS) and in vitro matured, fertilized and cultured to the blastocyst stage. 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 [26].

The methods for in vitro fertilization (IVF) and culture (IVC) described below have been reproduced from Di Francesco et al. 2012 [11]. 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 300g) on a Percoll discontinuous gradient (45% and 80%). The sperm pellet was resuspended to a final concentration of 2 × 106 mL−1 in the IVF medium, consisting of Tyrode albumin lactate pyruvate [27] 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, putative 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 [28]. Culture was carried out under humidified air with 5% CO2, 7% O2 and 88% N2 at 38.5 °C. On day 5 post-insemination (pi) the cleavage rate was assessed and the embryos transferred into fresh medium for further 2 days of IVC, when blastocyst rates were recorded.

1.2.2 RNA isolation

Samples for RNA isolation were obtained from pools (n = 20) of OOs and FCs for both conditions (BS and NBS). The methods described below have been reproduced in part from Lange-Consiglio et al. [29]. Total RNA was isolated by NucleoSpin miRNA kit (Macherey–Nagel, Germany), following the protocol in combination with TRIzol (Invitrogen, Carlsbad, CA, USA) lysis with small and large RNA in one fraction (total RNA). Concentration and quality of RNA were determined by Agilent 2,100 Bioanalyzer (RIN ≥ 6.5 and 7.5 for OOs and FCs, respectively) (Santa Clara, CA, USA). The isolated RNAs were stored at − 80 °C until use.

1.2.3 Library preparation and sequencing

In total, 20 libraries of small RNA and 20 libraries of RNA-Seq were obtained from five animals per group (n = 5) of two cellular types (OOs and FCs) in both seasons (BS and NBS). Small RNA libraries were prepared using TruSeq Small RNA Library Preparation kit, according to manu- facturer's instructions (Illumina). Small RNA (sRNAs) libraries were pooled together and purified with Agen- court AMPure XP (Beckman, Coulter, Brea, CA) (1 Vol. sample: 1.8 Vol. beads) twice [29]. The methods described below have been reproduced in part from Frattini et al. 2017 [30].

RNA-Seq libraries were generated using the Illumina TruSeq RNA Sample Preparation v2 Kit but with one-half of the recommended reagent volumes. Con- centration and profile of libraries were determined by Agilent 2100 Bioanalyzer before library sequencing on a single lane of Illumina Novaseq 6000 (San Diego, CA, USA).

1.2.4 Data analysis

1.2.4.1 miRNA analysis

Illumina raw sequences were quality checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed with Trimmomatic (version 0.32)[31], then miR- Deep2 (miRDeep2 (version 2.0.0.5)[32] was used for miRNA detection and discovery. Known miRNAs available at MirBase (https://www.mirbase.org/) were used to support miRNA identification. In particular, Bos taurus miRNAs were input to support known miRNA detection and miRNAs from related species (sheep, goat and human) were input to support novel miRNA identification. All the identified miRNAs were quantified using the miRDeep2 quantifier module. The Bioconductor edgeR package (version 2.4) was used to identify statistically significant differential expression between groups of samples (false discovery rate [FDR] < 0.05)[33]. Predicted miRNA gene targeting of differentially expressed Bos taurus miRNAs (DEmiRNAs) was performed with miR- Walk2.0 [34], using homologous human miRNAs as input identifiers. Target genes were submitted to GO analysis. GO classification of the DEGs was performed according to canonical GO categories, using the Cytoscape (version.3.2.1) plug-in ClueGO (version 2.3.5) which integrates GO and enhances biological interpretation of large lists of genes [35]. MicroRNA cluster analysis was performed with Genesis (version1.8.1) [36].

1.2.4.2 RNA-seq analysis

RNA-Seq raw data were trimmed using Trimmomatic (version 0.32) [31]. Sequences were aligned to the buffalo reference genome version UOA_WB_1 (GCF_003121395.1) using STAR_2.3.077. Sub- sequently, HTSeq-count (version 0.6.1p1) [38] was used to count sequences aligned to each gene. The software package EdgeR of Bioconductor (version 3.6) was used to estimate differential expression between groups of samples [33]. RNAseq cluster analysis was performed with Genesis (version1.8.1) [36].

Differentially expressed genes DEGs were submitted to GO analysis, using the Cytoscape (version.3.2.1) plug-in ClueGO (version 2.3.5) [35]. Venn diagrams for intersection between DEGs and miRNAs target genes, using the Venn Diagrams software (https://bioinfogp.cnb.csic.es/tools/venny/ version 2.1).

1.2.4.3 In vitro embryo production

Differences in cleavage and blastocyst rates between seasons were analyzed by Chi square test. The level of significance was set at $P < 0.05$.

1.2.5 Data availability

RNA-Seq data are available in the Sequence Reads Archive (SRA), BioProject accession number, PRJNA599337. Novel miRNA precursors and novel miRNA mature sequences are reported in Supplementary files S10 and S11.

1.3 Results

1.3.1 Cleavage and blastocyst rates

With regard to oocyte competence, cleavage rate was higher in the BS compared to the NBS (69.4%; vs. 60.7; $P < 0.05$). In addition, an improvement in blastocyst yields was recorded in the BS, both in relation to total COCs (26.5 vs. 16.3%; $P < 0.01$) and cleaved oocytes (38.7 vs. 27.4%; $P < 0.05$).

1.3.2 miRNAs

Twenty samples, i.e. 10 pools of OOs (sample OO1-OO5 from NBS and OO6-OO10 from BS) and 10 pools of FCs (sample FC1-FC5 from NBS and FC6-FC10 from BS), were characterized for their miRNA content.
About 10 million reads were sequenced for both OOs and FCs. About 1% and 20% of them were assigned to miRNAs in OOs and FCs, respectively.
In total, 769 miRNAs were identified in at least three samples in all conditions (468 Bos taurus bta-miRNAs, 279 novels, and 22 novels homologous to related species). Among them, 467 were detected in at least three OO samples and 635 in at least three FC samples. Principal Component

Analysis (PCA) clearly separates OOs and FCs according to their miRNA content, with 44% of the variance explained by component 1.
Few of the most expressed miRNAs showed a similar relative abundance in OOs and FCs (bta-miR-10b, bta- miR-148a and bta-miR-26a); on the contrary, expression rate of most miRNAs differed in the two cellular types (bta-miR-21-5p was highly expressed in FCs, whereas bta-miR-423-3p in OOs). In fact, there was a statistically significant difference in the expression of a high proportion of the miRNAs (n = 413) (False Discovery Rate FDR < 0.05) between OOs and FCs. When the two seasons were considered, the PCA produced a good distinction between oocytes collected in BS and NBS, whereas FCs from BS and NBS could not be clearly distinguished (Fig. 1.1). The number of differentially expressed miRNAs (DE-miRNAs, FDR < 0.05) between the two seasons was 13 for both OOs and FCs (Table 1.1). A view of the normalized expression of the most representative DE-miRNAs is shown in Fig. 1.2. Target prediction using human miRNAs homologous to buffalo DE-miRNAs led to the identification of 6,712 and 4,847 genes potentially regulated in OOs and FCs, respectively ($P < 0.05$). GO analysis using a subset of more significant target genes (n = 136 with $P < 0.0005$ for OOs, n = 139 with $P < 0.001$ for FCs) identified pathways related to triglyceride and cholesterol metabolism and transport, and mesoderm and epithelial cell morphogenesis differentiation for OOs, and related to photoperiodism, circadian clock regulation, and transforming growth factor beta signalling for FCs (Table 1.2).

1.3.3 RNASeq.

RNA-seq analysis was performed on the same samples used for miRNA profiling to evaluate the gene expression variation between the two cellular types and seasons. Approximately 23.5 ± 4.4 and 54.5 ± 10.5 millions of reads were obtained for OOs and FCs samples with a mapping rate of 93.5% and 92.4%, respectively. A total of 22,013 unique genes present in at least three samples from both cellular types were identified (19,240 counted in at least three OOs and 21,277 counted in at least three FCs samples). PCA considering the relative expression of these genes showed a clear separation between the two cellular types. There seems to be no seasonal effect in the overall transcript abundance for both OOs and FCs (Fig. 1.3).
The relative expression of mRNAs in the two cellular types was very different, with 14,680 (66.7%) DEGs between OOs and FCs. When DEGs were calculated between the two seasons, 22 mRNAs were found to differ

between NBS and BS for OOs, whereas only two DEGs were present in FCs (Table 1.3).
Although a limited number of DEGs was found to differ in OOs between the two seasons, GO analysis revealed that some of them were related to lipid storage and localization and regulation of interleukin-8 (IL8) production (Table 1.4).

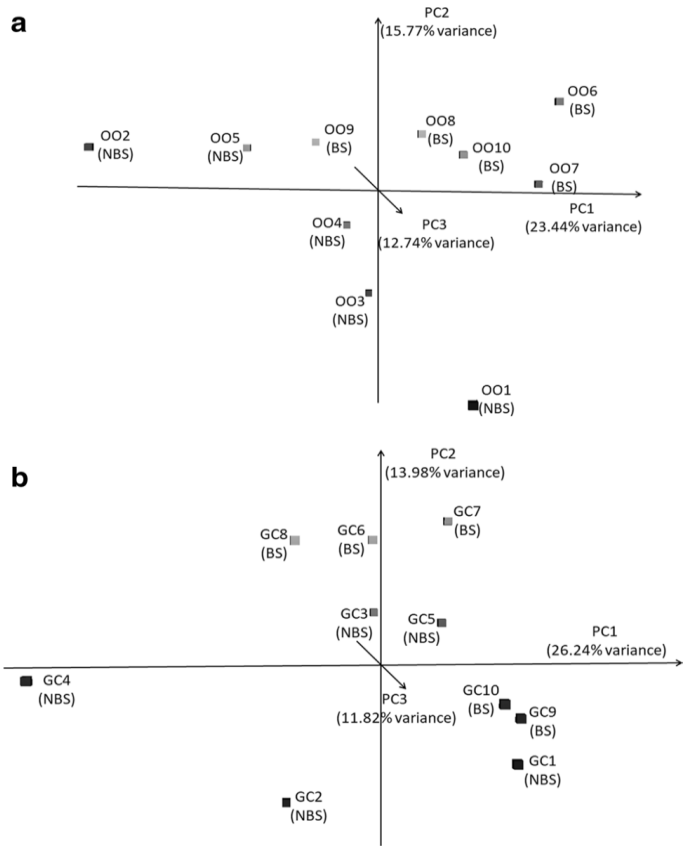

Fig. 1.1 Principal component analysis considering (a) the 467 miRNAs expressed at least in triplicate in the oocytes (OOs), (b) the 635 miRNAs expressed at least in triplicate in the Follicular cells (FCs). Samples 1–5 from non-breeding season (NBS), samples 6–10 from breeding season (BS).

OOs (NBS vs BS)			FCs (NBS vs BS)		
miRNAs	**logFC**	**FDR**	**miRNAs**	**logFC**	**FDR**
bta-miR-143	−2.07	5.42E−05	Novel:NC_037567.1_45577	2.65	1.69E−05
Novel:NC_037550.1_18643	1.58	7.24E−04	Novel:NC_037553.1_23674	2.09	3.78E−05
bta-miR-199a-3p	−2.10	7.24E−04	Novel:chi-miR-184	−4.24	1.54E−04
bta-miR-1468	−1.83	3.69E−03	bta-miR-2904	−2.90	2.50E−04
bta-miR-25	−0.91	1.44E−02	Novel:NC_037550.1_18643	−2.37	4.25E−04
bta-miR-1388-5p	−4.55	3.55E−02	bta-miR-2411-3p	−2.01	1.54E−03
bta-miR-296-3p	−1.41	3.91E−02	bta-miR-2440	−1.86	2.98E−03
Novel:NC_037557.1_30140	−1.36	3.91E−02	bta-miR-2332	−1.47	4.48E−03
Novel:NC_037569.1_47305	−1.36	3.91E−02	bta-miR-141	−3.73	4.48E−03
Novel:NC_037564.1_42998	−1.36	3.91E−02	bta-miR-2478	1.68	4.97E−03
bta-miR-331-5p	−4.19	4.12E−02	bta-miR-34b	−4.11	8.16E−03
bta-miR-199a-5p	−2.09	4.47E−02	bta-miR-34c	−4.02	8.16E−03
bta-miR-222	−1.35	4.95E−02	bta-miR-486	0.96	3.65E−02

Table 1.1 Differentially expressed miRNAs DE-miRNAs (false discovery rate (FDR) < 0.05) between the two seasons (NBS = non breeding season, BS = breeding season) for oocytes (OOs) and follicular cells (FCs).

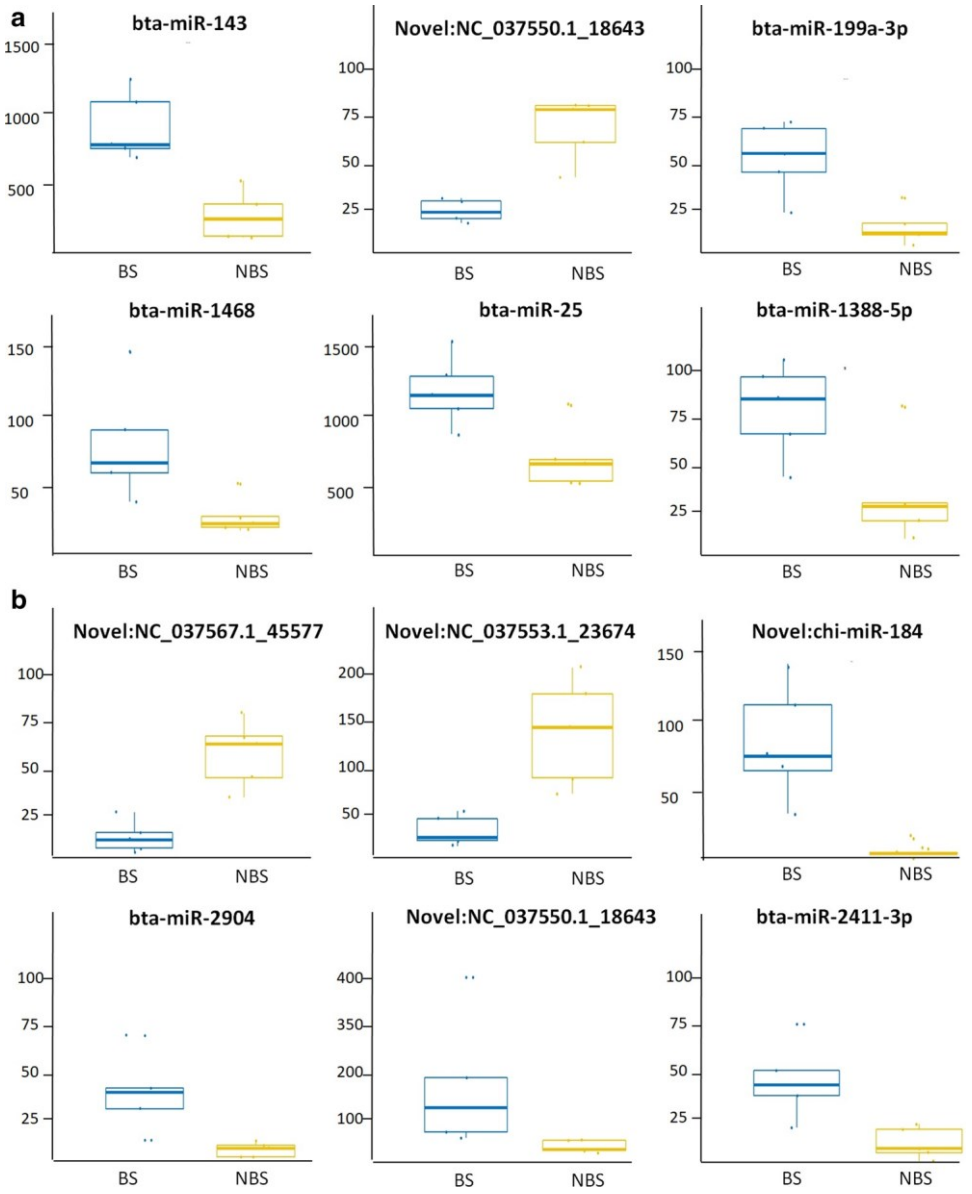

Figure 1.2 Box Plot of the most significant (top six sorted by FDR value) differentially expressed miRNAs (DE-miRNAs) in (a) oocyte and (b) follicular cells from animals between breeding season (BS) and non breeding season (NBS).

	GOID	Associated genes found	GO term	*P* value*
OOs	0010866	[DGAT2, FITM2, NR1H3]	Regulation of triglyceride biosynthetic process	0.004
	0060742	[NOTCH1, SFTPA1, SFTPA2]	Epithelial cell differentiation involved in prostate glanddevelopment	0.005
	0015918	[ABCG4, APOB, NR1H3, OSBPL6, PNLIP]	Sterol transport	0.009
	0030850	[NOTCH1, SFTPA1, SFTPA2, WNT5A]	Prostate gland development	0.012
	0048332	[AXIN1, GNPDA1, WNT5A]	Mesoderm morphogenesis	0.014
	0019915	[APOB, DGAT2, FITM2, NR1H3]	Lipid storage	0.026
	0001707	[AXIN1, GNPDA1, WNT5A]	Mesoderm formation	0.027
	0006536	[ATAT1, GGT1, GLUD2]	Glutamate metabolic process	0.028
	0006641	[APOB, DGAT2, FITM2, INSIG1, NR1H3]	Triglyceride metabolic process	0.028
	0042116	[IL13, NR1H3, WNT5A]	Macrophage activation	0.028
	0090207	[DGAT2, FITM2, NR1H3]	Regulation of triglyceride metabolic process	0.029
	0008206	[ACAA1, CYP27A1, OSBPL6]	Bile acid metabolic process	0.030
	0002637	[EXOSC3, GNPDA1, IL13]	Regulation of immunoglobulin production	0.033
	0070527	[FERMT3, MYH9, PRKCQ]	Platelet aggregation	0.033
	0002067	[IL13, NOTCH1, WNT5A]	Glandular epithelial cell differentiation	0.033
	0019217	[DGAT2, INSIG1, NR1H3, PDHB]	Regulation of fatty acid metabolic process	0.034
	0097006	[APOB, DGAT2, PLAGL2]	Regulation of plasma lipoprotein particle levels	0.035
	0055090	[DGAT2, FITM2, NR1H3]	Acylglycerol homeostasis	0.035
	0070328	[DGAT2, FITM2, NR1H3]	Triglyceride homeostasis	0.035
	0030301	[ABCG4, APOB, NR1H3, PNLIP]	Cholesterol transport	0.037
	0050830	[DROSHA, HIST1H2BK, PGLYRP1, PGLYRP3]	Defence response to Gram-positive bacterium	0.037
	0033344	[ABCG4, APOB, NR1H3]	Cholesterol efflux	0.038
	0002702	[EXOSC3, GNPDA1, IL13, WNT5A]	Positive regulation of production of molecular mediatorof immune response	0.038
	0030514	[CHRDL1, NOTCH1, WNT5A]	Negative regulation of BMP signaling pathway	0.038
	0010883	[APOB, FITM2, NR1H3]	Regulation of lipid storage	0.039
	0019432333 3	[DGAT2, FITM2, NR1H3]	Triglyceride biosynthetic process	0.039
	0043030	[IL13, NR1H3, WNT5A]	Regulation of macrophage activation	0.039
	0045599	[AXIN1, INSIG1, WNT5A]	Negative regulation of fat cell differentiation	0.040
	0002639	[EXOSC3, GNPDA1, IL13]	Positive regulation of immunoglobulin production	0.040
	0046460	[DGAT2, FITM2, NR1H3]	Neutral lipid biosynthetic process	0.040
	0046463	[DGAT2, FITM2, NR1H3]	Acylglycerol biosynthetic process	0.040
	0043153	[BHLHE40, PPP1CB, PPP1CC]	Entrainment of circadian clock by photoperiod	0.004
	1903844	[ING3, ONECUT2, SKI, STRAP, XBP1]	Regulation of cellular response to transforming growthfactor beta stimulus	0.008
	0017015	[ING3, ONECUT2, SKI, STRAP, XBP1]	Regulation of transforming growth factor beta receptorsignaling pathway	0.008
	0009648	[BHLHE40, PPP1CB, PPP1CC]	Photoperiodism	0.008
	0009649	[BHLHE40, PPP1CB, PPP1CC]	Entrainment of circadian clock	0.008
	1903845	[ONECUT2, SKI, STRAP, XBP1]	Negative regulation of cellular response to transforminggrowth factor beta stimulus	0.012
	0030512	[ONECUT2, SKI, STRAP, XBP1]	Negative regulation of transforming growth factor betareceptor signaling pathway	0.012

FCs	0010923	[FKBP1B, PPP1R1B, TMEM225]	Negative regulation of phosphatase activity	0.014
	0032755	[IL1RL2, TLR6, XBP1]	Positive regulation of interleukin-6 production	0.025
	0045582	[IL1RL2, ITPKB, XBP1]	Positive regulation of T cell differentiation	0.025
	0035304	[FKBP1B, PPP1R1B, SMPD1]	Regulation of protein dephosphorylation	0.035
	0071230	[CASTOR1, PDGFC, XBP1]	Cellular response to amino acid stimulus	0.035
	1903036	[FKBP1B, SCARF1, XBP1]	Positive regulation of response to wounding	0.040
	0010257	[NDUFAF6, NDUFC1, NDUFS7]	NADH dehydrogenase complex assembly	0.042
	0032981	[NDUFAF6, NDUFC1, NDUFS7]	Mitochondrial respiratory chain complex I assembly	0.042
	0097031	[NDUFAF6, NDUFC1, NDUFS7]	Mitochondrial respiratory chain complex I biogenesis	0.042
	0032922	[BHLHE40, PPP1CB, PPP1CC]	Circadian regulation of gene expression	0.047

*Table 1.2 GO terms identified for the target genes of differentially expressed miRNAs between the two seasons for oocytes (OOs) and Follicular Cells (FCs). Indicated are gene ontology IDs (GO-ID), gene ontology terms (GO-term), associated genes found and corrected P values as determined by ClueGO (https://apps.cytoscape. org/apps/cluego). *Term P value corrected with Bonferroni step down.*

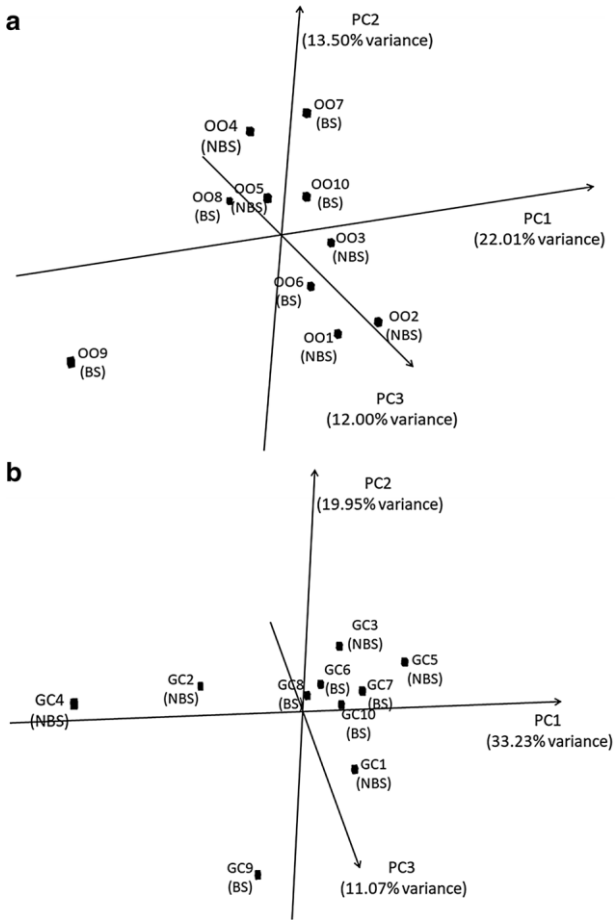

Figure 1.3 Principal component analysis considering (a) the 19,240 mRNAs expressed at least in triplicate in the oocytes (OOs), (b) the 21,277 mRNAs expressed at least in triplicate in the Follicular cells (FCs). Samples 1–5 from non-breeding season (NBS), samples 6–10 from breeding season (BS).

OOs (NBS vs BS)				FCs (NBS vs BS)			
GENE ID	**Human and cattle ortholog**	**logFC**	**FDR**	**GENE ID**	**Human and cattle ortholog**	**logFC**	**FDR**
APOE	APOE	−4.7	8.6E−07	LOC102409538	RNF213	−4.7	5.6E−04
LOC102397479	LOC102397479	−5.2	1.3E−05	COL26A1	COL26A1	2.1	2.3E−02
PLXNA4	PLXNA4	−2.3	2.6E−05				
IGF2	IGF2	−4.7	5.2E−04				
FOLR2	FOLR2	−7.5	8.0E−04				
CD14	CD14	−8.7	8.7E−04				
SPP1	SPP1	−3.2	2.5E−03				
LOC102409999	CD163	−4.7	2.8E−03				
CTSS	CTSS	−5.3	2.8E−03				
LOC102413141	GTF2IRD2	−0.7	3.9E−03				
LOC102392787	IL1B	−5.0	6.1E−03				
LOC112581169	LOC112581169	−0.9	7.6E−03				
CCL1	CCL1	−1.2	9.7E−03				
CTSK	CTSK	−3.8	9.7E−03				
LOC102415727	regakine 1	−5.3	1.1E−02				
MSR1	MSR1	−3.7	1.2E−02				
RUNX2	RUNX2	−1.3	1.9E−02				
LOC102404545	LOC102404545	2.3	2.1E−02				
LOC102400151	CYP11A1	−2.6	2.3E−02				
LOC102409533	HSPA1A	−0.8	3.6E−02				
NMB	NMB	−1.9	4.7E−02				
LOC112582161	LOC112582161	0.9	4.8E−02				

Table 1.3 Differentially expressed gene DEGs (false discovery rate (FDR) < 0.05) calculated between the two seasons (non breeding season NBS, breeding season) for oocytes (OOs) and follicular cells (FCs).

GOID	Associated genes found	GO term	*P* value*
GO:19915	[APOE, IL1B, MSR1]	Lipid storage	7.17E−05
GO:32370	[APOE, IL1B, SPP1]	Positive regulation of lipid transport	8.94E−05
GO:32677	[CD14, HSPA1A, IL1B]	Regulation of interleukin-8 production	4.09E−05
GO:32757	[CD14, HSPA1A, IL1B]	Positive regulation of interleukin-8 production	6.29E−05
GO:1905954	[APOE, IL1B, MSR1, SPP1]	Positive regulation of lipid localization	5.76E−06

*Table 1.4 GO terms identified for the differentially expressed gene (DEGs) between the two seasons for oocytes (OOs). Indicated are gene ontology IDs (GO-ID), gene ontology terms (GO-term), associated genes found and corrected P values as determined by ClueGO (https://apps.cytoscape.org/apps/cluego). * Term P value corrected with Bonferroni step down.*

1.3.5 miRNAs and mRNA interaction

In order to evaluate whether miRNAs could potentially regulate the expression of specific genes, the list of genes differentially expressed in oocytes between the two seasons was intersected with the list of DE-miRNA target genes observed in the same experimental condition. Six genes (CCL1, FOLR2, IGF2, HSPA1A, IL1B, CTSK) were found to be potentially regulated by specific DE-miRNAs. Interestingly, among the 8 DE-miRNAs (miR-143, miR-1468, miR-199a-3p, miR-199a-5p, miR-222, miR-25, miR-296-3p, miR-331-5p) targeting 6,712 genes, miR-296-3p targets 4 out of the 6 shared DEGs. In addition, all the miRNAs show a positive correlation with gene expression (Fig. 1.4).

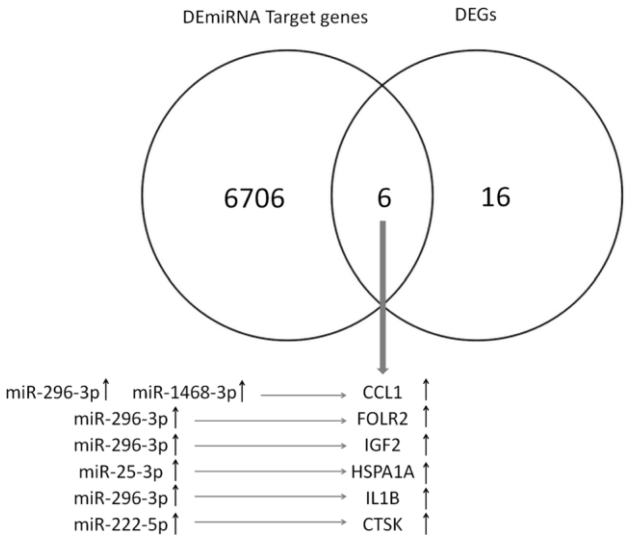

Figure 1.4 Venn diagram (https://bioinfogp.cnb.csic.es/tools/venny/ version 2.1) representing the intersection (n = 6) between differentially expressed genes (DEGs, n=22) and target genes of the differentially expressed miRNAs (DE-miRNAs, n=6,712) in oocytes collected in the NBS and BS. Shared genes with their relative target miRNAs are reported. ↑indicates overexpression in breeding season (both for genes and miRNAs).

1.4 Discussion

The present study aims to investigate the causes of the decreased oocyte competence during the NBS in buffalo. Many biological processes are required for developmental competence, with the exchange of information

between oocyte and follicular environment promoting oocyte maturation [39]. Therefore, the focus of this study was to evaluate differences in the miRNA and transcriptomic profiles of OOs and corresponding FCs from buffalo ovaries collected in the BS and NBS. To our knowledge, this is the first study reporting together the miRNA and mRNA profiling from pools of low numbers of oocytes and corresponding FCs collected from abattoir-derived ovaries in livestock, as well as the first time that the seasonal effects on miRNA and mRNA profiling of oocytes and FCs are investigated in buffalo. Unfortunately, the amount of RNA obtained from such a limited number of oocytes was not sufficient to perform further experiments to validate our results.

In accordance with previous findings [10,11], in the present study we observed a reduced oocyte developmental competence in the NBS, as indicated by decreased cleavage and blastocyst rates after in vitro fertilization (IVF), and this was associated to changes in miRNA and transcriptomic profiles both in OOs and FCs. Being miRNAs only one of the small RNA components, as expected those identified in buffalo represent only a fraction of the total small non-coding RNA present in both OOs and FCs [40,41]. The overall miRNA expression pattern was different between OOs obtained in the two seasons. However, FCs from the two seasons cluster together. Considering that different phenotypes of FCs can be observed within the follicle, it is likely that miRNA expression in FCs is mainly driven by cell position and function, thus masking seasonal effects [42]. Recently, Zhang et al. [43] reviewed miRNA profile studies related to ovarian development and function in mammals. Although many studies reported miRNA ovary profiling, only a few investigated miRNA variations in granulosa cells and only one in OOs [43].

Interestingly, many of the differentially expressed miRNAs in the two seasons for both OOs and FCs are involved in follicular maturation and development regulation. In our study seasonal changes modified the expression of miR-143, miR-25, miR-222 and miR-199a in buffalo OOs. In the mouse ovary, miR-143 is highly expressed and related to oestradiol production and steroidogenesis gene expression [44]. MiR-143 and miR-25 were also shown to promote progesterone release in human ovarian granulosa cells [45]. Furthermore, cyclic variations in the expression of miR-222 and miR-199a were reported in cattle during follicle maturation, with expression increasing until the mid-luteal phase, and decreasing in the late follicular phase in the bovine dominant follicle [13]. Some of the differentially expressed miRNAs identified between the NBS and BS in

buffalo FCs (miR-184, miR- 2411 and miR-34c) were also reported to exhibit expression modulation during the cycle in cattle. In particular, temporal miRNA expression dynamics were observed for miR-184 in FCs between days 3 and 7 of the bovine oestrous cycle and for miR-2411 and miR-34c between subordinate and dominant follicles during the early luteal phase [46]. MiR-34c was shown to exert anti-proliferative and pro-apoptotic effects in porcine granulosa cells by targeting Forkhead box O3a (FoxO3a) [47].

In addition, the expression of some of the DE-miRNAs detected in our study differs in several ovarian disorders. It was reported that miR-141 and miR-199a are respectively up and down regulated in human ovarian cancer [48], miR-184 is a potential predictor of recurrence in human ovarian granulosa cell tumours [48], and miR-486-5p is downregulated in cumulus cells collected from women affected by polycystic ovary syndrome [49]. Interestingly, GO analysis of the predicted target genes for DE-microRNAs uncovered pathways associated with OOs and FCs physiology. Oocytes collected from the BS and NBS showed DE-miRNAs able to regulate genes for triglyceride and sterol biosynthesis essential for lipid metabolism, which provides a potent source of energy during oocyte maturation [50]. In FCs, the DE-miRNA target genes were related to pathways involved in transformation of growth factor β (TGFβ) and circadian clock photoperiod. TGFβ promotes granulosa cell proliferation regulating the expression of luteinizing hormone receptor (LH-R) [51,52,53]. Altered photoperiod can affect mRNA expression in ovaries [15], in fact, transcriptome changes occurred between BS and NBS samples. Considering DEGs between seasons, it is interesting to note that although only two DEGs were found in FCs, many of the DEGs in the OOs are known to be related to oocyte competence. In the NBS, decreased oocyte competence in buffalo was associated to change in the expression of secreted phosphoprotein 1 (SPP1), RUNX family transcription factor 2 (RUNX2) and Cathepsin K (CTSK) in OOs. Both SPP1 and RUNX2 expression was observed to change in oocyte and-granulosa cell complexes at various stages of follicle develop- ment in pigs [54]. In addition, variations in the expression of SPP1 were recorded in cumulus cells derived from cumulus-oocyte-complexes (COCs) collected from cows undergoing FSH priming, as a model of high oocyte competence [55], and RUNX2 expression was associated with controlled ovarian stimulation outcome in assisted reproductive technology treatment in women [44]. Furthermore, CTSK in cumulus cells was suggested as a predic- tive marker for oocyte competence in bovine COC [56].

In buffalo OOs during the NBS, a decreased expression of heat shock protein family A (Hsp70) member 1A (HSPA1A), known to be related to oocyte survival and apoptosis, was also observed. The HSPA1A plays a critical role through its protective action against apoptosis and its expression is reduced in poor, as compared to competent, ovine COCs [57].
Another transcript downregulated in the NBS OOs is interleukin-1 beta (IL-1β). Although IL-1β deficiency in mice prolongs ovarian lifespan [58], IL-1β stimulates the growth and sustains maturation in mare [59] and bovine oocytes [60]. In addition, IL-1β was postulated to be involved in different ovulation associated events such as prostaglandin production and steroidogenesis [61]. Modulation of expression levels was observed in this study also for other genes related to gonadotropic hormone synthesis and metabolism, showing a reduced expression in buffalo OOs during NBS. Apolipoprotein E (APOE) is expressed in cultured ovarian granulosa cells, and is present in human follicular fluid where its relative levels are correlated with serum estrogen concentration [62]. In rats, APOE exerts a role in directing cholesterol during steroidogenesis and regulating follicular estrogenic production [63]. Insulin like growth factor 2 (IGF2) was observed to be expressed in bovine oocytes [64]. The expression of IGF2 is modulated by growth hormone (GH) in in vitro matured Rhesus macaque oocytes [65]. Furthermore, it is known that IGF2, in combination with follicle stimulating hormone (FSH), acts directly on oocyte competence in caprine follicles [66]. Finally, the folate receptor beta (FOLR2), also down regulated in OOs during the NBS, is a key gene linked to methionine/folate cycles in bovine oocyte [67], also involved in folate transport in mice oocytes during follicular development [68].
In our study, a positive correlation between DEGs and DE-miRNA target genes was observed. MiRNAs usually mediate repression of their target mRNAs by inhibiting their translation, therefore reducing the abundance of their products [69]. However, several studies reported a positive miRNA-mRNA regulation with a feed forward mechanism probably mediated by transcription factors [70]. Notably, among all DE-miRNAs, miR-296-3p is the most correlated with transcripts changing in OOs between BS and NBS. MicroR-296-3p was previously reported to be expressed in ovaries in mice [71] and to repress cell plasticity in different tumour lines [72], promoting apoptosis in liver [73] and in mammalian pancreatic α cells [74]. Recently, altered expression of bta-miR-296-3p was detected in muscle, kidney, and liver, in bovine foetuses with large offspring syndrome (LOS) [75]. In

addition, miR-296 was also observed to be epigenetically regulated as a part of the imprinted Gnas/GNAS clusters [76].

1.5 Conclusion

In conclusion, the reduced oocyte developmental competence recorded during the NBS in buffalo is associated with changes in miRNA and mRNA content in OOs and corresponding FCs. The GO analysis showed over-representation of key genes related to lipid and sterol biosynthesis and hormone regulation, crucial for folliculogenesis and acquisition of oocyte competence. These observations might help to explain the seasonal difference in the potential of buffalo oocytes, thus providing the basis for the development of strategies to improve oocyte competence in the NBS. Nevertheless, further efforts are still needed to validate expression modulation of miRNAs and key genes identified in our study and deeply investigate their role in seasonal reproduction in buffalo.

References

[1] Campanile, G. et al. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. Anim. Reprod. Sci. 121, 1–11 (2010).
[2] Zicarelli, L. Influence of seasonality on buffalo production. In The buffaloes (Bubalus bubalis)—production and research (ed. Presicce, G. A.) 196–224 (Bentham Science, Sharjah, 2016).
[3]. Gasparrini, B. Effects of reproductive season on embryo development in buffalo. Reprod. Fertil. Dev. 31, 68–81 (2019).
[4]. Campanile, G. et al. Embryonic mortality in buffaloes synchronized and mated by AI during the seasonal decline in reproductive function. Theriogenology 63, 2334–2340 (2005).
[5]. Campanile, G. et al. Corpus luteum function and embryonic mortality in buffaloes treated with a GnRH agonist, hCG and progesterone. Theriogenology 67, 1393–1398 (2007).
[6] Balestrieri, M. L. et al. Proteomic profiles of the embryonic chorioamnion and uterine caruncles in buffaloes (Bubalus bubalis) with normal and retarded embryonic development. Biol. Reprod. 88, 119 (2013).
[7] Strazzullo, M. et al. Global transcriptome profiles of Italian Mediterranean buffalo embryos with normal and retarded growth. PLoS ONE 9, e90027 (2014).
[8] Manjunatha, B. M., Ravindra, J. P., Gupta, P. S., Devaraj, M. & Nandi, S. Effect of breeding season on in vivo oocyte recovery and embryo production in non-descriptive Indian river buffaloes (Bubalus bubalis). Anim. Reprod. Sci 111, 376–383 (2009).
[9] Abdoon, A. S., Gabler, C., Holder, C., Kandil, O. M. & Einspanier, R. Seasonal variations in developmental competence and relative abundance of gene transcripts in buffalo (Bubalus bubalis) oocytes. Theriogenology 82, 1055–1067 (2014).
[10] Di Francesco, S. et al. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (Bubalus bubalis). Anim. Reprod. Sci. 123, 48–53 (2011).
[11] Di Francesco, S. et al. Ovum pick-up and in vitro embryo production (OPU-IVEP) in Mediterranean Italian buffalo performed in different seasons. Theriogenology 77, 148–154 (2012).
[12] Salzano, A. et al. Effect of photoperiod on follicular IGF-1 and oocyte quality independently of metabolic status in buffalo heifers. Ital. J. Anim. Sci. 18, 949–956 (2019).

[13] Tesfaye, D. et al. MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development. Reproduction 155, R121–R135 (2018).
[14] Mehta, N. & Cheng, H. Y. Micro-managing the circadian clock: the role of microRNAs in biological timekeeping. J. Mol. Biol. 425, 3609–3624 (2013).
[15] Salomon, A. K., Leon, K., Campbell, M. M. & Young, K. A. Folliculogenic factors in photoregressed ovaries: differences in mRNA expression in early compared to late follicle development. Gen. Comp. Endocrinol. 260, 90–99 (2018).
[16] Mara, L., Sanna, D., Casu, S., Dattena, M. & Muñoz, I. M. Blastocyst rate of in vitro embryo production in sheep is affected by season. Zygote 22, 366–371 (2013).
[17] Chen, L. et al. Identification of sheep ovary genes potentially associated with off-season reproduction. J. Genet. Genomics 39, 181–190 (2012).
[18] Di, R. et al. Characterization and comparative profiling of ovarian microRNAs during ovine anestrus and the breeding season. BMC Genomics 15, 899 (2014).
[19] Zhai, M., Xie, Y., Liang, H., Lei, X. & Zhao, Z. Comparative profiling of differentially expressed microRNAs in estrous ovaries of Kazakh sheep in different seasons. Gene 664, 181–191 (2018).
[20] Yang, J. et al. Comparative mRNA and miRNA expression in European mouflon (Ovis musimon) and sheep (Ovis aries) provides novel insights into the genetic mechanisms for female reproductive success. Heredity (Edinb). 122, 172–186 (2019).
[21] Sontakke, S. D., Mohammed, B. T., McNeilly, A. S. & Donadeu, F. X. Characterization of microRNAs differentially expressed during bovine follicle development. Reproduction 148, 271–283 (2014).
[22] Gebremedhn, S. et al. MicroRNA expression profile in bovine granulosa cells of preovulatory dominant and subordinate follicles during the late follicular phase of the estrous cycle. PLoS ONE 10, e0125912 (2015).
[23] Liu, S. et al. Identifying hub genes for heat tolerance in water buffalo (Bubalus bubalis) using transcriptome data. Front. Genet. 10, 209 (2019).

[24] Cai, X. et al. Identification and analysis of the expression of microRNA from lactating and nonlactating mammary glands of the Chinese swamp buffalo. J. Dairy Sci. 100, 1971–1986 (2017).
[25] Jerome, A., Thirumaran, S. M. K. & Kala, S. N. Identification of microRNAs in corpus luteum of pregnancy in buffalo (Bubalus bubalis) by deep sequencing. Iran. J. Vet. Res. 18, 287–290 (2017).
[26] Gasparrini, B., Neglia, G., Di Palo, R., Campanile, G. & Zicarelli, L. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 54, 1537–1542 (2000).

12 Vol:.(1234567890) Scientific Reports | (2020)
[27] Lu, K. H., Gordon, I., Gallagher, M. & Mc Govern, H. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. Vet Rec 121, 259–260 (1987).
[28] Tervit, H. R., Whittingham, D. G. & Rowson, L. E. Succesful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30, 493–497 (1973).
[29] Lange-Consiglio, A. et al. Amniotic microvesicles impact hatching and pregnancy percentages of in vitro bovine embryos and blastocyst microRNA expression versus in vivo controls. Sci. Rep. 10, 501 (2020).
[30] Frattini, S. et al. Genome-wide analysis of DNA methylation in hypothalamus and ovary of Capra hircus. BMC Genomics 18, 476 (2017).
[31] Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics 30, 2114–2120 (2014).
[32] Friedländer, M. R., Mackowiak, S. D., Li, N., Chen, W. & Rajewsky, N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucl. Acids Res. 40, 37–52 (2012).
[33] Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2009).
[34] Dweep, H. & Gretz, N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods 12, 697 (2015).
[35] Bindea, G. et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–1093 (2009).

[36] Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. Bioinformatics 18, 207–208 (2002).
[37] Dobin, A. et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
[38] Anders, S., Pyl, P. T. & Huber, W. HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169 (2015).
[39] Skowronski, M. T. et al. Pituitary hormones (FSH, LH, PRL, and GH) differentially regulate AQP5 expression in porcine ovarian follicular cells. Int J Mol Sci 20, E4914 (2019).
[40] Yang, Q. et al. Single-cell CAS-seq reveals a class of short PIWI-interacting RNAs in human oocytes. Nat. Commun. 10, 3389 (2019).
[41] Xu, Y. et al. TGF-β1 resulting in differential microRNA expression in bovine granulosa cells. Gene 663, 88–100 (2018).
[42] Dong, J. P. et al. CD24: a marker of granulosa cell subpopulation and a mediator of ovulation. Cell Death Dis. 10, 791 (2019).
[43] Zhang, J., Xu, Y., Liu, H. & Pan, Z. MicroRNAs in ovarian follicular atresia and granulosa cell apoptosis. Reprod. Biol. Endocrinol. 17, 9 (2019).
[44]. Zhang, L. et al. MiRNA-143 mediates the proliferative signaling pathway of FSH and regulates estradiol production. J. Endocrinol. 234, 1–14 (2017).
[45]. Sirotkin, A. V., Ovcharenko, D., Grossmann, R., Lauková, M. & Mlyncek, M. Identification of microRNAs controlling human ovarian cell steroidogenesis via a genome-scale screen. J. Cell Physiol. 219, 415–420 (2009).
[46]. Salilew-Wondim, D. et al. The expression pattern of microRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. PLoS ONE 9, e106795 (2014).
[48]. Xu, Y. et al. MicroRNA-34c regulates porcine granulosa cell function by targeting forkhead box O3a. J. Integr. Agric. 16, 2019–2028 (2017).
[49] Iorio, M. V. et al. MicroRNA signatures in human ovarian cancer. Cancer Res. 67, 8699–8707 (2007).
[50] Cheng, W. T. et al. MicroRNA profiling of ovarian granulosa cell tumours reveals novel diagnostic and prognostic markers. Clin. Epigenet. 9, 72 (2017).

[51] Shi, L., Liu, S., Zhao, W. & Shi, J. miR-483-5p and miR-486-5p are down-regulated in cumulus cells of metaphase II oocytes from women with polycystic ovary syndrome. Reprod. Biomed. Online 31, 565–572 (2015).
[52] Dunning, K., Russell, D. & Robker, R. Lipids and oocyte developmental competence: the role of fatty acids and β-oxidation. Reproduction 148, R15-27 (2014).
[53] Knecht, M., Feng, P. & Catt, K. J. Transforming growth factor-beta regulates the expression of luteinizing hormone receptors in ovarian granulosa cells. Biochem. Biophys. Res. Commun. 139, 800–807 (1986).
[54] Dorrington, J., Chuma, A. V. & Bendell, J. J. Transforming growth factor beta and follicle-stimulating hormone promote rat granulosa cell proliferation. Endocrinology 123, 353–359 (1988).
[55] Inoue, K. et al. Effect of transforming growth factor beta on the expression of luteinizing hormone receptor in cultured rat granulosa cells. Biol. Reprod. 67, 610–615 (2002).
[56] Munakata, Y. et al. Gene expression patterns in granulosa cells and oocytes at various stages of follicle development as well as in in vitro grown oocyte-and-granulosa cell complexes. J. Reprod. Dev. 62, 359–366 (2016).
[57] Sugimura, S. et al. Transcriptomic signature of the follicular somatic compartment surrounding an oocyte with high developmental competence. Sci. Rep. 7, 6815 (2017).
[58] Papamentzelopoulou, M. et al. Detection of RUNX2 gene expression in cumulus cells in women undergoing controlled ovarian stimulation. Reprod. Biol. Endocrinol. 10, 99 (2012).
[59] Bettegowda, A. et al. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. Biol. Reprod. 79, 301–309 (2008).
[60] Dhali, A. et al. Temporal expression of cumulus cell marker genes during in vitro maturation and oocyte developmental competence. J. Assist. Reprod. Genet. 34, 1493–1500 (2017).
[61] Uri-Belapolsky, S. et al. Interleukin-1 deficiency prolongs ovarian lifespan in mice. Proc. Natl. Acad. Sci. U. S. A. 111, 12492–12497 (2014).
[62] Caillaud, M., Duchamp, G. & Gérard, N. In vivo effect of interleukin-1beta and interleukin-1RA on oocyte cytoplasmic maturation,

ovulation, and early embryonic development in the mare. Reprod. Biol. Endocrinol. 3, 26 (2005).
[63] Lima, F. et al. Influence of interleukin 1 beta and tumour necrosis factor alpha on the in vitro growth, maturation and mitochondrial distribution of bovine oocytes from small antral follicles. Zygote 26, 381–387 (2018).
[64] Dang, X. et al. IL-1β Upregulates StAR and progesterone production through the ERK1/2- and p38-mediated CREB signaling pathways in human granulosa-lutein cells. Endocrinology 158, 3281–3291 (2017).
[65] Brown, S. A., Hay, R. V. & Schreiber, J. R. Relationship between serum estrogen and level of apolipoprotein E in human ovarian follicular fluid. Fertil. Steril. 51, 639–643 (1989).
[66] Polacek, D., Beckmann, M. W. & Schreiber, J. R. Rat ovarian apolipoprotein E: localization and gonadotropic control of messenger RNA. Biol. Reprod. 46, 65–72 (1992).
[66] Wang, L. M. et al. Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. Anim. Reprod. Sci. 114, 99–108 (2009).
[67] Nyholt de Prada, J. K., Kellam, L. D., Patel, B. G., Latham, K. E. & Vandevoort, C. A. Growth hormone and gene expression of in vitro-matured rhesus macaque oocytes. Mol. Reprod. Dev. 77, 353–362 (2010).
[68] Duarte, A. B. et al. Insulin-like growth factor II (IGF-II) and follicle stimulating hormone (FSH) combinations can improve the in vitro development of grown oocytes enclosed in caprine preantral follicles. Growth Horm. IGF Res. 23, 37–44 (2013).
[69] Kwong, W. Y., Adamiak, S. J., Gwynn, A., Singh, R. & Sinclair, K. D. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. Reproduction 139, 705–715 (2010).
[70] Meredith, M., MacNeil, A. H., Trasler, J. M. & Baltz, J. M. Growing mouse oocytes transiently activate folate transport via folate receptors as they approach full size. Biol. Reprod. 94, 125 (2016).
[71] Hutvágner, G. & Zamore, P. D. A microRNA in a multiple-turnover RNAi enzyme complex. Science 297, 2056–2060 (2002).
[72] Diaz, G., Zamboni, F., Tice, A. & Farci, P. Integrated ordination of miRNA and mRNA expression profiles. BMC Genomics 16, 767 (2015).

[73] Schneider, A. et al. Changes of ovarian microRNA profile in long-living ames dwarf mice during aging. PLoS ONE 12, e0169213 (2017).
[74] Vaira, V. et al. miR-296 regulation of a cell polarity-cell plasticity module controls tumor progression. Oncogene 31, 27–38 (2012).
[75] Cazanave, S. C. et al. A role for miR-296 in the regulation of lipoapoptosis by targeting PUMA. J. Lipid Res. 52, 1517–1525 (2011).
[76] Barbagallo, D. et al. miR-296-3p, miR-298-5p and their downstream networks are causally involved in the higher resistance of mammalian pancreatic α cells to cytokine-induced apoptosis as compared to β cells. BMC Genomics 14, 62 (2013).
[77] Li, Y. et al. Altered microRNA expression profiles in large offspring syndrome and Beckwith–Wiedemann syndrome. Epigenetics 14, 850–876 (2019).
[78] Robson, J. E., Eaton, S. A., Underhill, P., Williams, D. & Peters, J. MicroRNAs 296 and 298 are imprinted and part of the GNAS/Gnas cluster and miR-296 targets IKBKE and Tmed9. RNA 18, 135–144 (2012).

Chapter 2

Experiment 2: Variations of extracellular vesicles miRNAs content in relation to follicular development and season in buffalo (Bubalus bubalis)

2.1 Introduction

The importance of buffalo (Bubalus bubalis) breeding is clearly indicated by the world positive growth trend, due to specific features making the species an unreplaceable protein source particularly for tropical countries. A peculiar situation is described in Italy where the success of buffalo breeding is closely related to the production of mozzarella cheese, highly requested around the world. A major limiting factor is reproductive seasonality impeding continuity of milk production throughout the year and hence not allowing to meet the market demand. Buffalo is a short-day breeder, with an increased reproductive activity observed during decreasing day length months [1,2]. In Italy, where the Italian Mediterranean buffalo breed has been selected, the seasonality pattern shows an opposite trend to the market request. Therefore, the out of breeding mating strategy (OBMS), has been efficiently applied to distribute calving more evenly during the year [2]. However, forcing buffalo cows to conceive during the non-breeding season (NBS) may lead to extended post-partum anestrus, higher rates of embryonic mortality and overall reduced fertility [1,3,4]. In previous studies it was demonstrated that embryonic mortality is in part caused by impaired luteal function and consequently reduced progesterone secretion [5]. This in turn interferes with embryo growth that is accompanied by transcriptomic and proteomic changes at the level of embryos and chorioamnios/caruncles [6,7], definitively hampering embryo attachment. Embryonic mortality during the NBS is also in part due to reduced oocyte developmental competence, as shown by the decreased cleavage and blastocyst rates obtained after in vitro fertilization during increasing daylight months in Italian Mediterranean buffaloes [8,9]. A seasonal effect on follicular population and embryo outcomes was also reported by other authors [10,11]. A poorer oocyte quality was observed in Murrah buffalo heifers during long day months that was associated to lower intrafollicular levels of estradiol and IGF-1 [12], known to influence oocyte development [13,14,15]. However, the underlining biological causes of reduced oocyte competence in the NBS have not been unraveled yet in buffalo.

It is known that oocyte developmental competence is acquired during the last phase of oocyte growth that is strictly co-ordinated with follicular development [16]. Folliculogenesis involves complex paracrine interactions within ovaries and the bidirectional communication between oocytes and surrounding somatic cells is essential for oocyte maturation and acquisition of developmental competence, i.e. the capability of the oocyte to undergo

fertilization and embryogenesis. The acquisition of developmental competence is a gradual process requiring a precisely regulated spatio-temporal expression of various genes [16,17]. MicroRNAs (MiRNAs), known to regulate gene expression, play a significant role in mammalian follicular and oocyte development [18]. Many miRNAs were observed to change across ovarian developmental stages, and specific miRNAs were differentially expressed during follicular–luteal transition [19]. Equine follicular fluid contains different miRNAs whose expression changes between ovulatory and anovulatory follicles, in part reflecting changes observed in granulosa cells [20]. Follicular fluid hormones, metabolites and miRNAs content is correlated with follicular developmental stage [21,22]. Changes in follicular miRNAs levels during folliculogenesis have been reported in cattle, suggesting a regulatory role in oocyte growth [23]. Furthermore, in the same species it was recently demonstrated that differences in developmental competence are reflected in changes of miRNAs profile in follicular fluid and oocytes [24].

Follicular fluid contains extracellular vesicles (EVs) whose miRNAs cargo has been suggested to be implicated in cell communication in different species [25,26]. Co-incubation of bovine follicular EVs with cumulus-oocyte complexes (COC) was reported to promote cumulus expansion and increase the expression of key genes [27]. It was also demonstrated that EVs isolated from bovine follicular fluid enhance in vitro oocyte maturation and embryo development and influence miRNAs profile and developmental related genes in embryos [28].

In the sheep, another short-day breeder, seasonal effects were demonstrated on ovarian transcriptome and miRNAs profile between anestrus and breeding season [29,30]. Furthermore, seasonal-related variations in miRNAs involved in hormone regulation, follicular growth and angiogenesis were also found in estrous sheep [31]. Recently, we demonstrated that microRNAs (miRNAs) content and transcriptomic profile are altered both in oocytes and follicular by season in buffalo [32]. However, the follicular fluid, composed by serum-derived and locally produced factors, providing a specialized environment for oocyte growth and maturation, has not been characterized yet. The hypothesis of this work was that miRNAs content of EVs contained in buffalo follicular fluid might undergo seasonal variations that may account for the reduced oocyte developmental competence during the NBS. In order to test the hypothesis, we first investigated the miRNAs cargo of EVs isolated from follicular fluid collected from antral and pre-ovulatory follicles to identify those that are

implicated in regulation of folliculogenesis. Then the seasonal variations in EVs miRNAs content were assessed by characterizing follicular fluid from antral and pre-ovulatory follicles during the breeding season (BS) and the NBS.

2.2 Materials and Methods

2.2.1 Ethics

The experimental design and animal treatments were approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II, Italy (PG/2029/007004 of 2 July 2019).

2.2.2 Collection of Follicular Fluid

The study was carried out in Campania region, Southern Italy (latitude 40.5° - 41.5° N and longitude 13.5 - 15.5) in October, i.e. autumn (BS) and January, i.e. mid-winter (NBS). In order to collect follicular fluid (FF) from antral follicles and cumulus-oocyte-complexes for in vitro embryo production, 134 cyclic multiparous Italian Mediterranean Buffalo cows with a mean weight and age of 552.6. ± 12.1 kg and 5.3 ± 0.4 years, over a total of 10 replicates (5/season) were used (it is specified that the animals were the same enrolled in an earlier study, Capra et al. 2020 [32]. 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). In order to collect follicular fluid (FF) from pre-ovulatory follicles animals (n= 7 and 9, respectively in BS and NBS) with a mean weight and age of 555.2 ± 13.2 kg and 5.5 ± 0.3 years were synchronized by Ovsynch [33], consisting in GnRH administration of 12 mg buserelin acetate (Receptal,Intervet, Milan, Italy) im on Day 0, followed by 0.524 mg of synthetic prostaglandin (Cloprostenol, Estrumate, Schering-Plough Animal Health, Milan, Italy) on Day 7 and an additional 12 mg buserelin acetate on Day 9. Animals that ovulated after the first GnRH (n= 5/season) were slaughtered 18 h after the last GnRH, i.e. in proximity of ovulation.
Buffalo ovaries were collected at a local abattoir (Real Beef s.r.l., Flumeri (AV), Italy under national food hygiene regulations, and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30-35°C within 4 h after slaughter.

The FF of individual preovulatory follicles and antral follicles (pool of 20) was aspirated by a syringe with a 21 G gauge needle and poured into a petri dish for a quick search and assessment of the COC. Afterwards FF was transferred into a vial and centrifuged at 300 X g for 10 min at 4°C to separate the follicular fluid and the follicular cells. The FF was centrifuged again at 2000 g for 10 min and at 16500g x 30 min and supernatant was stored at -80°C until RNA isolation. After morphological assessment [Di Francesco et al. 2011 Grade A and B COCs, considered suitable for in vitro embryo production (IVEP), were in vitro matured, fertilized and cultured up to the blastocyst stage (n = 238 and 234, respectively in the BS and NBS – previous study).

2.2.3 Extracellular vesicle (EVs) isolation from Follicular Fluids

EVs were isolated from five biological replicates of FF collected from An (n=20) and pO (n=1) follicles, the in breeding (BS) and non-breeding (NBS) seasons, through Exoquick precipitation following previous reported method [34]. Size and concentration of EVs were evaluated by the NanoSight LM10 instrument.

2.2.4 RNA Isolation

Total RNA was extracted from isolated EVs. The methods described below have been reproduced in part from Lange-Consiglio et al. 2020 [35]. Total RNA was isolated by NucleoSpin miRNA kit (Macherey-Nagel, Germany), following the protocol in combination with TRIzol (Invitrogen, Carlsbad, CA, USA) lysis with small and large RNA in one fraction (total RNA). Concentration and quality of RNA were determined by Agilent 2100 (Santa Clara, CA, USA). The isolated RNAs were stored at – 80°C until use.

2.2.5 Library Preparation and Sequencing

In total, 20 libraries of small RNA were obtained from five isolated EVs per group (n=5) of two developmental stagse (An and pO) in both seasons (BS and NBS). Small RNA libraries were prepared using TruSeq Small RNA Library Preparation kit, according to manufacturer's instructions (Illumina). Small RNA (sRNAs) libraries were pooled together and purified with Agencourt AMPure XP (Beckman, Coulter, Brea, CA) (1 Vol. sample: 1.8 Vol. beads) twice [69]. Concentration and profile of libraries were

determined by Agilent 2100 Bioanalyzer before library sequencing on a single lane of Illumina Novaseq 6000 (San Diego, CA, USA).

2.2.6 Data Analysis

Illumina raw sequences were quality checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed with Trimmomatic (version 0.32) [36], then miRDeep2 (miRDeep2 (version 2.0.0.5) [37] was used for miRNA detection and discovery. Known miRNAs available at MirBase (http://www.mirbase.org/) were used to support miRNA identification. In particular, Bos taurus miRNAs were input to support known miRNA detection and miRNAs from related species (sheep, goat and human) were input to support novel miRNA identification. All the identified miRNAs were quantified using the miRDeep2 quantifier module. The Bioconductor edgeR package (version 2.4) was used to identify statistically significant differential expression between groups of samples (false discovery rate [FDR] < 0.05) [38]. Predicted miRNA gene targeting of differentially expressed Bos taurus miRNAs (DEmiRNAs) was performed with miRWalk2.0 [39], using homologous human miRNAs as input identifiers.
Target genes were submitted to GO analysis. GO classification of the DEGs was performed according to canonical GO categories, using the Cytoscape (version.3.2.1) plug-in ClueGO (version 2.3.5) which integrates GO and enhances biological interpretation of large lists of genes [40]. MicroRNA cluster analysis was performed with Genesis (version1.8.1) [41].

2.3 Results

2.3.1 Morpho-functional parameters of follicles and oocytes

The mean diameter of the pre-ovulatory follicles was 1.0 ± 0.05 and 1.3 ± 0.1 cm, respectively in the BS and NBS. The volume of FF of pre-ovulatory follicles was 1.5 ± 0.1 vs 1.1 ± 0.2 ml, respectively in BS and NBS. With the limitation of the low numbers, it was noted that during the BS 100% pre-ovulatory follicles (5/5) contained a COC with a nice expansion of cumulus cells while during the NBS only 2 out of 5 (40%) did. With regard to oocyte competence, a decrease of cleavage rate was recorded during the NBS compared to the BS (60.7 and 69.4 %, respectively; $P<0.05$). Likewise, a reduction of blastocyst yields was recorded in the NBS, both in

relation to total COCs (16.3 vs 26.5 %; P<0.01) and cleaved oocytes (27.4 vs 38.7 %; P<0.05).

2.3.2 EVs isolation and miRNAs characterization

EVs isolated from An and pO follicles in the two breeding seasons showed similar size distribution (mean size from 160 to 212 nm), but particle concentration was higher in antral follicles samples (An: 2.17e+11 particles/ml in BS and 1.01e+11 particles/ml in NBS; pO: 2.51e+10 particles/ml in BS and 5.40e+10 particles/ml in NBS), as shown in additional file X. About 37.7 and 23.8 million reads were sequenced for EVs isolated from An and pO follicles, of which 4.8 % and 1.6 % were assigned to miRNAs in An and pO FFs, respectively (additional file T). EVs isolated from An and pO FFs showed a total of 1335 miRNAs (538 known Bos taurus miRNAs, 324 homologous to known miRNAs from other species and 473 new candidate miRNAs). Principal component analysis of the 317 miRNAs, counted at least once in all 20 samples, clearly separated An and pO samples but only partially NBS and BS (Figure 2.1 A). However, within each developmental stage (An and pO), the analysis of 467 and 322 miRNAs respectively present in all An and pO samples showed a clear separation between BS and NBS, especially for pO (Figure 2.1 B and 2.1 C). FF isolated from follicles at different developmental stages contained EVs that showed a specific miRNAs cargo with 413 differentially expressed miRNAs (DE-miRNAs) (False Discovery Rate FDR<0.05) between An and pO (additional file W). Our results were in agreement with a previous study which characterized miRNA content of EVs in FF of large and small antral follicles in cattle [42], with half (43/81) of bovine DE-miRNA, that matched DE-bta-miRNAs detected between pO and An (Table 2.1). Interestingly, the expression of most of the DE-bta-miRNAs shared between the two studies, vary similarly in relation to follicle developmental stages (Table 2.1). Considering only the most significant DE-bta-miRNA (FDR < 10exp-6, LogFC>|2|, n= 18), 14 miRNAs (LogFC>2: bta-miR-132-3p, bta-miR-194, bta-miR-215, bta-miR-708, bta-miR-129-5p, bta-miR-193b , bta-miR-191; LogFC<2: bta-miR-101, bta-miR-130a, bta-miR-29c, bta-miR-378; bta-miR-148d, bta-miR-1246, bta-miR-335), homologous with human miRNAs, target 3'UTR of 273 genes with high confidence (P value <0.001), (additional file Y). Gene Ontology analysis of target genes identified pathways mainly related to glucuronidation, lipid and steroid metabolic process, response to steroid and estradiol stimulus (Table 2.2).

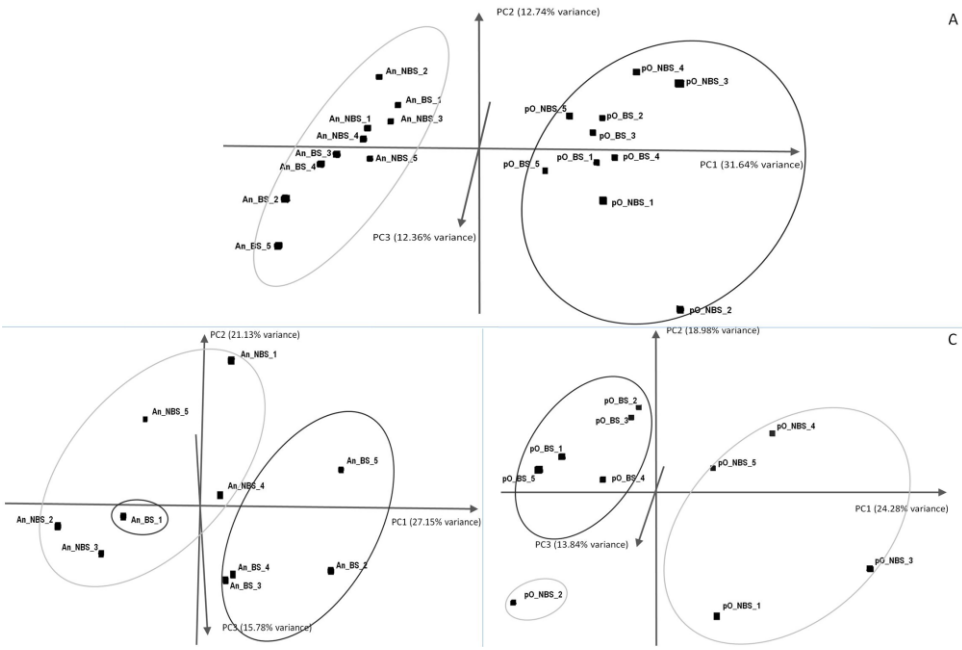

Figure 2.1 Principal Component Analysis (PCA) for: A) 317 miRNAs counted in all Antral (An) and preovulatory (pO) samples, B) 467 miRNAs counted in all An samples and C) 322 miRNAs counted in all pO samples

	This work	From Navakanitworakul et al., 2016	
Mir_name	Log_FC_pO_vs_An	FC_Large_vs_Small	Agree
bta-miR-132	5,93	17,1	+
bta-miR-215	5,09	-3,7	-
bta-miR-30d	1,94	3,2	+
bta-miR-542-5p	1,61	2,3	+
bta-miR-204	1,44	10,1	+
bta-miR-30b-5p	1,13	2,1	+
bta-miR-92b	1,09	12,5	+
bta-miR-192	1	-4,8	-
bta-miR-342	0,75	2	+

bta-miR-106b	0,71	-4,2	-
bta-miR-484	0,67	2,9	+
bta-miR-1839	-0,84	-4	+
bta-miR-148a	-0,86	-2,8	+
bta-miR-1307	-0,92	4,8	-
bta-miR-130b	-0,94	-3,2	+
bta-miR-222	-1,11	5,2	-
bta-miR-199a-3p	-1,15	-2,7	+
bta-miR-29a	-1,16	-4	+
bta-miR-152	-1,17	-2,6	+
bta-miR-10b	-1,19	-1,9	+
bta-miR-125b	-1,24	4,2	-
bta-miR-1388-5p	-1,31	-4,1	+
bta-miR-19a	-1,38	-14,6	+
bta-miR-143	-1,41	-2,4	+
bta-miR-210	-1,49	-3,4	+
bta-miR-1388-3p	-1,5	-419,9	+
bta-miR-378c	-1,65	-4,7	+
bta-miR-19b	-1,76	-6,1	+
bta-miR-103	-1,81	-2,3	+
bta-miR-107	-1,81	-3,1	+
bta-miR-592	-1,89	-3,1	+
bta-miR-660	-1,96	-2,3	+
bta-miR-142-5p	-1,99	4,8	-
bta-miR-101	-2,09	-3,9	+
bta-miR-130a	-2,14	-4,8	+
bta-miR-29c	-2,18	-5,8	+
bta-miR-378	-2,32	-2,3	+
bta-miR-451	-2,57	4,1	-
bta-miR-449a	-3,49	-8,7	+
bta-miR-335	-3,69	-20,3	+
bta-miR-2284z	-4,19	-3,6	+

Table 2.1 Comparison of DE-miRNAs between pO and An Follicle identified in our study with DE-miRNAs from previous study (Navakanitworakul et al., 2016) comparing, miRNA content of extracellular vesicles in follicular fluid of large and small antral follicles.

GOID	Associated Genes Found	GOTerm	Term P value*
GO:0052696	[UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]	flavonoid glucuronidation	3,98E-14
GO:0052697	[UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]	xenobiotic glucuronidation	6,19E-12
GO:0006789	[UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	bilirubin conjugation	2,18E-10
GO:0070980	[UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	biphenyl catabolic process	2,18E-10
GO:0033013	[ABCB6, CPOX, TCN1, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	tetrapyrrole metabolic process	1,73E-05
GO:0045833	[CRTC3, NCOR1, SIRT4, SORL1, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	negative regulation of lipid metabolic process	4,38E-05
GO:0045939	[UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	negative regulation of steroid metabolic process	1,36E-04
GO:0045471	[CHRNB2, GNRH1, HPGD, POLG2, TUFM, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	response to ethanol	2,14E-04
GO:0071392	[UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	cellular response to estradiol stimulus	6,41E-04
GO:0006953	[CD163, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	acute-phase response	6,53E-04
GO:0008210	[UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	estrogen metabolic process	7,14E-04
GO:0071385	[DDIT4, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	cellular response to glucocorticoid stimulus	1,09E-03
GO:0001523	[KDM5A, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]	retinoid metabolic process	1,15E-03

GO:0032355	[HPGD, KCNJ11, MBD1, MYCBP2, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	response to estradiol	1,65E-03
GO:0032466	[CHMP4C, E2F8, TEX14]	negative regulation of cytokinesis	2,22E-03
GO:0051187	[AHCY, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	cofactor catabolic process	3,97E-03
GO:0031960	[CPNE1, DDIT4, GNRH1, MYCBP2, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	response to corticosteroid	7,33E-03
GO:0021952	[CDH11, CHRNB2, DCC, MYCBP2]	central nervous system projection neuron axonogenesis	9,26E-03
GO:0016999	[CBR4, MTHFD1L, UGT1A1, UGT1A10, UGT1A4, UGT1A6, UGT1A7, UGT1A8]	antibiotic metabolic process	1,34E-02
GO:0006474	[NAA25, NAA30, NAA50]	N-terminal protein amino acid acetylation	3,17E-02
GO:0032465	[BIRC6, CALM2, CHMP4C, E2F8, SPAST, TEX14]	regulation of cytokinesis	3,26E-02
GO:0071466	[DDIT4, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9]	cellular response to xenobiotic stimulus	3,28E-02

*Corrected with Bonferroni step down

Table 2.2 Gene Ontology (GO) analysis result of target genes for the most significant differentially expressed miRNAs (DE-bta-miRNAs) between An and pO (FDR < 10exp-6, LogFC>|2|, n= 18). Gene ontology IDs (GO-ID), gene ontology terms (GO-term), associated genes found and corrected p-values as determined by ClueGO (http://apps.cytoscape.org/apps/cluego) are indicated.

Although a subset of the most significant DE-miRNAs between pO and An targets genes which function is directly related to estrogen response, these miNAs did not show a common variation in both development stages in relation to breeeding seasons. Comparison of miRNAs cargo of EVs isolated in the BS and NBS in both developmental stages did not show any DE-miRNAs, probably due to the high heterogeneity of miRNA expression between An and pO follicles. However, when only An or pO developmental

stage was considered separately, an alteration in miRNAs expression between seasons was found, with 14 and 12 DE-miRNAs between NBS and BS for An and pO respectively (table 2.3). Seven out of the 14 De-miRNAs in An follicles and 10 of the 12 DE-miRNAs in pO follicles were reduced in NBS.Surprisingly, the variation of these DE-miRNAs in EVs in the two breeding seasons showed mainly an opposite direction in An and pO follicles, i.e. when a specific miRNAs increases from NBS to BS in An follicle the same miRNAs decreases from NBS to BS in pO follicle and viceversa (Figure 2.2).
Finally, GO analysis of target genes for human homologous DE-miRNAs between NBS and BS (P value <0.001), identified pathways related to interleukin-6 production and secretion in An follicles and synaptic transmission and cell adhesion in pO follicles, respectively (Table 2.4).

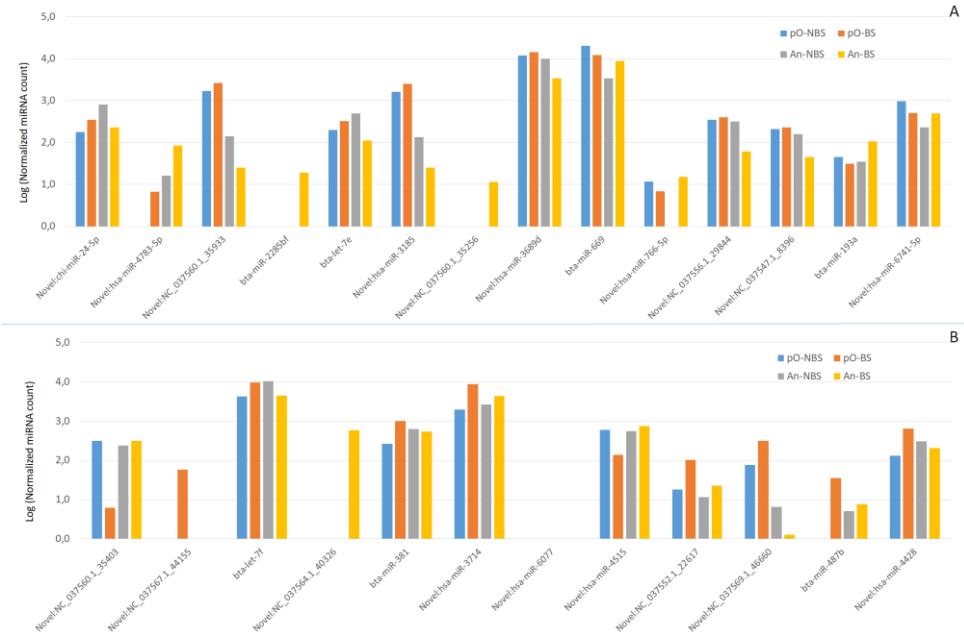

Figure 2.2 Average expression of differentially expressed miRNAs (DE-miRNAs) differing in the non-breeding season (NBS) and breeding season (BS) in A) antral follicles and B) pO follicles. For each combination An and pO follicles in NBS and BS, the Log normalized miRNAs count was reported.

An (NBS vs BS)				pO (NBS vs BS)			
miRNA	**Log FC**	**FDR**	**Homologous human miRNA (miRBase)**	**MiRNA**	**Log FC**	**FDR**	**Homologous human miRNA (miRBase)**
Novel:chi-miR-24-5p	1,8	0,001	hsa-miR-24-1-5p	Novel:NC_037560.1_35403	5,3	0,002	none
Novel:hsa-miR-4783-5p	-2,3	0,006	hsa-miR-4783-5p	Novel:NC_037567.1_44155	-5,2	0,016	none
Novel:NC_037560.1_35933	2,3	0,011	none	bta-let-7f	-1,4	0,016	hsa-let-7f-5p
bta-miR-2285bf	-5,7	0,012	none	Novel:NC_037564.1_40326	-1,9	0,016	none
bta-let-7e	2,0	0,012	hsa-let-7e-5p	bta-miR-381	-1,9	0,016	hsa-miR-381-3p
Novel:hsa-miR-3185	2,2	0,012	hsa-miR-3185	Novel:hsa-miR-3714	-2,1	0,043	hsa-miR-3714
Novel:NC_037560.1_35256	-5,0	0,019	none	Novel:hsa-miR-6077	-3,9	0,043	hsa-miR-6077
Novel:hsa-miR-3689d	1,5	0,024	hsa-miR-3689d	Novel:hsa-miR-4515	1,9	0,043	hsa-miR-4515
bta-miR-669	-1,3	0,027	hsa-miR-574-3p	Novel:NC_037552.1_22617	-2,6	0,043	none
Novel:hsa-miR-766-5p	-3,3	0,033	hsa-miR-766-5p	Novel:NC_037569.1_46660	-2,1	0,043	none
Novel:NC_037556.1_29844	2,2	0,034	none	bta-miR-487b	-4,6	0,043	hsa-miR-487b-3p
Novel:NC_037547.1_8396	1,8	0,037	none	Novel:hsa-miR-4428	-2,2	0,048	hsa-miR-4428
bta-miR-193a	-1,6	0,045	hsa-miR-193a-5p				
Novel:hsa-miR-6741-5p	-1,0	0,045	hsa-miR-6741-5p				

Table 2.3 Differentially expressed miRNAs (DE-miRNAs) between non-breeding (NBS) and breeding season (BS) in antral (An) and preovulatory (pO) follicles. For each DEmiRNAs, logFC = log Fold Change, False Discovery Rate FDR < 0.05 and homologous human miRNA were reported.

An (NBS vs BS)			
GOID	**Associated Genes Found**	**GOTerm**	**Term Pvalue***
GO:0072604	[C1QTNF3, C1QTNF4, DDX58, SYT11, TLR8]	interleukin-6 secretion	0.011
GO:0001755	[NRTN, PHACTR4, SEMA3A, SEMA4C, SEMA4G]	neural crest cell migration	0.012
GO:0150079	[SYT11, TAFA3, TNFRSF1B]	negative regulation of neuroinflammatory response	0.012
GO:0032675	[C1QTNF3, C1QTNF4, DDX58, MYD88, SYT11, TLR1, TLR8]	regulation of interleukin-6 production	0.029
GO:0050710	[C1QTNF3, SYT11, TLR8]	negative regulation of cytokine secretion	0.033
GO:0061900	[SYT11, TAFA3, TLR8]	glial cell activation	0.04
GO:0032635	[C1QTNF3, C1QTNF4, DDX58, MYD88, SYT11, TLR1, TLR8]	interleukin-6 production	0.045
GO:0015459	[KCNAB2, KCNE5, SYNGAP1]	potassium channel regulator activity	0.047
pO (NBS vs BS)			
GOID	**Associated Genes Found**	**GOTerm**	**Term Pvalue***
GO:0051966	[ADORA1, DISC1, DRD2]	regulation of synaptic transmission, glutamatergic	0.033
GO:0051895	[BCAS3, DUSP22, LRP1]	negative regulation of focal adhesion assembly	0.049
GO:0150118	[BCAS3, DUSP22, LRP1]	negative regulation of cell-substrate junction organization	0.049
*Corrected with Bonferroni step down			

2.4 Discussion

The present study aimed to investigate whether season might influence the EVs miRNAs profile in FF of both antral and pre-ovulatory follicles in buffalo. A further objective was to investigate the FF-EVs-miRNA cargo of antral (An) and preovulatory (pO) buffalo follicles in order to understand possible changes occurring in different developmental stages and their potential role in modulating follicle development. This is the first report on the characterization of miRNAs contained in EVs of FF in this species, demonstrating that specific miRNAs may be involved in regulating follicular development and modulating seasonal effects on oocyte competence.

In order to achieve these goals, we isolated the EVs using a previously published method based on polymer precipitation of EVs from bovine FF that showed a high degree of purity in EVs [34]. In accordance with earlier

findings in bovine [28,42], a similar size distribution in buffalo FF-EVs was observed. Interestingly, An follicles showed a higher EVs concentration compared to pO follicles, that was in accordance with a previous study that reported a higher EVs concentration in FF isolated from small versus large bovine follicle [42]. A high similarity of buffalo and bovine FF-EVs-miRNA cargo has also been observed with several shared miRNAs whose expression similarly varies during different stages of follicular development [42]. GO analysis of the target genes for the miRNAs that showed the most significant variation in EVs isolated from An e pO follicles showed alteration in genes which function is related to hormone regulation such as response to estradiol and estrogen metabolism. It is known that estradiol supports follicular and oocyte growth, antrum formation, and follicular function [43]. Interestingly, miR-132-3p and miR193-b, which are highly enriched in EVs from pO follicles, were observed to be highly abundant in human follicular fluids and to regulate estradiol and progesterone concentration, respectively, in a steroidogenic human granulosa-like tumor cell line [44]. Other indirect evidence depicted some of these DE-miRNAs essential for a correct follicle maturation. MiR-132 expression was significantly reduced in the follicular fluid of polycystic ovary patients [44]. In addition, other three miRNAs (miR191, miR-29c and miR378) whose expression vary consistently between An and pO follicles were differentially expressed in the follicular fluid of women with endometriosis compared to healthy patient [45] and other three miRNAs (miR132-3p, miR-708, and miR-335) were differentially expressed between the follicular fluid of preovulatory dominant and subordinate follicles [46], indicating a potential role in the selection of follicles. Interestingly, the expression of these miRNAs varies similarly also in granulosa cells and theca cells [46].

MiRNAs profiling of oocytes and follicular cells was observed to change during different breeding season in buffalo [32]. The present study also characterized miRNAs content of EVs isolated from FF in An and pO follicles in the BS and NBS in order to evaluate if a seasonal effect in FF-EVs-miRNA content exist. Indeed, EVs isolated from An and pO follicles were enriched in specific miRNAs in the two breeding seasons. In particular, 14 and 12 DEmiRNAs were identified respectively in An follicles and pO follicles. Interestingly, the GO analysis revealed that seasonal variation in An follicles involves miRNAs targeting genes which function is related to interleukin-6 (IL-6) production and secretion. This is very interesting as cytokines, in addition to their role in inflammation processes, are known to regulate physiological reproductive events like follicular development and

ovulation. Among cytokines IL-6, characterized by both pro-inflammatory and anti-inflammatory activities, modulates intraovarian functions at different levels, by regulating steroidogenesis, angiogenesis, as well as granulosa cell function [47]. IL-6 is highly expressed in pig granulosa cells and is secreted in the follicular fluid [48]. IL-6 is also present in human FF and increases during oocyte maturation potentially contributing to follicular growth and development [49]. Recently, IL-6 in FF was observed to reduce embryo fragmentation and to improve the rates of clinical pregnancy in human [50]. In the mouse IL-6 exerts a regulatory role on cumulus expansion and oocyte developmental competence [51]. Therefore, we speculate that the altered IL-6 secretion may be one of the factors interfering with the acquisition of oocyte competence during the NBS in buffalo. Despite the limitations of the low numbers observed, it is intriguing that cumulus expansion was impaired in pre-ovulatory follicles during NBS.

In this study let-7e and let7f was differentially expressed in An and pO follicles during beween two breeding seasons. In an earlier work seasonal alterations of miRNAs ovarian profiling were also demonstrated in the sheep, with few members of let7 family identified among DEmiRNAs [52]. A role of the let-7miRNA family in granulosa cell programmed death and follicular atresia was demonstrated in swine [53].

In pO follicles, GO analysis of target gene for DE-miRNAs showed involvement in the regulation of focal adhesion assembly and cell-substrate junction organization. These pathways are important to ensure the establishment of a correct bidirectional communication between the oocyte and surrounding somatic cells, essential for proper oocyte growth and maturation [54,55,56]. Indeed, in addition to support oocyte metabolic needs, somatic cells are involved in the complex signal transduction necessary to control the resumption of oocyte meiosis. This is possible through paracrine factors and direct cell-cell communication via gap junctions located at the sites of cell contact. Coupling between germ and somatic cells is a complex sequential process, starting with adherens contacts that develop into adherens junctions and finally into gap junction [57]. It was demonstrated that a focal adhesion kinase is involved in regulating the adherens junctions formation between oocyte and somatic cells in the mouse, hence playing a pivotal role on oocyte-follicle communication. Finally, it is worth noting that the analysis of the DE-miRNAs expression level between NBS and BS has shown an opposite pattern in An and pO follicle. Some DE-miRNA were observed to increase in An while decreasing in pO from NBS to BS and viceversa. Taking into

account that a cyclic temporal miRNAs expression has been observed during follicle development in bovine, with several miRNAs increasing in expression until the mid-luteal phase, and decreasing in the late follicular phase [58], the alteration observed in NBS and BS for An and pO follicle could probably be related to an acceleration or delay of follicular development in the two breeding seasons.

2.5 Conclusion

In conclusion, it was demonstrated that buffalo follicular fluid contains EVs which miRNA cargo is strictly related to follicular developmental stage. Passing from An to pO different miRNAs could modulate the expression of genes which function was mainly associated to estradiol and stereoid metabolism. It was also shown that season influences the miRNAs content of EVs isolated from both An and pO follicles. In particular, EVs isolated from An follicles showed also misregulation of miRNAs that potentially influence IL-6 expression and secretion in FF, while those isolated from pO follicles contained miRNAs targeting genes involved in regulation of focal adhesion assembly and cell-substrate junction organization. This is the first report on the characterization of miRNAs contained in EVs isolated from buffalo follicular fluid and opens the way to future studies to develop in vitro corrective strategies for improving oocyte developmental competence during NBS in this species.

References

[1] Campanile, G. et al. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. Anim. Reprod. Sci. 121, 1-11 (2010).
[2]Zicarelli, L. Influence of Seasonality on Buffalo Production in The buffaloes (Bubalus bubalis) – Production and Research (ed. Giorgio A. Presicce) 196-224. (Bentham Science, 2016).
[3]Campanile, G. et al. Embryonic mortality in buffaloes synchronized and mated by AI during the seasonal decline in reproductive function. Theriogenology 63, 2334-2340 (2005).
[4] Gasparrini, B. Effects of reproductive season on embryo development in buffalo. Reprod Fertil Dev, 31, 68-81 (2019).
[5] Campanile, G. et al. Corpus luteum function and embryonic mortality in buffaloes treated with a GnRH agonist, hCG and progesterone. Theriogenology 67, 1393–1398 (2007).
[6] Balestrieri, M. L. et al. Proteomic Profiles of the Embryonic Chorioamnion and Uterine Caruncles in Buffaloes (Bubalus bubalis) with Normal and Retarded Embryonic Development. Biology of Reproduction 88, 119 (2013).
[7] Strazzullo, M. et al. Global transcriptome profiles of Italian Mediterranean buffalo embryos with normal and retarded growth. Plos One; 9, e90027 (2014).
[8] Di Francesco, S. et al. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (Bubalus bubalis). Anim. Reprod. Sci. 123 , 48-53 (2011).
[9] Di Francesco, S. et al. Ovum pick-up and in vitro embryo production (OPU-IVEP) in Mediterranean Italian buffalo performed in different seasons. Theriogenology. 77,148-54. (2012b).
[10] Manjunatha, B. M., Ravindra, J. P., Gupta, P. S., Devaraj, M. & Nandi, S. Effect of breeding season on in vivo oocyte recovery and embryo production in non-descriptive Indian river buffaloes (Bubalus bubalis). Anim. Reprod. Sci; 111, 376-383 (2009).
[11] Abdoon, A. S., Gabler, C., Holder, C., Kandil, O. M. & Einspanier, R. Seasonal variations in developmental competence and relative abundance of gene transcripts in buffalo (Bubalus bubalis) oocytes. Theriogenology. 82, 1055-1067 (2014).
[12] Salzano, A. et al. Effect of photoperiod on follicular IGF-1 and oocyte quality independently of metabolic status in buffalo heifers. Italian Journal of Animal Science. 18, 949-956 (2019).

[13] Glister C, Tannetta DS, Groome NP, Knight PG. 2001. Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. Biol. Reprod. 65(4):1020-8.
[14] Gong JG, McBride D, Bramley TA, Webb R. 1994. Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on bovine granulosa cell steroidogenesis in vitro. J. Endocrinol. 143(1):157-64.
[15] Walters KA, Binnie JP, Campbell BK, Armstrong DG, Telfer EE. 2006. The effects of IGF-I on bovine follicle development and IGFBP-2 expression are dose and stage dependent. Reproduction. 131(3):515-23
Izadyar F, Van Tol HT, Colenbrander B, Bevers MM. 1997. Stimulatory effect of growth hormone on in vitro maturation of bovine oocytes is exerted through cumulus cells and not mediated by IGF-I. Mol. Reprod. Dev. 47(2):175-80
[16] Labrecque R, Sirard MA. The study of mammalian oocyte competence bytranscriptome analysis: progress and challenges. Mol Hum Reprod 2014;20:103e16.
[17] Khan DR, Landry DA, Fournier E, Vigneault C, Blondin P, Sirard MA. Transcriptome meta-analysis of three follicular compartments and its correlation with ovarian follicle maturity and oocyte developmental competence in cows. Physiol Genom 2016;48:633e43
[18]Tesfaye D, Gebremedhn S, Salilew-Wondim D, Hailay T, Hoelker M, Grosse-Brinkhaus C, Schellander K.MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development. Reproduction. 2018 Mar;155(3):R121-R135. doi: 10.1530/REP-17-0428.
[19] Donadeu FX, Schauer SN, Sontakke SD.Involvement of miRNAs in ovarian follicular and luteal development. J Endocrinol. 2012 Dec;215(3):323-34. doi: 10.1530/JOE-12-0252.
[20] Donadeu FX, Schauer SN. Differential miRNA expression between equine ovulatory and anovulatory follicles. Domest Anim Endocrinol. 2013 Oct;45(3):122-5. doi: 10.1016/j.domaniend.2013.06.006.
[21] Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol. 2009 May 4;7:40. doi: 10.1186/1477-7827-7-40.
[22] Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D.Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs

in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. PLoS One. 2013 Nov 4;8(11):e78505. doi: 10.1371/journal.pone.0078505.
[23] Zielak-Steciwko AE, Browne JA, McGettigan PA, Gajewska M, Dzieciol M, Szulc T, et al. Expression of microRNAs and their target genes and pathways associated with ovarian follicle development in cattle. Physiol Genom 2014;46:735e45]
[24] Pasquariello R, Manzoni E F M,Fiandanese N, ViglinoA, Pocar P, Brevini T A L, Williams J L, Gandolfi F. Implications of miRNA expression pattern in bovine oocytes and follicular fluids for developmental competence. Theriogenology 2020;145:77-85
[25] da Silveira JC, Veeramachaneni DN, Winger QA, Carnevale EM, Bouma GJ. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle.
Biol Reprod. 2012 Mar 19;86(3):71. doi: 10.1095/biolreprod.111.093252.
[26] Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, Xing Q, Jin L, He L, Wu L, Wang L. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. J Clin Endocrinol Metab. 2013 Jul;98(7):3068-79. doi: 10.1210/jc.2013-1715.
[27] Hung WT, Hong X, Christenson LK, McGinnis LK. Extracellular Vesicles from Bovine Follicular Fluid Support Cumulus Expansion. Biol Reprod. 2015 Nov;93(5):117. doi: 10.1095/biolreprod.115.132977.
[28] da Silveira JC, Andrade GM, Del Collado M, Sampaio RV, Sangalli JR, Silva LA, Pinaffi FVL, Jardim IB, Cesar MC, Nogueira MFG, Cesar ASM, Coutinho LL, Pereira RW, Perecin F, Meirelles FV. Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One. 2017 Jun 15;12(6):e0179451. doi: 10.1371/journal.pone.0179451.
[29] Lange-Consiglio, A. et al. Amniotic microvesicles impact hatching and pregnancy percentages of in vitro bovine embryos and blastocyst microRNA expression versus in vivo controls. Sci. Rep. 10, 501 (2020).
[30] Frattini, S. et al. Genome-wide analysis of DNA methylation in hypothalamus and ovary of Capra hircus. BMC Genomics 18, 476 (2017).

[31] Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics 30, 2114–2120 (2014).
[32] Capra E, Lazzari B, Russo M, Kosior MA, Valle GD, Longobardi V, Stella A, Consiglio AL, Gasparrini B. Seasonal effects on miRNA and transcriptomic profile of oocytes and follicular cells in buffalo (Bubalus bubalis). Sci Rep. 2020 Aug 11;10(1):13557. doi: 10.1038/s41598-020-70546-5.
[33] Neglia G, Gasparrini B, Di Palo R, De Rosa C, Zicarelli L, Campanile G. Comparison of pregnancy rates with two oestrus synchronization protocols in Italian Mediterranean Buffalo cows. Theriogenology 2003;60:125–33
[34] Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D. 2013. Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. PLoS One. 8(11):e78505.
[35] Lange-Consiglio, A. et al.. Amniotic microvesicles impact hatching and pregnancy percentages of in vitro bovine embryos and blastocyst microRNA expression versus in vivo controls. Sci Rep. 10,501 (2020).
[36] Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina Sequence Data. Bioinformatics. 30, 2114-2120 (2014).
[37] Friedländer, M. R, Mackowiak, S. D., Li, N., Chen, W. & Rajewsky, N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res. 40, 37-52 (2012).
[38] Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26, 139-40 (2009).
[39] Dweep, H. & Gretz, N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods. 12, 697 (2015).
[40] Bindea, G. et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 25, 1091-1093 (2009).
[41] Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. Bioinformatics. 18, 207-208 (2002).
[42] Navakanitworakul R, Hung WT, Gunewardena S, Davis JS, Chotigeat W, Christenson LK. Characterization and Small RNA Content of

Extracellular Vesicles in Follicular Fluid of Developing Bovine Antral Follicles. Sci Rep. 2016 May 9;6:25486. doi: 10.1038/srep25486.
[43] Itoh T, Kacchi M, Abe H, Sendai Y, Hoshi H. Growth, antrum formation, and estradiol production of bovine preantral follicles cultured in a serum-free medium. Biol Reprod. 2002 Oct;67(4):1099-105. doi: 10.1095/biolreprod67.4.1099.
[44] Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, Xing Q, Jin L, He L, Wu L, Wang L. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. J Clin Endocrinol Metab. 2013 Jul;98(7):3068-79. doi: 10.1210/jc.2013-1715.
[45] Li X, Zhang W, Fu J, Xu Y, Gu R, Qu R, Li L, Sun Y, Sun X. MicroRNA-451 is downregulated in the follicular fluid of women with endometriosis and influences mouse and human embryonic potential. Reprod Biol Endocrinol. 2019 Nov 19;17(1):96. doi: 10.1186/s12958-019-0538-z.
[46] Gebremedhn S, Salilew-Wondim D, Ahmad I, Sahadevan S, Hossain MM, Hoelker M, Rings F, Neuhoff C, Tholen E, Looft C, Schellander K, Tesfaye D. MicroRNA Expression Profile in Bovine Granulosa Cells of Preovulatory Dominant and Subordinate Follicles during the Late Follicular Phase of the Estrous Cycle. PLoS One. 2015 May 19;10(5):e0125912. doi: 10.1371/journal.pone.0125912. eCollection 2015.
[47] Bornstein S.R., Rutkowski H., Vrezas I. Cytokines and steroidogenesis.Molecular and Cellular Endocrinology 215 (2004) 135–141
[48] Maeda A, Inoue N, Matsuda-Minehata F, Goto Y, Cheng Y, Manabe N. The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. J Reprod Dev. 2007 Jun;53(3):481-90. doi: 10.1262/jrd.18149. Epub 2007 Feb 2.
[49] Kawasaki F, Kawano Y, Kosay Hasan Z, Narahara H, Miyakawa I. The clinical role of interleukin-6 and interleukin-6 soluble receptor in human follicular fluids. Clin Exp Med. 2003 May;3(1):27-31. doi: 10.1007/s102380300012.
[50] Yang J, Yang X, Yang H, Bai Y, Zha H, Jiang F, Meng Y. Interleukin 6 in follicular fluid reduces embryo fragmentation and improves the clinical pregnancy rate. J Assist Reprod Genet. 2020 May;37(5):1171-1176. doi: 10.1007/s10815-020-01737-2. Epub 2020 Mar 18.
[51] Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S & Richards JS 2009 Interleukin-6: an autocrine regulator of the mouse cumulus cell–

oocyte complex expansion process. Endocrinology 150 3360–3368. (doi:10.1210/en.2008-1532).
[52] Di R, He J, Song S, Tian D, Liu Q, Liang X, Ma Q, Sun M, Wang J, Zhao W, Cao G, Wang J, Yang Z, Ge Y, Chu M. Characterization and comparative profiling of ovarian microRNAs during ovine anestrus and the breeding season. BMC Genomics. 2014 Oct 15;15(1):899. doi: 10.1186/1471-2164-15-899.
[53] Cao R, Wu WJ, Zhou XL, Xiao P, Wang Y, Liu HL. Expression and Preliminary Functional Profiling of the let-7 Family during Porcine Ovary Follicle Atresia. Mol. Cells 2015; 38(4): 304-311.
[54] Kidder GM, Vanderhyden BC. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. Can. J. Physiol. Pharmacol. 2010; 88:399–413
[55] Wigglesworth K, Lee KB, O'Brian MJ, Peng J, Matzuk MM, Eppig JJ. Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes. Proc. Nat. Acad. Sci. USA. 2013; 110:E3723–9.
[56] Banerjee S, Banerjee S, Saraswat G, Bandyopadhyay SA, Kabir SN. Female reproductive aging is master-planned at the level of ovary. PLoS One 2014;9:e96210.
[57] Mora JM, Fenwick MA, Castle L, Baithun M, Ryder TA, Mobberley M, Carzaniga R, Franks S, Hardy K. Characterization and Significance of Adhesion and Junction-Related Proteins in Mouse Ovarian Follicles. Biol Reprod. 2012
[58] Tesfaye D, Gebremedhn S, Salilew-Wondim D, Hailay T, Hoelker M, Grosse-Brinkhaus C, Schellander K. MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development. Reproduction. 2018 Mar;155(3):R121-R135. doi: 10.1530/REP-17-0428.

Chapter 3

Experiment 3: Seasonal variations in the metabolomic profile of ovarian follicle in Italian Mediterranean Buffalo

3.1 Introduction

The different gene regulation observed at the follicular level between the BS and NBS likely results in different metabolites production, influencing indeed the metabolic profile of the follicle. Therefore, it is important to characterize the metabolome of the follicular environment, that undoubtedly impacts on the oocyte during its development, in relation to season. Metabolites are the most reliable indicators of phenotypic traits, as end products of metabolic pathways. The knowledge of metabolic profile of the follicle is fundamental to unveil the causes of reduced oocyte competence during the NBS and develop corrective strategies. Recently, advances in omics technologies, among which the latest developed is metabolomics, have been achieved and consequently these technologies have been applied to different field of research, including reproduction.Metabolomics allows a broad identification of low molecular weight metabolites, that are the downstream products of genome, transcriptome and proteome expression [1,2], in biological fluids, cells and tissues, providing a picture of their dynamic variations in response to environmental or genetic factors [3].
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, considered one of the strongest for biological fluid research [4]. As metabolome is related to the genome, physiology and environment, it represents a valid tool to identify fertility biomarkers [5,6].
The follicular microenvironment where the oocyte grows certainly influences oocyte competence. The follicular fluid is in part composed by plasma-derived elements, in part by secretion products of the somatic cells of the follicle [7]. The follicular fluid protects the oocyte against proteolysis and has a buffering role against negative blood conditions [8]. It is known that in post-partum cows there is a correlation between the concentration of some metabolites in plasma and follicular fluid, affecting the quality of granulosa cells and oocyte [9]. The chemical composition of the follicular fluid has been evaluated by several authors [10,11,12] and has been proposed to predict oocyte competence. It is known that follicular fluid composition, influenced by climate [13] and nutrition [14] affects the oocyte maturation process [15, 16]. Follicular fluid contains steroids, glycoproteins,

fatty acids, amino acids, growth factors, regulatory molecules, contributing to the metabolism of somatic cells and of the oocyte [17,18,19].
Metabolomic analysis of the follicular fluid to predict oocyte developmental competence has been carried out in cattle [20]. This study revealed a positive correlation between some amino acids like L-alanine, glycine and L-glutamate and blastocyst development and a negative correlation with urea levels. In addition, a lower content of total fatty acid and palmitic acid and a higher amount of linolenic acid was detected in the follicular fluid of competent oocytes. The beneficial effect of alanine and glycine on oocyte potential was confirmed by other studies [21,22] (21: Cetica et al., 2003). These amino acids were also found to be predictive markers of competence in another work whereas fatty acids were uninfluential [23]. In contrast, differences were recorded in the fatty acid composition of the follicular fluid of the pre-ovulatory follicle in cows vs heifers, representing respectively models of low vs high fertility; in particular higher concentrations of saturated fatty acids (palmitic and stearic acid), known to be detrimental for oocyte maturation and development, and lower levels of docosahexaenoic acid were observed in the follicular fluid of cows [24]. It is known that the abundance of saturated fatty acids, like palmitic and stearic acids negatively affects oocyte competence, while linoleic and linolenic acids are beneficial [25,26]. The lipic composition of the follicular fluid may be influenced by the diet, as plasmatic changes in fatty acids are in part reflected in the follicular fluid. However, it is known that cumulus cells take up more fatty acids, acting as a protective barrier for the oocyte [27].
In the same study that compared cows vs heifers, the amino acid profile also showed variations, with higher levels of glycine and L-glutamine and lower levels of L-alanine and oxoproline in follicular fluid from cows compared with heifers. Another noninvasive approach to predict oocyte competence is the analysis of the spent medium (SM) after IVM; amino acid profiling could be an index of bovine oocyte viability, with lower consumption of glutamine and lower production of alanine associated to competent oocytes [28]. Despite increasing studies on follicular fluid and SM [17,18,19,28] the metabolome of the oocyte has never been investigated, likely due to more emphasis given to noninvasive assessments. Several studies looked at potential markers of oocyte competence using cumulus cells but mainly limited to gene expression [29,30,31,32].
The reduced oocyte competence recorded in the NBS in buffalo is one of the main limitations of buffalo farming profitability and undoubtedly limits the utilization of IVEP technology. To the best of our knowledge,

metabolomics has not been yet applied to buffalo reproduction. Therefore, the purpose of this experiment was to evaluate whether season influences the metabolites content and metabolic pathways within the ovarian follicle in this species, to unveil the causes of the reduced competence during NBS and to lay the ground for further studies to develop corrective strategies, based on either diet modulation or the addition of specific key components during in vitro maturation of oocytes collected during the NBS. In order 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, oocytes and cumulus cells before and after IVM, as well as the spent maturation medium. To our knowledge this is the first time a metabolomic approach is used to study the ovarian follicle and the COC in buffalo in its complexity, by analyzing all its components.

3.2 Matherials and Methods

3.2.1 Experimental design

In this work, samples collected in two different seasons were analyzed.
The trial was carried out in October i.e during the breeding season (BS) and in January, i.e. during the non-breading season (NBS). The samples were collected at a local slaugherhouse (Real Beef s.r.l., Flumeri (AV), Italy), under national food hygiene regulations. In this experiment, all samples came from carcasses of cyclic multiparous Italian Mediterranean buffalo cows (Bubalus bubalis), grown under controlled nutrition and housed inside barns in intensive farms located in the province of Caserta (Italy). 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).
At the slaughterhouse, a trained technician collected the ovaries, after a macroscopic inspection of the genital tract performed by the veterinarian to exclude all animals with pathologies of the uterine tract. The ovaries of healthy and cyclic animals were then detached and stored in saline solution supplemented with 150 mg/L kanamycin at a temperature of about 30-35 degrees, then transported to the laboratory within 4 hours after slaughter.
During each season five replicates were carried out to collect follicular fluid, follicular cells, oocytes and cumulus cells both before and after in vitro maturation, as well as the in vitro maturation (IVM) spent medium. Briefly, on the day of slaughter follicular fluid was aspirated, separated from

follicular cells and one third of the COCs found was denuded in order to collect both oocytes and cumulus cells; then these samples were stored until analyses. In addition, the remaining COCs were matured in vitro and 22 h post- IVM half of the matured COCs was denuded to separately collect and store oocytes and cumulus cells, as well as the spent medium, with the remaining half used as a control for in vitro embryo production. Finally, to better comprehend the analysis of the spent medium, for each season and for each maturation step, samples of the IVM medium were also stored straight after preparation and after 22 h incubation in the presence or absence of oocytes.

3.2.2 Collection of follicular fluid and follicular cells

Once they arrived in the laboratory, the ovaries were transferred into a beaker and washed repeatedly with physiological saline with 150 mg/L kanamycin to remove blood residues. Then 2-8 mm diameter follicles were aspirated under controlled pressure to collect follicular fluid and oocytes. Follicular fluid was aspirated using an 18 G needle under vacuum (40–50 mm Hg) in Falcon tubes and poured into a petri dish for oocyte recovery. The ovaries were aspirated in groups of 8-10 ovaries. All the COCs that were found were transferred to a dish with medium H199 and evaluated according to morphology and classified according to Di Francesco et al. [33]. The remaining fluid was transferred into 1.5 ml eppendorf 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 x 10 min), then the supernatant was removed and the pellet stored at -80 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.

3.2.3 Collection of immature oocytes and cumulus cells

For each replicate, A and B COCs were splitted into three 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 minutes. Then the content of the tubes was moved to a pedri dish, the oocytes were removed and transferred into eppendor tubes in a volume of about 20 µL and stored at -80°C as immature oocytes (IO). The remaining liquid was subjected to

centrifugation (2500g x 15 min), then after removing the supernatant, the pellet was stored at -80°C as cumulus cells from immature oocytes (CC-IO).

3.2.4 Collection of oocytes, cumulus cells and spent medium after IVM

For each replicate, Grade A and B COCs recovered by follicular aspiration were rinsed in HEPES-buffered TCM199 supplemented with 10% fetal calf serum (FCS) and in vitro matured. The methods for in vitro maturation (IVM) described below have been reproduced in part from Gasparrini et al. 2003 [34]. 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-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.
After IVM half of the COCs was denuded, processed as previously described for IO and CC-IO, and stored at -80°C as mature oocytes (MO) and cumulus cells from matured oocytes (CC-MO).

3.2.5 In vitro fertilization and culture

The methods for in vitro fertilization (IVF) and culture (IVC) described below have been reproduced from Di Francesco et al. 2012 [11]. Frozen straw from a bull previously tested for IVF were thawed at 37 °C for 40 sec and sperm was selected by centrifugation (25 minutes at 300 g) on a Percoll discontinuous gradient (45% and 80%). The sperm pellet was re-suspended to a final concentration of 2 × 106 mL-1 in the IVF medium, consisting of Tyrode albumin lactate pyruvate [35] 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, putative 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 [36]. Culture was carried out under humidified air with 5% CO2, 7% O2 and 88% N2 at 38.5° C. On day 5 post-insemination the cleavage rate was assessed and the embryos transferred into fresh medium for further 2 days of IVC, when blastocyst rates were recorded.

3.3.6 Extraction of the polar and lipophilic fractions from samples

To separate the polar and lipophilic fractions, each type of samples was first re-suspended in 170 mL of H2O and 700 mL of methanol. For cell samples (FC, IO, CC-IO, MO and CC-MO) the pellet-containing solution was sonicated for 30 sec 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 and media). Then, 350 mL of chloroform was added and the samples were mixed on an orbital shaker in ice for 10 min. After this, 350 mL of a 1:1 (v/V) H2O/chloroform solution was added to each sample, that was vortexed for 5 sec and centrifuged at 4000 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, the aqueous (polar) and lipophilic (apolar) phases were collected separately and evaporated.

3.2.6 H-NMR metabolomic analysis

The polar fractions were dissolved in 630 mL of PBS-D2O with the pH adjusted to 7.2, and 70 mL of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D2O) was used as the internal standard. On the other hand, the lipophilic fractions were dissolved in 700 mL of deuterated chloroform. A 600 MHz Avance Bruker spectrometer with a TSI probe was used to acquire 1H-NMR spectra at 300 K. In the case of polar fractions 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. The spectral 0.50–8.60 ppm region of the 1H-NMR spectra was integrated in buckets of 0.04 ppm using the AMIX package (Bruker, Biospin, Germany). In detail, we excluded in the case of the spectra obtained from the polar fractions the water resonance region (4.5–5.2 ppm) during the analysis and normalized the bucketed region to the total spectrum area using Pareto scaling.

3.2.7 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 type of samples because it can more effectively cope with chemical shift variation in full-resolution 1H-NMR datasets without requiring binning or alignment steps [37]. Furthermore, the Variable Importance in Projection (VIP) method was used to identify specific metabolites that show the greatest variations between seasons and their pattern. Finally, pathway analysis on polar metabolites was performed using the Metabo Analyst tool [38].
Differences in cleavage and blastocyst rates between seasons were analyzed by Chi square test. The level of significance was set at $P < 0.05$.

3.3 Results

3.3.1 In vitro embryo production

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

3.3.2 Metabolomic analysis of the polar phase of follicular fluid

The OPLS-DA plot evidenced that the FF samples from different seasons clustered separately, even though NMR spectra slightly overlapped, suggesting the presence of significant differences in proton signals (metabolites) between seasons (Figure 3.1).

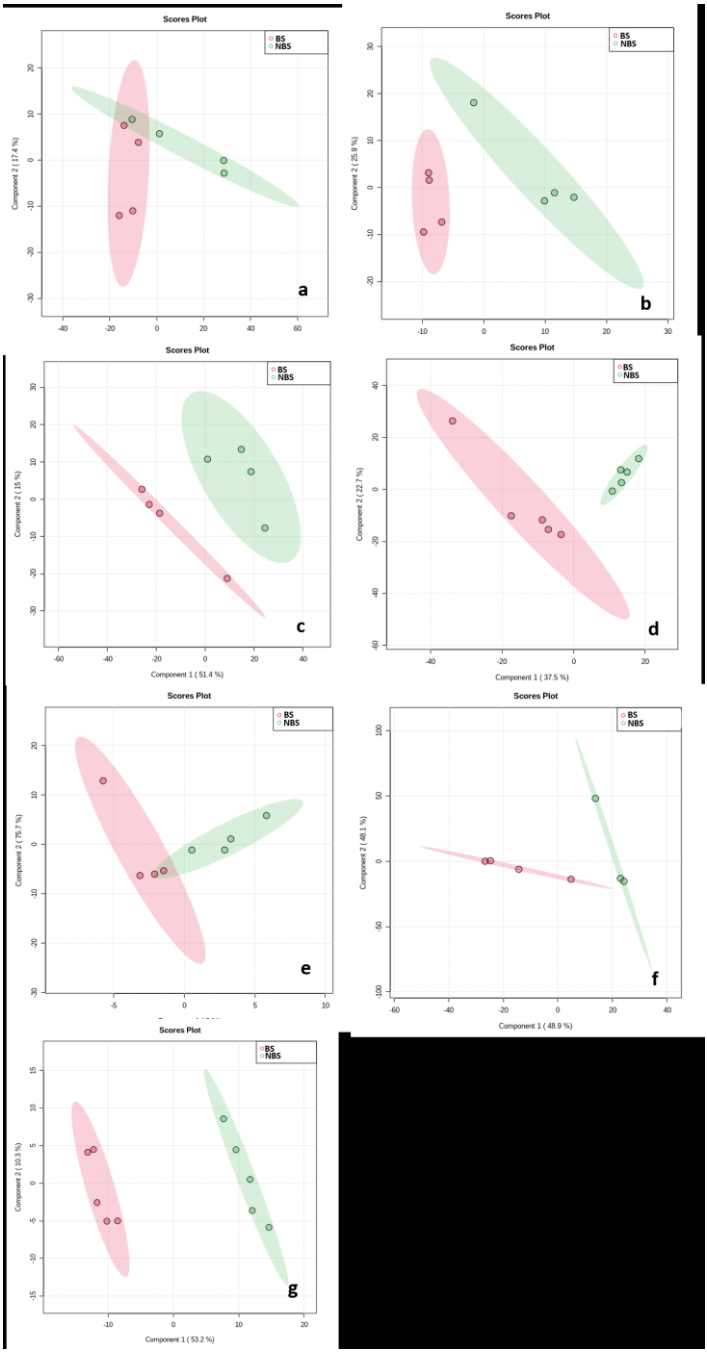

Figure 3.1. Score plot showing the differences in proton signals between bs and NBS in: a. Follicular Fluid; b. Follicular Cells; c. immature oocytes, d. oocytes after in vitro

maturation; e. Cumulus cells from immature oocytes; f. Cumulus cells from mature oocytes; g. Spent medium.

The VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in FF (Figure 3.2). Interestingly, most of the metabolites (12/15) were in lower amount during the NBS. More specifically, in the NBS reduced levels of lactate, trimethylamine, lysine, serine, cysteine, glutamate, glutathione, glycerophosphocholine, proline, coline and phosphocoline were detected. In contrast, β-hydroxybutyrate, leucine and isoleucine were more abundant during the NBS.
As shown in Figure 3.3, the pathway analysis evidenced that differentially expressed metabolites between seasons are involved in several metabolic pathways, such as metabolism of glutathione, methionine, glutamate, glycine/serine and cysteine; degradation of homocysteine, valine/leucine/isoleucine and lysine; biosynthesis of phospholipids, phosphatidylethanolamine and phosphatidilcoline.

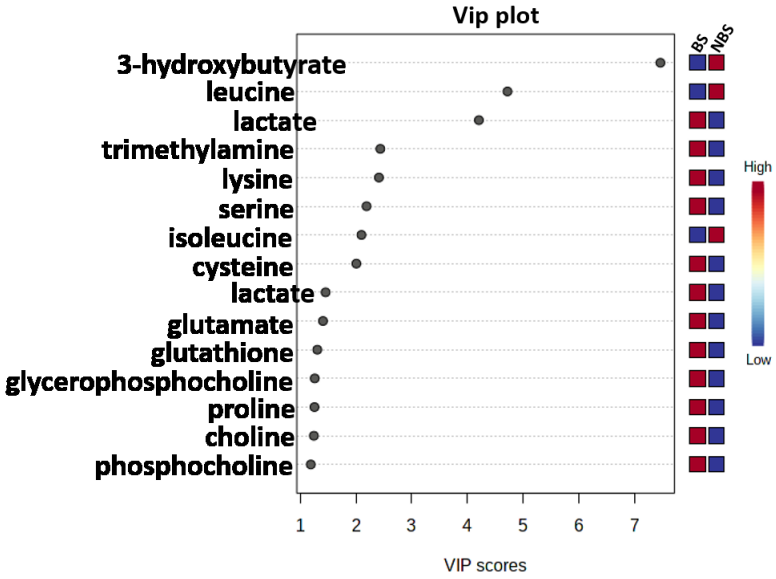

Figure 3.2. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in FF

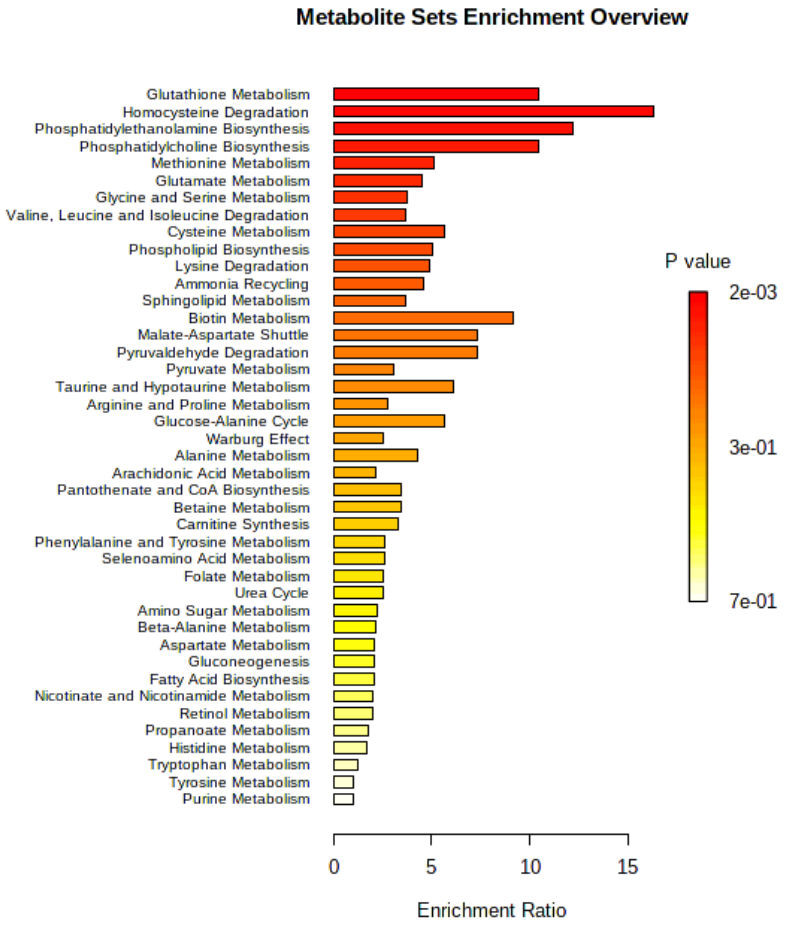

Figure 3.3 Enrichment pathway analysis of differentially abundant metabolites between seasons in follicular fluid.

3.3.3 Metabolomic analysis of the polar phase of follicular cells

The OPLS-DA evidenced that samples of follicular cells collected in different seasons clustered into two distinct groups, suggesting significant differences in the metabolic profile also in the somatic cells of the follicle in relation to season (Figure 3.1). 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 (Figure 3.4).
The pathways enrichment analysis revealed that the main metabolic pathways influenced by season are degradation of valine/leucine/isoleucine and lactose, gluconeogenesis, sphingolipid metabolism, la phosphatidylcholine biosynthesis, pyruvate metabolism, lactate synthesis, glutathione metabolism, Warburg effect, transfer of acetyl groups into mitochondria and glycolysis (Figure 3.5).

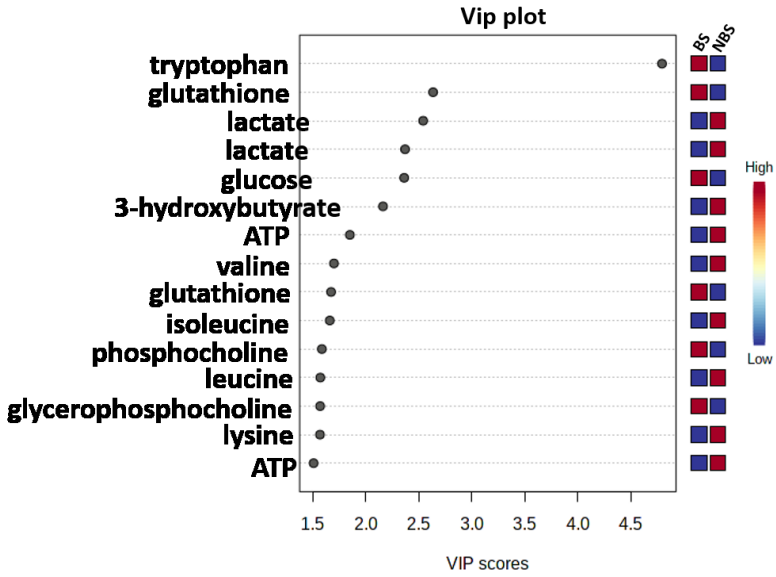

Figure 3.4. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in FC

3.3.4 Metabolomic analysis of the polar phase of cumulus cells and immature oocytes

The OPLS-DA plot showed a clear separation of cumulus cells (CC-IO) and immature oocytes (IO) into the two different seasonal classes, indicating differences in metabolites content (Figure 3.1).

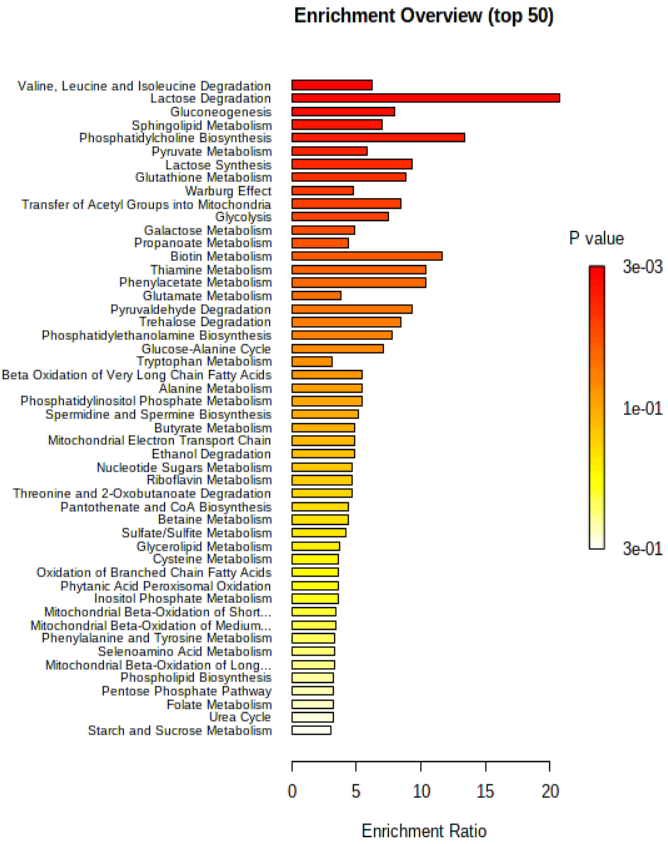

Figure 3.5. Enrichment pathway analysis of differentially abundant metabolites between seasons in follicular cells.

With regard to cumulus cells, 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 (Figure 3.7).
In the IO decreased levels of formate, ornithine, threonine, glucose and hydroxylysine and increased content of glutathione, ATP, sarcosine, trimethylamine and aspartate were observed during the NBS compared to the BS (Figure 3.6).
The main metabolic pathways influenced by season in cumulus cells were: lactose degradation, gluconeogenesis, sphingolipid metabolism, phosphatidylcholine biosynthesis, Warburg effect, lactose synthesis,

threonine and 2-oxobutanoate degradation, valine, leucine and isoleucine degradation, transfer of acetyl groups into mitochondria, glycolysis, galactose metabolism and propanoate metabolism (Figure 3.9).

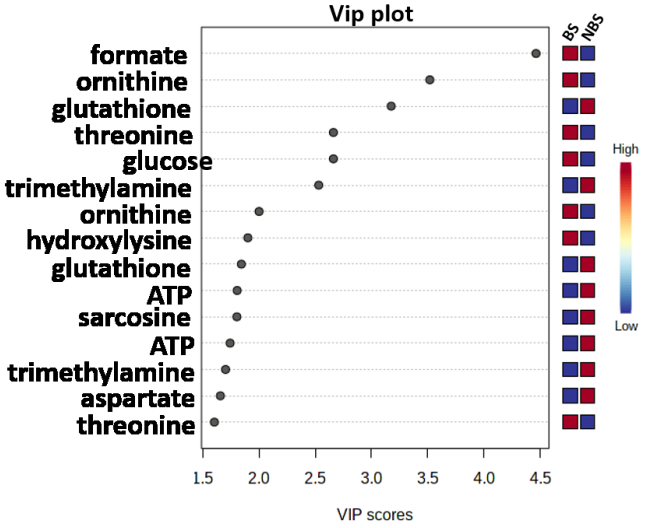

Figure 3.6. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in OI

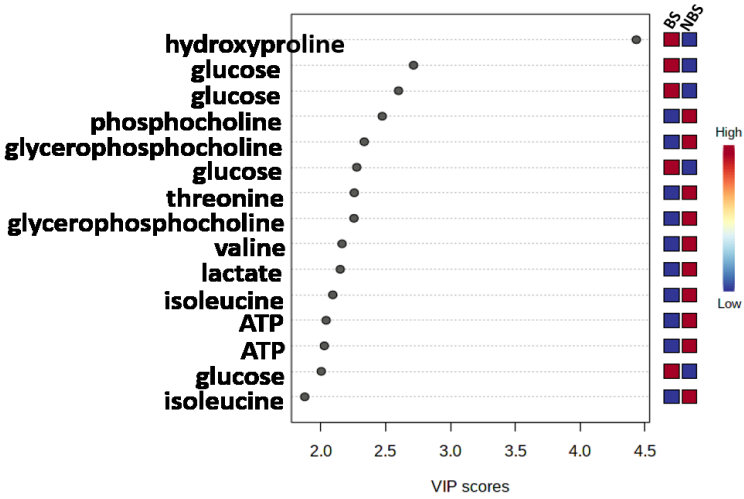

Figure 3.7. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in CC-IO

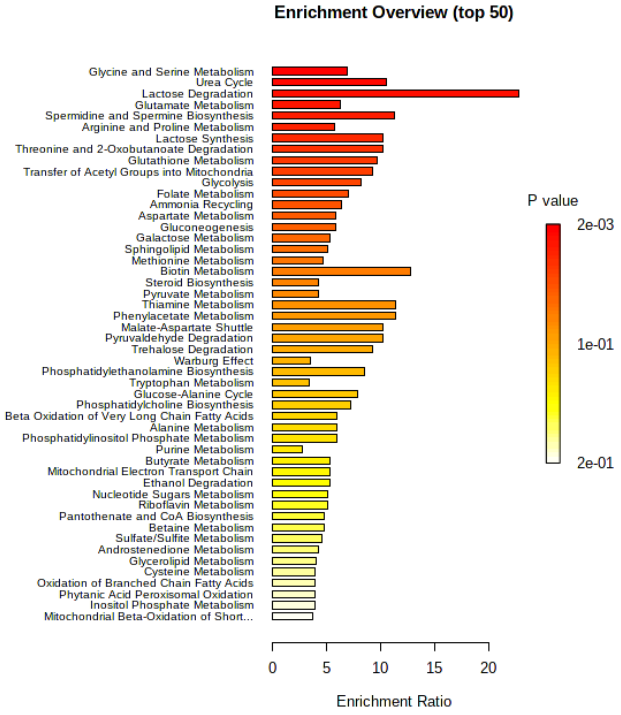

Figure 3.8. Enrichment pathway analysis of differentially abundant metabolites between seasons in IO.

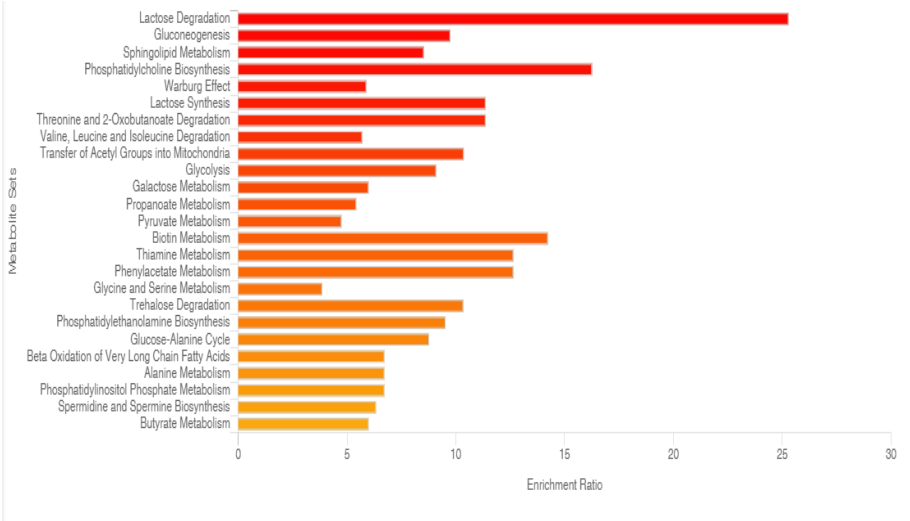

Figure 3.9. Enrichment pathway analysis of differentially abundant metabolites between seasons in CC-IO.

The enrichment analysis revealed that the main metabolic pathways influenced by season at the level of the oocyte were glycine and serine metabolism, urea cycle, lactose degradation, glutamate metabolism, spermidine and spermine biosynthesis, arginine and proline metabolism, lactose synthesis, threonine and 2-oxobutanoate degradation, glutathione metabolism, transfer of acetyl groups into mitochondria, glycolysis, folate metabolism, ammonia recycling, aspartate metabolism and gluconeogenesis (Figure 3.8).

3.3.5 Metabolomic analysis of the polar phase of cumulus cells and oocytes after in vitro maturation

Also cumulus cells and oocytes after IVM clustered separately into two distinct seasonal classes (Figure 3.1). In cumulus cells post-IVM the content of citrulline, aspartate, asparagine, glutathione, glucose, trimethylamine, sarcosine was reduced, while that of ATP, lactate, glycerophosphocholine, phosphocholine and hydroxyproline was increased during the NBS (3.11).

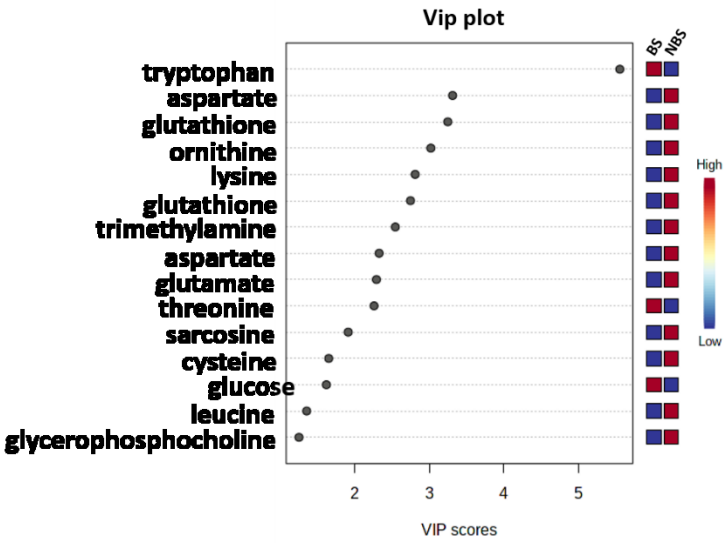

Figure 3.10. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in MO.

The VIP plot revealed that the oocyte levels of tryptophan, threonine and glucose were reduced, while the levels of aspartate, glutathione, ornithine, lysine, trimethylamine, glutamate, sarcosine, cysteine, leucine and glycerophosphocholine were increased during the NBS compared to the BS (Figure 3.10).

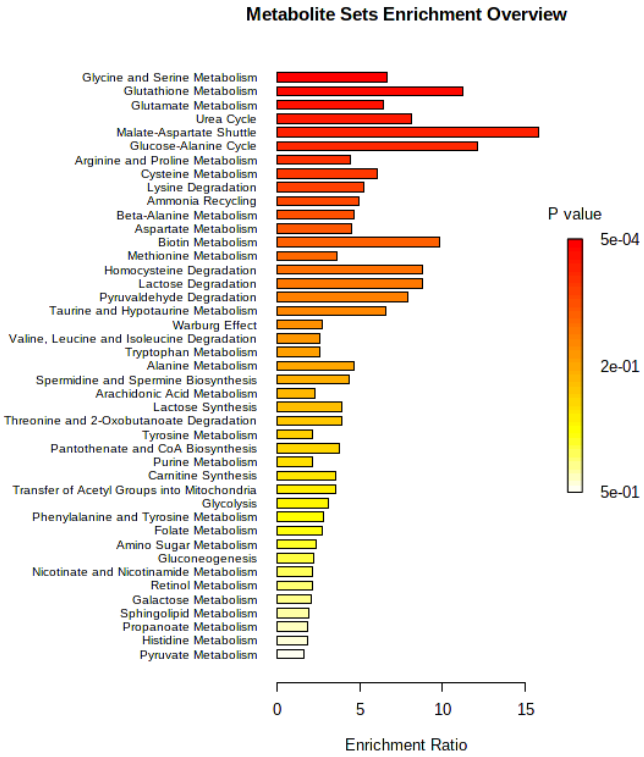

Figure 3.11. Enrichment pathway analysis of differentially abundant metabolites between seasons in MO.

The main metabolic pathways affected by season in cumulus cells post-IVM were: aspartate metabolism, urea cycle, lactose degradation, ammonia recycling, gluconeogenesis, sphingolipid metabolism, phosphatidylcholine biosynthesis, pyruvate metabolism, glutamate metabolism, arginine and proline metabolism, lactose synthesis, glutathione metabolism, transfer of acetyl groups into mitochondria, Warburg effect and glycolysis (3.12).

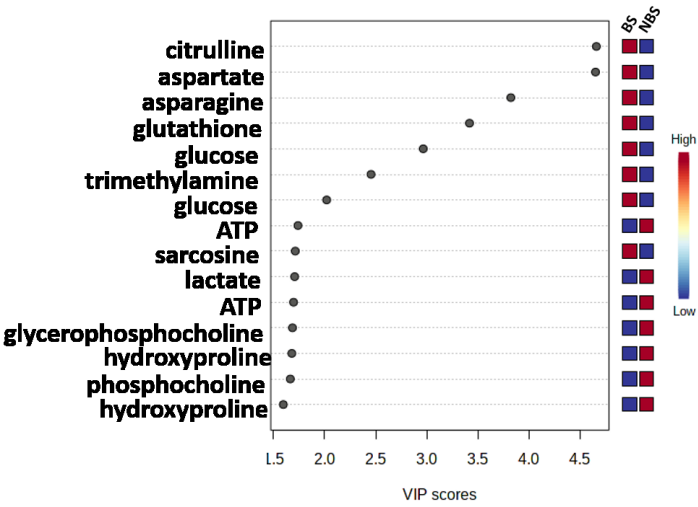

Figure 3.12. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in CC-MO.

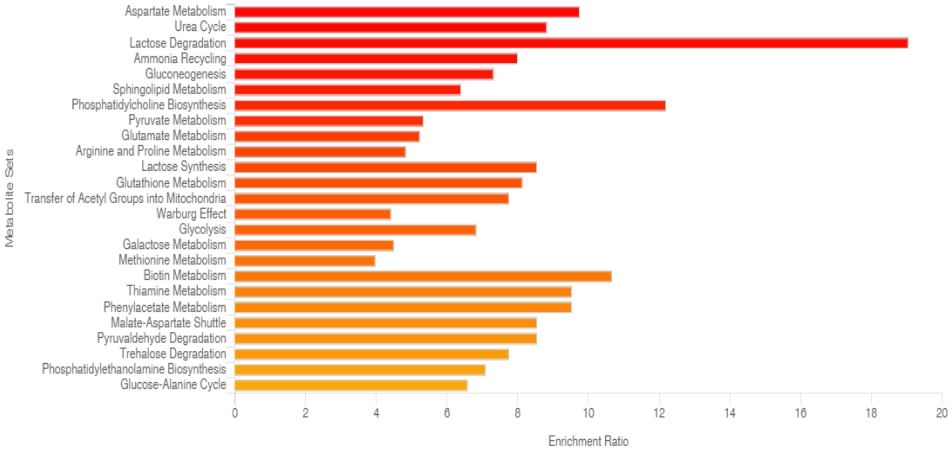

Figure 3.13. Enrichment pathway analysis of differentially abundant metabolites between seasons in CC-MO.

In the post-IVM oocytes the metabolic pathways mainly affected by season were glycine and serine metabolism, glutathione metabolism, glutamate metabolism, urea cycle, malate-aspartate shuttle, glucose-alanine cycle, arginine and proline metabolism, cysteine metabolism (Figure 3.11).

3.3.6 Metabolomic analysis of spent medium

The content in metabolites in the spent medium was also markedly different according to season, as shown by samples clustering into distinct classes (Figure 3.1).

Figure 3.1 Score plot of spent medium (SM) samples collected during the breeding (BS) and non-breeding (NBS) seasons

During the NBS higher levels of methionine, leucine, aspartate, glutamate, ornithine and alanine were detected in the SM, while lower levels were found of phosphocholine, choline, lactate, lysine, trimethylamine, glutathione, sarcosine and asparagine (Figure 3.14).

The main metabolic pathways affected by season in post-IVM spent medium were urea cycle, glycine and serine metabolism, glutathione and glutamate metabolism, malate-aspartate shuttle, ammonia recycling, aspartate metabolism, glucose-alanine cycle, phspahtydilcholine biosynthesis etc (Figure 3.15).

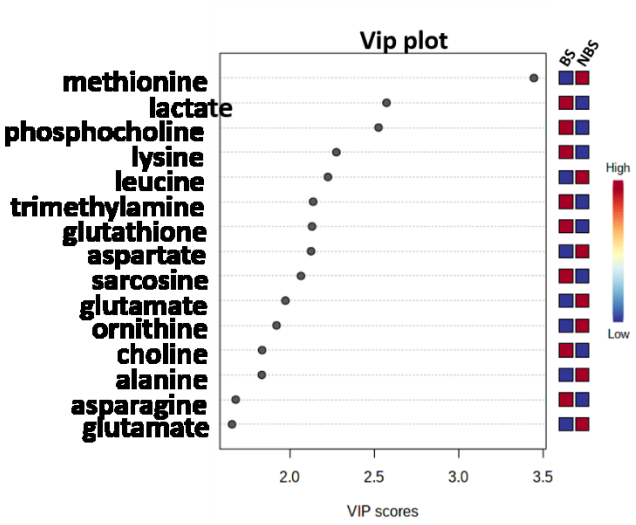

Figure 3.14. VIP plot shows the 15 proton signals corresponding to metabolites that showed the greatest seasonal variations in SM.

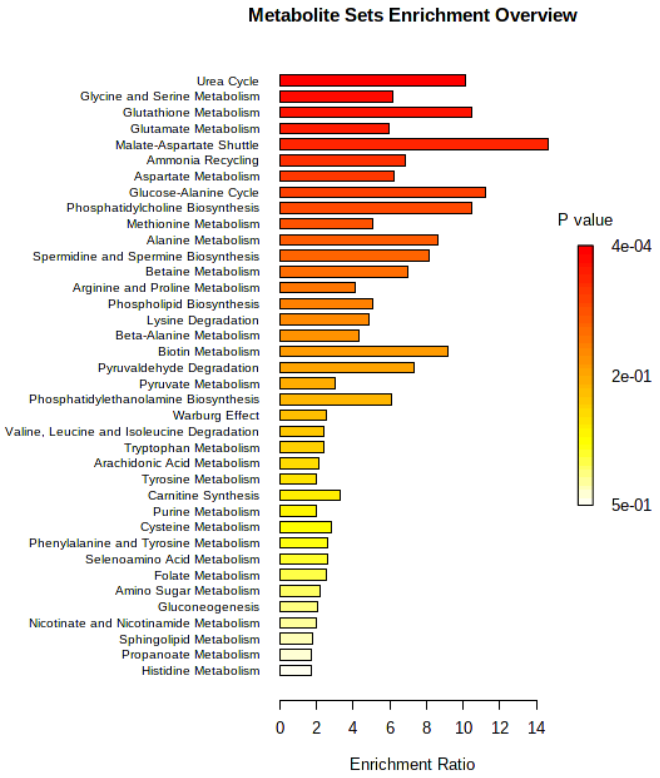

Figure 3.15. Enrichment pathway analysis of differentially abundant metabolites between seasons in SM.

3.3.7 Metabolomic analysis of the apolar phase of follicular fluid

The OPLS-DA revealed significant differences in the lipid profile of FF between seasons, as indicated by the score plot reported in Figure 3.16, showing a clear separation of samples collected in the BS and NBS. In Figure 3.17 the 15 proton signals corresponding to metabolites with the highest seasonal variation are reported. In particular, during the NBS the FF contained lower levels of cholesterol, phospholipids and some fatty acids. such as omega-3, linoleic acid and others non identified, with higher levels of triglycerides and arachidonic acid (figure 3.17). The metabolite that showed the greatest variation, with more abundance in the NBS, was a fatty acid that the analysis did not allow to identify.

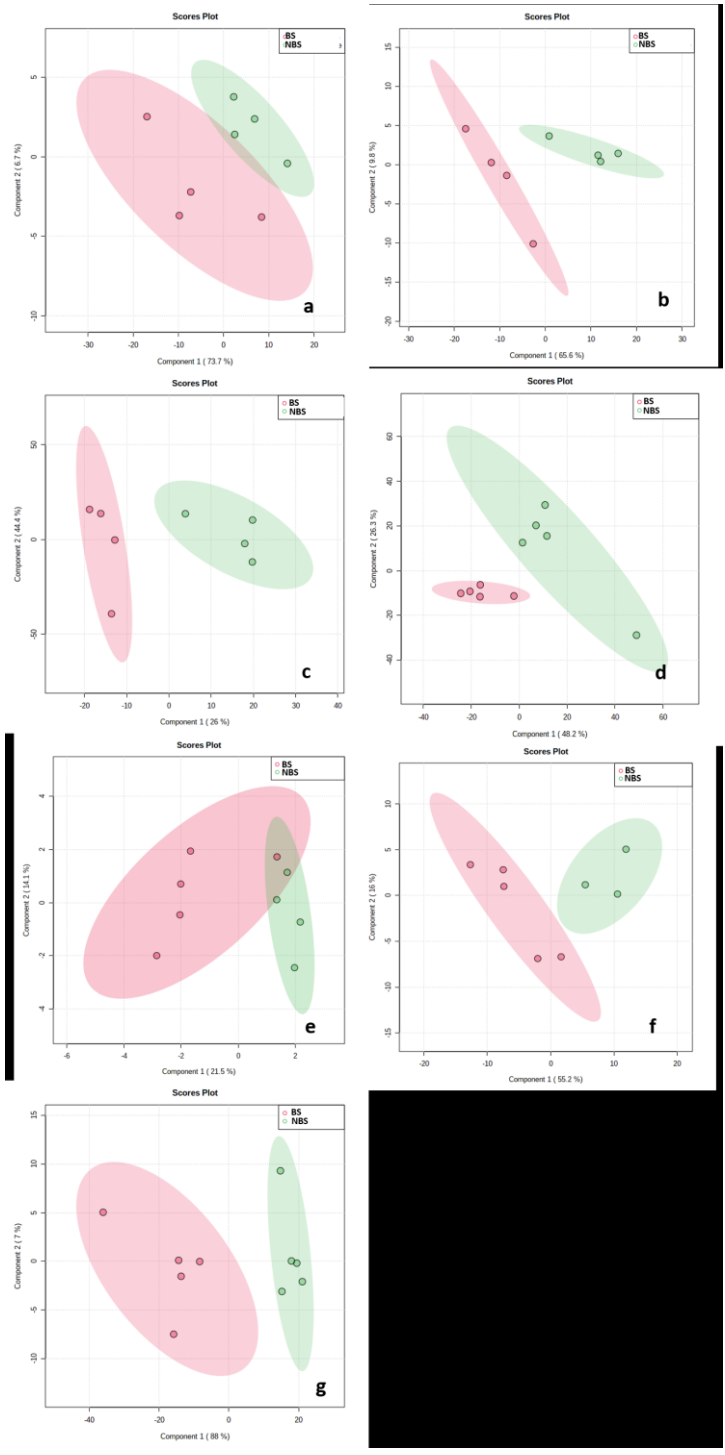
Scores Plot
BS
NBS
Component 1 (73.7 %)
Component 2 (6.7 %)
a
Component 1 (65.6 %)
Component 2 (9.8 %)
b
Component 1 (26 %)
Component 2 (44.4 %)
c
Component 1 (48.2 %)
Component 2 (26.3 %)
d
Component 1 (21.5 %)
Component 2 (14.1 %)
e
Component 1 (55.2 %)
Component 2 (16 %)
f
Component 1 (88 %)
Component 2 (7 %)
g

Figure 3.16. Score plot showing the differences in proton signals between bs and NBS in: a. Follicular Fluid; b. Follicular Cells; c. immature oocytes, d. oocytes after in vitro maturation; e. Cumulus cells from immature oocytes; f. Cumulus cells from mature oocytes; g. Spent medium.

3.3.8 Metabolomic analysis of the apolar phase of follicular cells

A clear separation of spectra was also observed for follicular cells samples collected in different seasons (Figure 3.16). The VIP plot evidenced a decreased lipid content during the NBS, with 14 out of 15 proton signals less abundant, like cholesterol, phospholipids, triglycerides and fatty acids, while the only lipid that increased, showing the greatest seasonal variation, was a non-identified fatty acid (Figure 3.18).

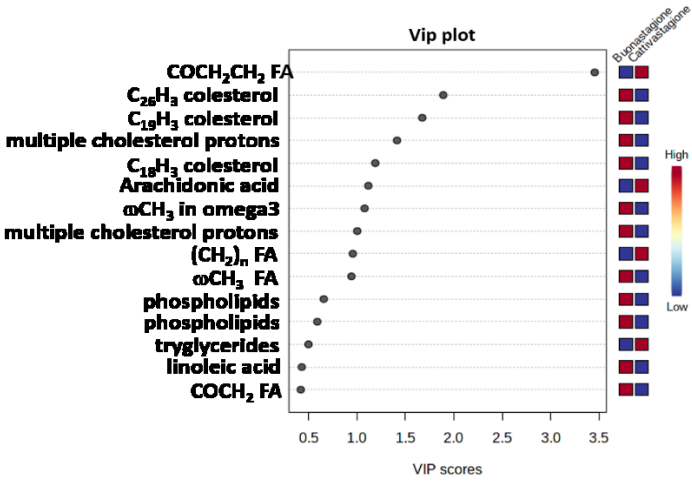

Figure 3.17. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in FF

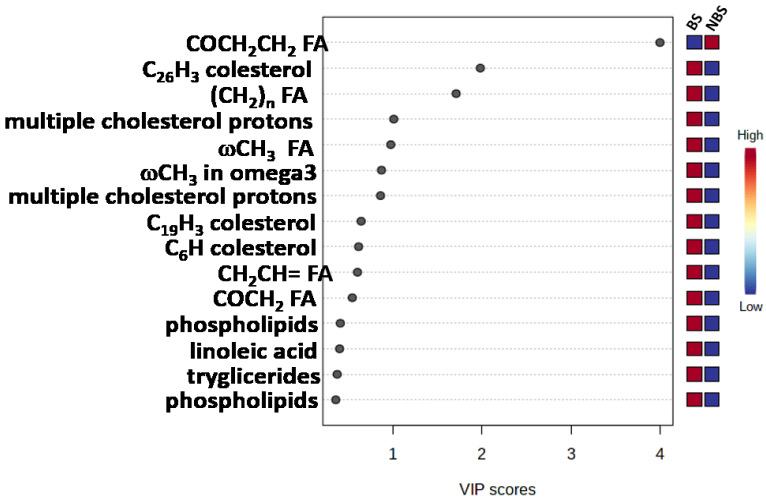

Figure 3.18. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in FC

3.3.9 Metabolomic analysis of the apolar phase of immature oocytes and cumulus cells

The OPLS-DA plot also showed a distinct separation of the lipophilic extracts of both cumulus cells and immature oocytes between NBS and BS, according to the metabolic content (3.16). The proton signals corresponding to metabolites showing the greatest seasonal variation are reported in the VIP plot (Figure 3.19 and 3.20). In particular, in cumulus cells (CC-IO) cholesterol and some fatty acids, including -CH3 in omega3 and arachidonic acid, were more abundant, while other fatty acids and phospholipids were less abundant in the NBS (Figure 3.20). During the NBS IO had reduced content of cholesterol and some fatty acids, including -CH3 FA, but increased glycerophospholipids, phospholipids, triglycerides and linoleic acid (Figure 3.19).

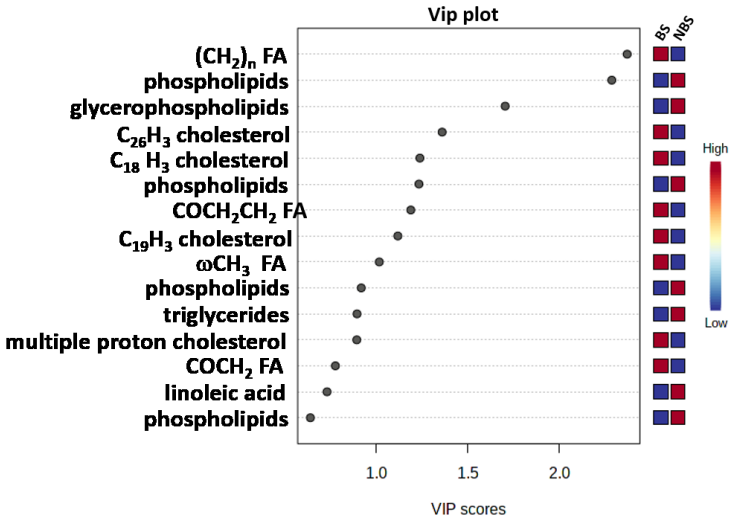

Figure 3.19. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in IO.

3.3.10 Metabolomic analysis of the apolar phase of cumulus cells and oocytes after in vitro maturation

The OPLS- DA analysis of the apolar phase of both cumulus cells and oocytes after IVM also revealed that there is a clear separation of the samples according to season (3.16).
In the cumulus cells (CC-MO) cholesterol, triglycerides and some fatty acids including arachidonic acid and -CH3 in omega3 were less abundant, whereas phospholipids and other fatty acids including docosahexaenoic acid were more abundant during the NBS compared to the BS (Figure 3.21).
In the MO the metabolites showing the greatest seasonal variation were phospholipids, glycerophospholipids, triglycerides, phosphatidylcholine and fatty acids, like -CH3 in omega3 and linoleic acid, that increased during the NBS, as well as cholesterol that decreased during the NBS compared to the BS (Figure 3.20).

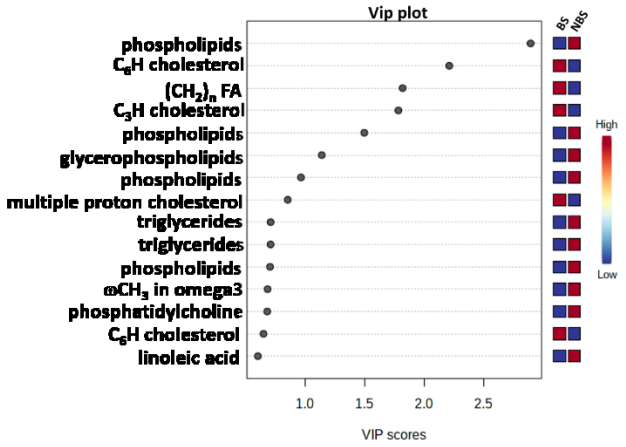

Figure 3.20. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in MO.

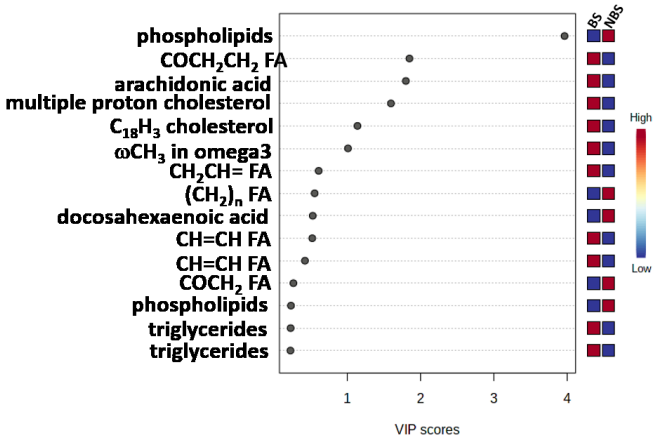

Figure 3.21. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in CC-MO.

3.3.11 Metabolomic analysis of apolar phase of spent medium (SM)

The lipid content in the spent medium was also markedly different according to season, as shown by samples clustering into distinct classes (Figure 3.16). During the NBS higher levels of some fatty acids, including arachidonic and eicosapentaenoic were detected in the SM, while lower levels of cholesterol, glycerophospholipids and other fatty acids, among which a fatty acid of omega3 family were observed (Figure 3.22).

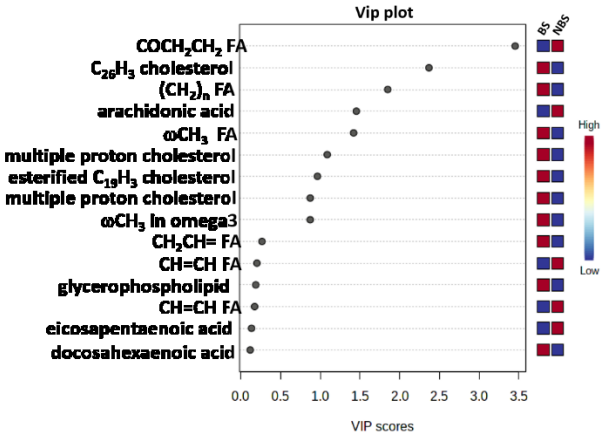

Figure 3.22. VIP plot shows the 15 proton signals corresponding top apolar metabolites that showed the greatest seasonal variations in SM.

An overall representation of seasonal differences in metabolites content in the different type of samples is provided in Tables 3.1 (polar metabolites) and 3.2 (apolar metabolites).

3.4 discussion

To the best of our knowledge this is the first time that a metabolomic approach has been used to study the ovarian follicle in the Italian Mediterranean buffalo, and the first to have simultaneously analyzed all its components, in relation to season. The rational of this work derives from the hypothesis that during NBS the oocyte grows in a suboptimal environment, resulting in impaired growth and acquisition of competence. Indeed, the results of IVEP confirmed, in agreement with literature [33] and with previous experiment, a marked influence of season on oocyte competence in Italian Mediterranean buffalo, as shown by reduced cleavage and blastocyst rates.

	FF	FC	IO	CC -IO	MO	CC-MO	SM
3-hydroxydroxybutyrate	▲	▲					
Lysine	▼	▲			▲		▼
Serine	▼						
Cysteine	▼				▲		
Proline	▼						
Hydroxyproline						▲	
Leucine	▲	▲			▲		▲
Iso-Leucine	▲	▲		▲			
Sarcosine			▲		▲	▼	▼
Methionine							▲
Alanine							▲
Valine		▲		▲			
Threonine			▼	▲	▼		
Ornithine			▼		▲		▲
Citrulline						▼	
Trimethylamine	▼		▲		▲	▼	▼
Tryptophan		▼			▼		
Aspartate			▲		▲	▼	▲
Asparagine						▼	▼
Formate			▼				
glutamate	▼				▲		▲
Glutathione	▼	▼	▲		▲	▼	▼
Choline	▼						▼
phosphocholine	▼	▼		▲		▲	▼

Glycerophosphocholine	▼	▼		▲	▲	▲	
Glucose		▼	▼	▼	▼	▼	
Lactate	▼	▲		▲		▲	▼
ATP		▲	▲	▲		▲	

Table 3.1 Differences in metabolites content (polar) between seasons in follicular fluid (FF), follicular cells (FC), immature oocytes (IO) and corresponding cumulus cells (CC_IO), mature oocytes (MO) and corresponding cumulus cells (CC-MO) and spent medium (SM). ▲ higher concentration in the NBS vs BS; ▼ lower concentration in the NBS vs BS.

	FF	FC	IO	CC -IO	MO	CC-MO	SM
Arachidonic acid	▲			▲		▼	▲
Linoleic acid	▼	▼	▲		▲		
Docosahexaenoic acid						▲	▼
Omega -3	▼	▼	▼	▲	▲	▼	▼
Eicosapentaenoic acid							▲
Cholesterol	▼	▼	▼	▲	▼	▼	▼
Phospholipids	▼	▼	▲	▼	▲	▲	
Phosphatidylcholine					▲		
Glycerophospholipids			▲		▲		▲
Triglycerides	▲	▼	▲		▲	▼	

Table 3.2 Differences in metabolites content (apolar) between seasons in follicular fluid (FF), follicular cells (FC), immature oocytes (IO) and corresponding cumulus cells (CC_IO), mature oocytes (MO) and corresponding cumulus cells (CC-MO) and spent medium (SM). ▲ higher concentration in the NBS vs BS; ▼ lower concentration in the NBS vs BS

Seasonal differences in metabolites content were demonstrated, that may account for the reduced oocyte developmental competence observed during NBS. Therefore, this study allowed the identification of potential fertility

markers. Moreover, the identification and subsequent quantification of some key molecules that play an active role in the metabolism / regulation of oocyte and associated cell activity can lay the ground for the development of corrective strategies based on the addition of such substances in the IVM medium or in vivo through dietary supplements during the non-breeding season.

3.4.1 Metabolomic analysis of the polar metabolites in relation to season

With regard to the analysis of the polar phase of reproductive samples, seasonal differences were recorded in several amino acids, energy substrates, and cholines at different levels.
Interestingly, most of the metabolites showing seasonal variations in the FF were in lower amounts during the NBS, with the exception of hydroxybutyrate (HB), leucine and isoleucine (see table n. 3.1). An intriguing finding of the study is indeed the higher amount of HB, which is the metabolite showing the greatest seasonal variation, in both the FF and FC collected during the NBS. It is worth reminding that at the Italian latitudes, where the trial was carried out, the NBS substantially falls in winter, when the environmental temperatures are low (range 0-15 °C). Given the correlation existing between the intrafollicular and serum concentration of HB [39], this result agrees with the observations that buffalo, unlike cattle, tends to be in a condition of negative energy balance (NEB) especially in the coldest periods of the year rather than at the beginning of lactation (unpublished data). The low adaptability of buffalo to cold temperatures, due to the tropical origin of the species, and particularly to severe thermic excursions, aggravated by exposure to cold winds, may result in inappropriate thermoregulation, causing a NEB condition, particularly during the transition from mild to cold periods. The NEB is determined by an insufficient supply of energy with respect to the metabolic needs of the animal, resulting in a temporary decrease of blood glucose values, to which the organism responds by raising GH levels and lowering those of T4 (functional hypothyroidism)[40]. The main role of GH during NEB is to reduce the susceptibility of adipose tissue and muscles to insulin and to change the insulin:glucagon ratio towards the latter hormone. This basically leads to a different distribution of hematic glucose, mainly destined to tissues that are physiologically less sensitive to insulin (central nervous system, erythrocytes, intestine, mammary gland), and activates the neoglucogenesis pathway, starting from other molecules as pyruvate,

lactate, amino acids and NEFA. In addition, this results in the mobilization of adipose reserves and muscular proteins in order to provide energy and the precursors necessary to maintain glucose levels within physiological ranges. The increased lipomobilization is responsible for the increase of hematic levels of NEFAs and beta-hydroxybutyrate. In buffalo this may also occur at the end of lactation, when the animals make an additional energetic effort to keep high the milk fat concentration.

It has been hypothesized that the serum changes occurring during NEB in dairy cattle, such as elevated NEFA and beta-hydroxybutyrate (β-HB) levels, low glucose levels, as well as increased urea levels, may adversely affect the quality of the follicular environment and consequently the oocyte, leading to the ovulation of a less competent oocyte [41]. In fact, there is a correlation between the composition of plasma and follicular fluid regarding the levels of glucose, NEFA, β-HB, urea and total cholesterol [42,43,44]. It is known that the somatic cells of the follicle (granulosa and cumulus cells) protect the oocytes, providing a sort of barrier from toxic compounds [45,46], resulting in the oocyte being isolated within the follicle from adverse conditions to a certain extent. It was demonstrated in cattle that during NEB glucose levels decrease and NEFA levels increase in the follicular fluid [47]. Furthermore, the toxic effects of high concentrations of urea [48,49], low glucose levels [50] and high levels of NEFA, particularly palmitic and stearic acid, in follicular fluid have been demonstrated on the bovine oocytes, as well as an additive toxic effect of β HB under moderate hypoglicemic conditions has been hypothesized [51].

In our study, however, the only indicator of NEB at the FF level was β-HB, while neither elevated levels of NEFA and urea, nor reduced levels of glucose were found in the FF. With regard to NEFA it is worth specifying that the type of analysis did not allow the identification of various fatty acids that showed different distribution according to season. However, during the NBS lower glucose contents were recorded in FC, as well as in oocytes and cumulus cells both before and after IVM. This last observation, together with increased lactate production in the NBS in follicular and cumulus cells (before and after IVM), suggests a modification of the microenvironment in which the oocyte develops, reflecting the serum changes that occur during the unfavorable season, in which sub-clinical metabolic disorders seem to be more frequent, which may justify the reduced oocyte competence. The abundance of leucine and isoleucine in the FF during NBS may also be compatible with NEB, in which protein mobilization occurs, as these amino acids are markers of protein turnover [52]. The hypothesis that during the

NBS, coinciding with colder months, buffaloes are predisposed to undergo NEB that may account at least partially, for the decreased oocyte function, is certainly suggestive but requires further investigation. In any case, seasonal changes were also detected in the concentrations of glucose, lactate and ATP, suggesting an impact of the environment on the energy metabolism of the follicle, confirmed by the pathway enrichment analysis. Glycolysis is the main energy pathway of the COC, with glucose representing the main substrate [53], and pyruvate, lactate and ATP the main products that accumulate at the end of this process. Indeed, while the granulosa\cumulus cells of the follicle are mainly glycolytic, the energy metabolism of the oocyte depends mainly on the mitochondrial oxidation of pyruvate for the ATP production. The oocyte can be considered a poor user of glucose, or at least in a direct way. It is established that the cumulus cells absorb glucose from the follicular environment, metabolize it into pyruvate and transfer it to the oocyte through the gap junctions and that therefore the oocyte uses pyruvate as an energy source [54,55,56]. Furthermore, the consumption of pyruvate is higher in mouse oocytes actively engaged in meiotic maturation [57] and oocytes in active meiotic division have higher ATP consumption rate than those in GV stage [58]. The reduced glucose levels in FC, CC and OO are in line with the hypothesized katabolic status of the animals during the NBS. However, the lower levels of glucose and the higher levels of lactate detected in the FC and CC may also suggest an increased anaerobic glycolytic during the NBS which is, however, not reflected in the concentration of lactate in the follicular fluid which is, in contrast, reduced. There should be, in fact, a positive correlation between the use of glucose and the production of lactate and it has been hypothesized that when the follicle grows its energy needs increase in a condition of reduced oxygen availability (due to the thickening of the avascular epithelium), resulting in an increase in glycolytic activity and, therefore, of lactate production [59,60].

Lactate, which is normally in high concentration in follicular fluid and reproductive tract [61], playing a regulatory role of the cytosolic redox state, can be oxidized by lactate dehydrogenase into pyruvate. An increased lactate production in vitro has been associated to decreased oocyte competence in the mouse [62,63]. In contrast, reduced lactate levels, together with increased glucose values, have been negatively correlated to oocyte competence in humans [64]. Likewise, in an earlier work impaired lactate production by granulosa cells was described in women with polycystic ovarian syndrome with ovulation problems [65], suggesting the importance

of lactate on oocyte development. Therefore, alterations in the production/consumption of lactate can exert deleterious effects on oocyte maturation, due to the important role played by lactate in the follicle metabolism.

The metabolism of glucose within the follicle can occur through different pathways: 1) glycolysis, producing ATP and pyruvate/lactate; 2) pentose phosphate pathway, providing precursors of purine nucleotides and NADH for synthesis pathway and antioxidant activity; 3) hexosamine biosynthetic pathway, important for protein glycosylation and hyaluronic acid synthesis for cumulus expansion; and 4) the polyol pathway producing sorbitol and fructose [66]. The oocyte metabolizes pyruvate through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, providing the greatest amount of the ATP [67,68]. The larger amount of ATP observed during NBS 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. Despite this the hypothesis that during the NBS, the somatic cells of the follicle increase the glycolytic activity, as suggested by reduced glucose and increased lactate levels, to provide fuel to the oocyte cannot be rulled out. The increased ATP content detected in the oocytes during the NBS is in line with this hypothesis. It is likely, as described in other species, that the increased ATP mainly derives from the entry of pyruvate, transferred via gap junctions from cumulus cells, into the TCA cycle and oxidative phosphorylation. However, the reduced glucose oocyte content may suggest that this substrate can also be directly utilized by the oocyte. On the other hand, the lower glucose content may be simply a consequence of the deficiency of the substrate in follicular/cumulus cells. Indeed, although pyruvate is undoubtedly the preferred energy substrate, oocytes possess the machinery for glucose metabolism [69,70] and glucose can be transported into the oocyte via sodium-coupled glucose transporters or facilitative glucose transporters (GLUTs), as well as through gap junctions [71]. Another suggestive hypothesis that derives from the observation of the metabolites involved in energy supply is that the greater amount of ATP in somatic cells and oocytes observed during the NBS could suggests that under adverse conditions there is a compensatory increase of energy metabolism, in agreement with the “quiet embryo” hypothesis [72,73], i.e. that non- or sub- viable embryos exhibit a greater demand of nutrients, to implement reparative processes [74,75].

Certainly, to satisfy energy needs, an adequate content of ATP in oocytes and embryos is critical for development, affecting nucleic acid and protein

synthesis, and it has been suggested to be an indicator of the developmental potential of mouse [76,77] and bovine embryos [78]. However, the relationship between ATP content and oocyte developmental competence is controversial, with contradictory findings in different species. In fact, variations in ATP concentrations appear to be associated with developmental competence in humans; in particular, lower levels were found in oocytes unable to be fertilized and advance to zygote stages [79], as well as in oocytes and embryos failing to establish pregnancies [80]. However, no significant differences in ATP content were observed between more and less competent porcine oocytes after IVM [81,82]. A seasonal influence was also observed on the profile of amino acids and peptides of the follicle. With regard to amino acid content, the discussion will focus on those previously suggested as competence markers in other species. Amino acids are essential for numerous functions. They represent the substrates for the synthesis of various substances such as proteins (glutamine, aspartate, glycine), glutathione (glutamate, cysteine and glycine), glycoproteins and hyaluronic acid (glutamine linked to glucose), and signal molecules such as nitric oxide (arginine). They also play important roles as energy substrates (glutamine, glycine and others), regulators of pH and osmolarity (glycine, alanine, glutamine and others), chelators of heavy metals (glycine), donors of methyl groups (methionine), in the secretion of ammonia (alanine) and in anaplerosis (aspartate, glutamate, leucine, lysine and isoleucine) [67,83].
Among differentially abundant metabolites in the two seasons, glutathione (GSH) and its precursors cysteine and glutamate, as well as serine, involved in the synthetic pathway of cysteine, are of particular interest, due to the known role played in antioxidant cell defense and oocyte development. During the NBS all these metabolites are less abundant in the FF. In addition, glutathione levels are also reduced in FC, CC-MO and SM but unexpectedly higher in both IO and MO.
Glutathione (GSH) is an intra and extracellular non-protein sulphydryl compound, composed by three amino acids (cysteine, glutamate and glycine), playing a major role in the protection of mammalian cells from oxidative damages. In addition, GSH is also involved in many other biological processes such as amino acid transport, protein and DNA synthesis and disulfide reduction [84]. Furthermore, GSH, as co-enzymes constituent, participates in energy metabolism and is engaged in embryonic events like cell proliferation and differentiation at later preimplantation stages in the mouse and bovine [85,86,87]. It is known that GSH contributes to sperm decondensation and to the formation of male pronucleus after

fertilization [88,89]. It has been also demonstrated that oocytes are capable to synthesize GSH during maturation in several species [90,91,92,93], including buffalo [94,95]. Indeed, GSH concentration increases during oocyte maturation [96], contributing to create a reservoir pool that will protect the embryos during development until embryonic gene activation occurs [97]. The synthesis of GSH highly depends on the availability of cysteine [98], which is the precursor that can be taken up by the oocyte through the alanine-serine-cysteine transport system [99]. Another amino acid that was reduced in the FF of the NBS is serine, that enters the synthetic pathway of cysteine. In an in vitro trial it was found that after 15 h of IVM all amino acids increased both in cumulus cells and in SM, with the exception of serine that decreased [100], and it was hypothesized that oocytes need serine in the environment to reduce oxidative stress by stimulating GSH synthesis, similarly to tumoral cells [101].
In our study the reduced levels of serine, cysteine and glutamate in the FF during the NBS are reflected in the reduced GSH content, suggesting that the oocyte grows in an environment deficient in antioxidant defense, that may account for the lower competence. What is unexpected is the elevated intraoocyte concentration of GSH both before and after IVM, which is considered a marker of oocyte developmental competence [102]. Indeed, the measurement of GSH concentration in oocytes after IVM has been proposed as a valuable indicator of oocyte cytoplasmic maturation [103]. Furthermore, the importance of elevated intraoocyte GSH levels was confirmed by improved embryo yields obtained supplementing IVM medium with thiol compounds, known to stimulate GSH synthesis [104,105]. The majority of cells are not able to uptake GSH from extracellular space, but they can take up the constituent amino acids after GSH is broken down for either GSH synthesis or other pathways [106]. Another source of GSH for the oocyte may be GSH ethyl ester, which is hydrolysed to GSH by intracellular esterase [107]. Addition of GSH ethyl ester during bovine IVM indeed resulted in increased GSH oocyte content and blastocyst cell number [108]. In another work supplementation of culture medium with GSH was beneficial on mouse embryo development, without though affecting the intracellular GSH content in the embryo [109]. Therefore, it may be that the beneficial effects of GSH supplementation are due to the modulation of the extracellular environment, to the protective action on cell membrane or to the release of cysteine for protein synthesis. In fact, it was suggested that extracellular GSH may non-enzymatically

scavenge reactive oxygen species in the medium or on the extracellular surface of the embryos, preventing oxidative damages [110].
Based on these observations, we may speculate that the reduced GSH content in the FF results in an impaired protection within the follicle. Nevertheless, it is very hard to interpret the increased GSH concentration in less competent oocytes, that undoubtedly requires further investigations.
The higher GSH content in oocytes during NBS may be related to the suggested higher nutrient supply at the level of the follicle, due to a presumed increased level of NEFAs and a high β-HB level and is in line with the increased ATP content, suggesting a high energy status or decreased consumption of energy by the oocyte. While the hypothesis the during NBS GSH content is higher due to lower consumption by the oocytes that are "metabolically less active" is in contrast with the intensified energy metabolism suggested by other findings, including the increased ATP content. It is not possible to rule out that during NBS the oocytes, growing in an environment lacking antioxidants, uptake cysteine from the FF to synthesize more GSH in the attempt to counteract oxidative stress. This would be more in line with an accelerated metabolism. It is known that intracytoplasmic GSH levels are somehow related to the energy balance of the oocyte and ROS levels. The analysis of ROS levels, that unfortunately was not carried out, would certainly help to understand the phenomenon. What is unquestionable is that during the NBS the metabolism of glutathione and its main precursors is disrupted at all levels within the follicle, as confirmed by the pathway enrichment analysis, and this undoubtedly may affect oocyte competence.
With regard to other amino acids, our results agree with those of a previous study on amino acid profile of follicular fluid in cattle, in which the levels of glutamate were higher in the follicular fluid corresponding to oocytes that develop into blastocysts than in those that do not cleave or undergo developmental arrest [111].
In the same study a negative correlation was found between levels of leucine and isoleucine in FF and oocyte competence, in agreement with the results of our study in which higher levels of leucine and isoleucine were observed during NBS. Both leucine and isoleucine were also increased in FC during the NBS and, with some differences, also in other matrices analyzed.
These metabolites, i.e. glutamate and leucine also showed a good correlation with the quality of bovine oocytes assessed on morphological basis [111]. In another work performed in bovine, glutamate, as well as L-alanine and glycine were identified as metabolic markers of competence [112]. It

follows that some FF amino acids considered predictive markers of oocyte competence in cattle, such as glutamate, leucine and isoleucine, can be candidate markers also in buffalo.
On the other hand, the lower follicular levels of proline, lysine and serine found in our study during NBS do not agree with what is described in cattle. In the latter species, in fact, lysine and proline decreased at the follicular level with increasing competence, while serine was irrelevant. However, in humans, follicular proline levels were related to pregnancy success, in agreement with our results [64]. In porcine, intrafollicular levels of L-alanine and L-arginine were associated with blastocyst production. Moreover, the amino acids which in the bovine FF were more predictive of competence, such as alanine and glycine [111] did not show variations in our study according to the season. These observations confirm the importance to take into account species-specific differences.
The amino acid turnover during maturation has also been proposed as an indirect assessment of oocyte developmental competence [113]. In particular, the turnover of alanine, glutamine, arginine, tryptophan and leucine were associated to bovine oocyte competence. In our study 10 amino acids showed seasonal differences in the SM, suggesting a potential role as competence markers. In particular, lower levels of lysine, sarcosine, trimethylamine, asparagine and higher levels of leucine, methionine, alanine, ornithine, aspartate and glutamate in the SM were associated to reduced developmental competence. The increased amount of alanine during NBS is in agreement with the previous study in cattle [113]. This may be related to the role played by this amino acid to dispose ammonia during in vitro culture [114]. In contrast, the leucine levels in the SM showed an opposite pattern [113].
Another interesting result is the finding of lower concentration, at the level of the follicular fluid, during the NBS, of choline and its derivatives phosphocholine and glycerophosphocholine. The choline derivatives were also less expressed in the somatic cells of the follicle. These data are in line with previous work carried out in the human field in which there was a reduction of choline, phosphocholine and glycerophosphocholine in the follicular fluid of follicles containing oocytes that did not cleave after in vitro fertilization [64]. Choline and phosphocholine are the precursors of phosphatidylcholine which is a key component of biological membranes. The importance of these metabolites is confirmed by the compromised follicular development and oocyte maturation recorded in transgenic mice in which the phosphatidylcholine production pathway is altered [115]. In

fact, the pattern of choline metabolites is similar to that of phospholipids known to be constituents of membranes (see Table n). It follows that the decrease in choline metabolites recorded in the FF and FC during the NBS may be related to reduced developmental competence.
The pathway enrichment analysis revealed the main metabolic pathways affected by season. Among these, undoubtedly metabolism of glutathione and its precursors was disrupted at all levels within the follicle (FF, somatic cells and oocytes). An effect of season was also observed on phosphatidylcholine biosynthesis and valine/leucine/isoleucine degradation both in the FF and all somatic cells. Furthermore, metabolic pathways involved in energy metabolism, like lactose degradation/synthesis, glycolysis, gluconeogenesis, Warburg effect, transfer of acetyl groups into mitochondria were mainly affected in the somatic cells. These were also in part affected in the immature oocytes that also showed alterations in urea cycle, ammonia recycling, as well metabolism of several amino acids and folate. The urea cycle and amino acids metabolism were also influenced by season in the oocytes after IVM, as well as in SM, where ammonia recycling and phosphatydilcholine biosynthesis were also affected.

3.4.2 Metabolomic analysis of the apolar metabolites in relation to season

The NMR analysis of the apolar phase undoubtedly showed significant differences in the lipid profile of the different biological matrices between seasons. Overall, most of the changes in the lipid composition of FF were reflected in FC but not in the other components of COCs and SM, confirming a complex metabolic interaction, in part modulated by cumulus cells (see Table 3.2).
Lipids are hydrophobic organic molecules that play a central role in cell metabolism, representing a primary energy source for cells, are involved in cell signaling and are essential constituents of cell membranes [116,117]. In addition, lipids are the precursors for synthesis of steroids and eicosanoids in COCs [118].
A peculiar feature was observed concerning triglycerides abundance, with higher levels in FF, lower levels in the FC and greater amounts recorded both in IO and MO, despite lower concentrations in CC-MO.
The greater amounts of tryglycerides (TAG) recorded in FF during NBS, corresponding to a reduced oocyte competence, is in agreement with previous studies in humans. In a recent study higher TAG content in FF were associated to negative pregnancy outcome in women [119].

Furthermore, higher tryglicerides content in human FF have been correlated with levels of adipokines and pro-inflammatory cytokines, indicating a TAG-induced inflammatory response that may affect oocyte development [120].
The lower content of tryglycerides, as well as of most other lipids, in the FC during the NBS may suggest the activation of the lipolytic pathway, in the attempt to satisfy energy demands, as supported by the higher ATP levels found (see Table n.2). An acceleration of metabolic processes according to Leese's "quiete embryo" hypothesis [72,73] may indicate a stress condition of cells that make a compensatory effort to cope with a suboptimal microenvironment. Generally, triglycerides are stored inside the oocytes and cells in the form of lipid droplets. These represent an important energetic substrate for the production of ATP.
The dark appearance of the cytoplasm of oocytes of various species [121,122], including buffalo [123,124,125], is due to the presence of a great number of lipid droplets. Buffalo oocytes present a high quantity of intracytoplasmic lipid droplets, suggesting a higher triglyceride content, that has not yet been measured. The lipid droplets have dynamic functions during the oocyte maturation and embryonic development; they can be synthesized, degraded and can change shape and dimensions [126]. In buffalo oocytes and embryos the lipid droplets show variations in distribution and structure during maturation and development, according to cell metabolic needs [124,125,127]. These lipid droplets are associated to endoplasmic reticulum and mitochondria [128], constituting metabolic units [129], playing a role for mitochondrial ATP production [130]. Triglycerides contained in lipid droplets represent the main endogenous energy source for oocytes and embryos in different species [131,132]. The greater amount of lipid droplets in the oocytes compared to other cells is likely due to the fact that the embryo depends on its energy stores during the development until placentation [122,132,133,134], that in buffalo takes place around 30-35 days [135].
The lipid composition of the extracellular medium has been shown to influence the lipid composition of oocytes [136]. In fact, bovine oocytes matured in vitro in the presence of fetal serum, rich in lipids, show a higher intracytoplasmic content of triglycerides and cholesterol than those matured in the absence of serum [137], indicating that incorporation of lipids within the cytoplasm from the environment is taking place.
An unexpected finding is the higher concentration of triglycerides found inside the oocytes collected during NBS as in bovine the amount of

intracellular lipid droplets, known to contain mainly triglycerides, is positively associated with oocyte competence [138]. On the other hand, an excessive accumulation of lipids is known to exert a negative effect on oocyte viability and cryotolerance [116]. It is worth noting that the MNR analysis provided a relative quantifications of the various components between seasons, with the BS representing the most physiological condition in the species, associated to a higher degree of oocyte competence. The hypothesis that higher TAG levels inside the oocytes are a consequence of their inability to use these energy sources correctly, due to a disruption of the lipolytic pathways for energy production, is not supported by the findings of higher ATP content (see Table 3.1) during NBS. It is therefore likely that there may be a threshold level, beyond which the quantity of triglycerides becomes harmful to the oocyte. This hypothesis appears more realistic and supported by the finding of lower concentrations of triglycerides both in the FC and in CC-MO during the NBS. These cytotypes are known to mediate the quantity of some molecules that reach the oocyte from the FF [140,141]. Therefore, it seems reasonable that somatic cells allow the oocyte to accumulate only the quantity of triglycerides that is optimal for its metabolism, retaining the surplus in their cytoplasm and that this mechanism is partly impaired in NBS.

Differences in cholesterol and phospholipids content were observed at different levels between seasons. Cholesterol and phospholipids are essential elements for the formation of cell membranes, which play a crucial role during the rapid cell division process occurring after fertilization. Anomalies of cholesterol levels at the membrane level can negatively influence fertilization and subsequently embryonic development; this can occur both in case of excess and defect. In fact, in an experiment conducted in mice it was observed that an excess of cholesterol, while not interfering with meiotic progression, can induce activation of the oocyte and cause infertility [142]. On the contrary, sub-physiological levels of cholesterol in the mouse oocyte have been associated with a delay in the extrusion of the second polar body and a reduction in fertilization rates [143].

Cholesterol plays a role both as a substrate for the synthesis of cell membranes and as a substrate for the production of steroid hormones by the follicle [144]; therefore, the lower concentration of cholesterol and its esters in the FF and FC of the NBS can be interpreted as a reduced availability of substrate useful for the synthesis of these components by the granulosa cells and cumulus cells. However, granulosa cells and cumulus cells are known to play a role in cholesterol biosynthesis in various species, including bovine

[145,146,147,148]. Furthermore, the cumulus cells appear to be fundamental in modulating the lipid metabolism of the oocyte during its maturation [148]. Therefore, the finding of higher cholesterol concentrations in cumulus cells during NBS may be interpreted as a compensatory biosynthetic activity, in the attempt to ensure physiological events to take place despite the deficiencies in the environment. Nevertheless, the oocyte content in cholesterol was reduced before and after IVM, as well as in the spent medium. It is known that oocytes are capable to uptake cholesterol from culture medium [149,150] and hence from the follicular environment. Potential exogenous sources of cholesterol, as well as fatty acids, for the oocyte are present in the follicular fluid in the form of high-density lipoproteins (HDL), undoubtedly the most abundant in FF, because they are the only subclass able to pass the blood-follicle barrier [151]. It was previously reported that HDL of FF are a primary source of cholesterol for steroid synthesis [145] and have angiogenic function on the ovarian follicle [152]. Therefore, the reduced levels in the oocytes here reported are likely the result of reduced incorporation.

Taken together these findings suggest that, although both cholesterol and phospholipids were lower during NBS in the follicular environment (FF and FC), in the oocyte the phospholipid : cholesterol ratio is higher, mainly due to increased phospholipids. As cholesterol and phospholipids are essential components of cell membrane, an unbalanced ratio may result in altered membrane stability and fluidity in the NBS, likely accounting for decreased competence. On the other hand, the higher amounts of phospholipids and glycerophospholipids in both IO and MO during NBS may be due to the increased lipid droplets, suggested by higher TAG, as constituents of their membranes.

The abundance of TAG in the oocytes, together with an increased amount of β-hydroxybutyrate in FF and FC, suggests an overload of fatty acids in the FF during the NBS [153]. Unfortunately, we lack information on the total amount of fatty acids in the two seasons, as the analysis only showed relative amounts and was not able to identify all fatty acids. Among the prevalent fatty acids in bovine FF, i.e. linoleic (LA), oleic, stearic and palmitic [154], only LA showed seasonal variations in our study.

Some fatty acids, such as LA and arachidonic (AA), which we found differently abundant in relation to season, may have not only an energetic but also a direct role in the regulation of definite functions in the oocyte, cumulus and granulosa cells. The different concentration of LA between the two seasons, in some of matrices examined is one of the most interesting

findings. Lower concentrations of LA in FF and FC collected in the NBS were observed, while opposite outcome was detected in immature and mature oocytes. Linoleic acid is a polyunsaturated fatty acid from the omega-6 series; it is considered an essential fatty acid as the body is not normally able to produce it independently. The involvement of LA in oocyte development is well known and studied. In cattle, LA is the most abundant fatty acid in the follicular fluid, accounting for about 30% of the total fatty acids of the follicular fluid [155]. Furthermore, the content of LA varies according to the state of follicular growth, with higher concentrations in the smaller follicles, suggesting a role of LA in oocyte maturation [156]. It has been shown that the addition of LA during the in vitro maturation of bovine oocytes has a dose-dependent effect on the reversible inhibition of nuclear maturation, while it has no effect in the advanced stages of culture [155]. The finding of lower concentrations of LA in the FF and FC and higher levels in oocytes in the NBS may suggest that follicular cells are capable to modulate the transfer of substances that reach the oocyte and that an altered metabolism of these in NBS somehow translates into a non-ideal environment. LA can be converted by a desaturation process into AA, a precursor of prostaglandin synthesis, which was found to be more abundant in the FF during the NBS. The AA is a polyunsaturated fatty acid that accounts for approximately 2.5% of the lipid component of bovine follicular fluid [157]. In addition to its role as a precursor of some eicosanoids, AA plays a significant role in modulating functions during the maturation of the oocytes and embryonic development. In our study higher concentrations of AA were found in FF of samples collected in the NBS, as well as in CC-IO. After IVM lower levels were detected in CC-MO, with higher levels in the SM, while no differences were found in FC as well as in oocytes. A certain beneficial effect of AA on oocyte maturation is described in the literature, but it seems that it is strictly dose dependent [157]. Therefore, the higher concentration of AA in the FF in the NBS may be consistent with the decrease in oocyte competence. In a study conducted on bovine granulosa cells it was observed that the addition of low doses of AA to the culture medium increases cell survival, while at higher doses the AA suppresses survival and induces apoptosis, probably correlated with oxidative stress [154,157]. To confirm this, it has been shown that cell death induced by AA is attributable to mitochondrial damage caused by oxidative stress [158]. Furthermore, the previously cited study showed that low doses of AA facilitate the accumulation of lipid droplets in granulosa cells, while higher concentrations inhibit this function, which is essential both to provide an

energy reserve and to provide a “barrier” against the lipotoxic effects of saturated fatty acids on the oocyte [153]. For this reason, the presence of higher concentrations of AA could be the cause of a reduced functionality of the granulosa cells and of the general reduction of the lipid components found during the NBS. The direct role of AA on the oocyte is still not well known, but high concentrations of compounds derived from arachidonic acid in follicular fluid at the time of oocyte retrieval have been associated with a reduced ability to produce pronuclei after ICSI in women [159]. The role of AA on oocyte metabolism is still unclear; however, it appears to be involved in the regulation of membrane channels [160] and of the kinase activity which is necessary for the direct cell-to-cell communication and paracrine signaling during oocyte maturation [161]. The lower concentrations of AA in CC-MO, in contrast to higher levels in the SM, suggest that during the NBS cumulus cells are less efficient to modulate the AA concentration in the surrounding milieu, likely due to reduced uptake from the medium.

3.5 Conclusions.

In conclusion, this study demonstrated relevant differences in metabolites content in all the follicular components analyzed between seasons in buffalo, suggesting that during the NBS several metabolic pathways are altered, resulting in decreased oocyte competence. The metabolites differentially abundant between seasons were involved mainly in glutathione metabolism, energy generating metabolism, phospholipid biosynthesis, amino acid metabolism. Lipid composition was also affected by season at different levels. Therefore, during NBS metabolic changes occur, likely due to a condition of NEB, influencing the follicular environment and are in part reflected at the somatic cells and the oocyte. The results may also suggest that the oocyte changes its metabolism to respond to these adverse conditions.

Furthermore, this work allowed the identification of potential markers of oocyte competence in the FF; among these, potential positive markers are glutathione, glutamate, lactate and cholines, while negative markers are leucine, isoleucine and β-hydroxybutyrate. These results suggest potential strategies to modulate the follicular environment or, more easily the IVM medium to improve the competence of oocytes collected during the NBS. However, a limitation of our study is related to the fact that the assay only allowed a relative quantification of differentially expressed metabolites and,

especially for the apolar phase, information on the total content of fatty acids and on the identification of some of the fatty acids showing variation is lacking. Undoubtedly, the study provided a complex intriguing picture of the seasonal impact on the follicle and consequently on the oocyte. However, due to the complexity of the topic, to better comprehend what occurs within the follicle during NBS further studies are certainly needed, first of all the absolute quantification of targeted metabolites. In addition, evaluation of the antioxidant capacity and ROS levels in the oocytes would also help to understand the oocyte response to the suboptimal conditions experienced during NBS.

[1] Nicholson, J. K., & Lindon, J. C. (2008). Metabonomics. Nature, 455(7216), 1054-1056.
[2] Deepinder, F., Chowdary, H. T., & Agarwal, A. (2007). Role of metabolomic analysis of biomarkers in the management of male infertility. Expert Review of Molecular Diagnostics, 7(4), 351-358.
[3] Nicholson, J. K., Lindon, J. C., & Holmes, E. (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica, 29(11), 1181-1189.
[4] Lindon, J. C., Holmes, E., & Nicholson, J. K. (2003). Peer reviewed: so what's the deal with metabonomics?. Analytical chemistry, 75(17), 384-A.
[5] Baka, S., & Malamitsi-Puchner, A. (2006). Novel follicular fluid factors influencing oocyte developmental potential in IVF: a review. Reproductive biomedicine online, 12(4), 500-506.
[6] Sinclair, K., & Dudley, E. (2019). Metabolomics and biomarker discovery. Advancements of Mass Spectrometry in Biomedical Research, 613-633.
[7] Guerreiro, T. M., Gonçalves, R. F., Melo, C. F. O. R., de Oliveira, D. N., Lima, E. D. O., Visintin, J. A., ... & Catharino, R. R. (2018). A metabolomic overview of follicular fluid in cows. Frontiers in veterinary science, 5, 10.
[8] Gosden, R. G., Hunter, R. H. F., Telfer, E., Torrance, C., & Brown, N. (1988). Physiological factors underlying the formation of ovarian follicular fluid. Reproduction, 82(2), 813-825.
[9] Vanholder, T., Leroy, J. L. M. R., Van Soom, A., Opsomer, G., Maes, D., Coryn, M., & de Kruif, A. (2005). Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. Animal reproduction science, 87(1-2), 33-44.
[10] Bender, K., Walsh, S., Evans, A. C. O., Fair, T., & Brennan, L. (2010). Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction, 139(6), 1047-1055.
[11] Nandi, S., Kumar, V. G., Manjunatha, B. M., & Gupta, P. S. P. (2007). Biochemical composition of ovine follicular fluid in relation to follicle size. Development, growth & differentiation, 49(1), 61-66.
[12] Nishimoto, H., Hamano, S., HILL, G. A., Miyamoto, A., & Tetsuka, M. (2009). Classification of bovine follicles based on the concentrations of steroids, glucose and lactate in follicular fluid and the status of

accompanying follicles. Journal of Reproduction and Development, 0902020130-0902020130.
[13] Lew, B. J., Meidan, R., & Wolfenson, D. (2006). Hormone concentration and follicular development in dairy cows under seasonal and acute hyperthermia. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 58, 816-822.
[14] Santos, J. E. P., Bilby, T. R., Thatcher, W. W., Staples, C. R., & Silvestre, F. T. (2008). Long chain fatty acids of diet as factors influencing reproduction in cattle. Reproduction in Domestic Animals, 43, 23-30.
[15] Da Broi, M. G., Giorgi, V. S. I., Wang, F., Keefe, D. L., Albertini, D., & Navarro, P. A. (2018). Influence of follicular fluid and cumulus cells on oocyte quality: clinical implications. Journal of assisted reproduction and genetics, 35(5), 735-751.
[16] Orsi, N. M., Gopichandran, N., Leese, H. J., Picton, H. M., & Harris, S. E. (2005). Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle. Reproduction, 129(2), 219-228.
[17] Nandi, S., Kumar, V. G., Manjunatha, B. M., Ramesh, H. S., & Gupta, P. S. P. (2008). Follicular fluid concentrations of glucose, lactate and pyruvate in buffalo and sheep, and their effects on cultured oocytes, granulosa and cumulus cells. Theriogenology, 69(2), 186-196.
[18] Lonergan, P., Monaghan, P., Rizos, D., Boland, M. P., & Gordon, I. (1994). Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. Molecular reproduction and development, 37(1), 48-53.
[19] Gérard, N., Loiseau, S., Duchamp, G., & Seguin, F. (2002). Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (^ 1H NMR). REPRODUCTION-CAMBRIDGE-, 124(2), 241-248.
[20] Matoba, S., Bender, K., Fahey, A. G., Mamo, S., Brennan, L., Lonergan, P., & Fair, T. (2014). Predictive value of bovine follicular components as markers of oocyte developmental potential. Reproduction, Fertility and Development, 26(2), 337-345.
[21] vedi tesi espostio
[22] Lee, E. S., & Fukui, Y. (1996). Synergistic effect of alanine and glycine on bovine embryos cultured in a chemically defined medium and amino acid uptake by in vitro-produced bovine morulae and blastocysts. Biology of Reproduction, 55(6), 1383-1389.
[23] Sinclair, K. D., Lunn, L. A., Kwong, W. Y., Wonnacott, K., Linforth, R. S. T., & Craigon, J. (2008). Amino acid and fatty acid composition of

follicular fluid as predictors of in-vitro embryo development. Reproductive biomedicine online, 16(6), 859-868.
[24] Bender, K., Walsh, S., Evans, A. C. O., Fair, T., & Brennan, L. (2010). Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction, 139(6), 1047-1055.
[25] Aardema, H., Vos, P. L., Lolicato, F., Roelen, B. A., Knijn, H. M., Vaandrager, A. B., ... & Gadella, B. M. (2011). Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biology of reproduction, 85(1), 62-69.
[26] Marei, W. F., Wathes, D. C., & Fouladi-Nashta, A. A. (2009). The effect of linolenic acid on bovine oocyte maturation and development. Biology of Reproduction, 81(6), 1064-1072.
[27] Aardema, H., Lolicato, F., van de Lest, C. H., Brouwers, J. F., Vaandrager, A. B., van Tol, H. T., ... & Gadella, B. M. (2013). Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biology of reproduction, 88(6), 164-1.
[28] Hemmings, K. E., Leese, H. J., & Picton, H. M. (2012). Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. Biology of reproduction, 86(5), 165-1.
[29] Zhang, X., Jafari, N., Barnes, R. B., Confino, E., Milad, M., & Kazer, R. R. (2005). Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. Fertility and sterility, 83(4), 1169-1179.
[30] Caixeta, E. S., Ripamonte, P., Franco, M. M., Junior, J. B., & Dode, M. A. N. (2009). Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. Reproduction, Fertility and Development, 21(5), 655-664.
[31] Assidi, M., Dufort, I., Ali, A., Hamel, M., Algriany, O., Dielemann, S., & Sirard, M. A. (2008). Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. Biology of reproduction, 79(2), 209-222.
[32] Kussano, N. R., Leme, L. O., Guimarães, A. L. S., Franco, M. M., & Dode, M. A. N. (2016). Molecular markers for oocyte competence in bovine cumulus cells. Theriogenology, 85(6), 1167-1176.
[33] Di Francesco, S., Novoa, M. V. S., Vecchio, D., Neglia, G., Boccia, L., Campanile, G., ... & Gasparrini, B. (2012). Ovum pick-up and in vitro

embryo production (OPU-IVEP) in Mediterranean Italian buffalo performed in different seasons. Theriogenology, 77(1), 148-154.
[34]. Gasparrini, B., Neglia, G., Di Palo, R., Campanile, G. & Zicarelli, L. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 54, 1537–1542 (2000).
[35]. Lu, K. H., Gordon, I., Gallagher, M. & Mc Govern, H. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. Vet Rec 121, 259–260 (1987).
[36]. Tervit, H. R., Whittingham, D. G. & Rowson, L. E. Succesful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30, 493–497 (1973).
[37] Savorani, F., Tomasi, G., & Engelsen, S. B. (2013). Alignment of 1D NMR data using the ICOSHIFT tool: A tutorial. Magnetic resonance in food science: Food for thought, 343, 14.
[38] Xia, J., Psychogios, N., Young, N., & Wishart, D. S. (2009). MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic acids research, 37(suppl_2), W652-W660.
[39] Leroy, J. L. M. R., Van Soom, A., Opsomer, G., & Bols, P. E. J. (2008). The consequences of metabolic changes in high-yielding dairy cows on oocyte and embryo quality. Animal, 2(8), 1120-1127.
[40] Campanile, G., Avallone, L., d'Angelo, A., Di Palo, R., & Di Meo, C. (1994, June). Influence of the season and of the number of days after calving on the pattern of thyroid hormones in buffalo cows. In Proceedings of the Fourth World Buffalo Congress, São Paulo, Brazil (pp. 564-566).
[41] Britt, J. H. (1992, August). Influence of nutrition and weight loss on reproduction and early embryonic death in cattle. In American Association of Bovine Practitioners Proceedings of the Annual Conference (pp. 143-148).
[42] Jorritsma, R., De Groot, M. W., Vos, P. L. A. M., Kruip, T. A. M., Wensing, T., & Noordhuizen, J. P. T. M. (2003). Acute fasting in heifers as a model for assessing the relationship between plasma and follicular fluid NEFA concentrations. Theriogenology, 60(1), 151-161.
[43] Tabatabaei, S., & Mamoei, M. (2011). Biochemical composition of blood plasma and follicular fluid in relation to follicular size in buffalo. Comparative Clinical Pathology, 20(5), 441-445.
[44] Alkalby, J. M., Bushra, F. H., & Fahad, T. A. (2012). Study on some hormonal and biochemical constituents of follicular fluid and blood plasma in buffaloes. Bas. J. Vet. Res, 11, 90-102.
[45] Aardema, H., van Tol, H. T., Wubbolts, R. W., Brouwers, J. F., Gadella, B. M., & Roelen, B. A. (2017). Stearoyl-CoA desaturase activity

in bovine cumulus cells protects the oocyte against saturated fatty acid stress. Biology of Reproduction, 96(5), 982-992.
[46] Albertini, D. (2004). Oocyte-granulosa cell interactions. In Essential IVF (pp. 43-58). Springer, Boston, MA.
[47] Leroy, J. L. M. R., Vanholder, T., Delanghe, J. R., Opsomer, G., Van Soom, A., Bols, P. E. J., ... & de Kruif, A. (2004). Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. Theriogenology, 62(6), 1131-1143.
[48] Ocon, O. M., & Hansen, P. J. (2003). Disruption of bovine oocytes and preimplantation embryos by urea and acidic pH. Journal of Dairy Science, 86(4), 1194-1200.
[49] Iwata, H., Inoue, J., Kimura, K., Kuge, T., Kuwayama, T., & Monji, Y. (2006). Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. Animal reproduction science, 91(3-4), 215-223.
[50] Sutton-McDowall, M. L., Gilchrist, R. B., & Thompson, J. G. (2004). Cumulus expansion and glucose utilisation by bovine cumulus–oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. Reproduction, 128(3), 313-319.
[51] Leroy, J. L. M. R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., ... & Van Soom, A. (2005). Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction, 130(4), 485-495.
[52] Neff, N. T., Ross, P. A., Bartholomew, J. C., & Bissell, M. J. (1977). Leucine in cultured cells: its metabolism and use as a marker for protein turnover. Experimental cell research, 106(1), 175-183.
[53] Sutton-McDowall, M. L., Gilchrist, R. B., & Thompson, J. G. (2010). The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction, 139(4), 685.
[54] Donahue, R. P., & Stern, S. J. D. J. (1968). Follicular cell support of oocyte maturation: production of pyruvate in vitro. Reproduction, 17(2), 395-398.
[55] Leese, H. J., & Barton, A. M. (1985). Production of pyruvate by isolated mouse cumulus cells. Journal of Experimental Zoology, 234(2), 231-236.
[56] Leese, H. J., & Barton, A. M. (1985). Production of pyruvate by isolated mouse cumulus cells. Journal of Experimental Zoology, 234(2), 231-236.

[57] Downs, S. M., Humpherson, P. G., & Leese, H. J. (2002). Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. Molecular Reproduction and Development: Incorporating Gamete Research, 62(1), 113-123.
[58] Dalton, C. M., Szabadkai, G., & Carroll, J. (2014). Measurement of ATP in single oocytes: impact of maturation and cumulus cells on levels and consumption. Journal of cellular physiology, 229(3), 353-361.
[59] Boland, N. I., Humpherson, P. G., Leese, H. J., & Gosden, R. G. (1993). Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles in vitro. Biology of reproduction, 48(4), 798-806.
[60] Gull, I., Geva, E., Lerner-Geva, L., Lessing, J. B., Wolman, I., & Amit, A. (1999). Anaerobic glycolysis: the metabolism of the preovulatory human oocyte. European Journal of Obstetrics & Gynecology and Reproductive Biology, 85(2), 225-228.
[61] Harris, S. E., Gopichandran, N., Picton, H. M., Leese, H. J., & Orsi, N. M. (2005). Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology, 64(4), 992-1006.
[62] Herrick, J. R., Lane, M., Gardner, D. K., Behboodi, E., Memili, E., Blash, S., ... & Krisher, R. L. (2006). Metabolism, protein content, and in vitro embryonic development of goat cumulus–oocyte complexes matured with physiological concentrations of glucose and L-lactate. Molecular Reproduction and Development: Incorporating Gamete Research, 73(2), 256-266.
[63] Lane, M., & Gardner, D. K. (2005). Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reproduction, Fertility and Development, 17(3), 371-378.
[64] Wallace, M., Cottell, E., Gibney, M. J., McAuliffe, F. M., Wingfield, M., & Brennan, L. (2012). An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. Fertility and sterility, 97(5), 1078-1084.
[65] Rice, S., Christoforidis, N., Gadd, C., Nikolaou, D., Seyani, L., Donaldson, A., ... & Franks, S. (2005). Impaired insulin-dependent glucose metabolism in granulosa-lutein cells from anovulatory women with polycystic ovaries. Human Reproduction, 20(2), 373-381.
[66] Sutton-McDowall, M. L., Gilchrist, R. B., & Thompson, J. G. (2010). The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction, 139(4), 685.

[67] Dumollard, R., Duchen, M., & Carroll, J. (2007). The role of mitochondrial function in the oocyte and embryo. Current topics in developmental biology, 77, 21-49.
[68] Dumollard, R., Carroll, J., Duchen, M. R., Campbell, K., & Swann, K. (2009, May). Mitochondrial function and redox state in mammalian embryos. In Seminars in cell & developmental biology (Vol. 20, No. 3, pp. 346-353). Academic Press.
[69] Rieger D, Loskutoff NM (1994) Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. J Reprod Fertil 100(1):257–262
[70] Saito T, Hiroi M, Kato T (1994) Development of glucose utilization studied in single oocytes and preimplantation embryos from mice. Biol Reprod 50(2):266–270
[71] Wang Q, Chi MM, Schedl T, Moley KH (2012) An intercellular pathway for glucose transport into mouse oocytes. Am J Phys Endocrinol Metab 302(12):E1511–E1518. doi:10.1152/ajpendo.00016.2012
[72] Leese, H. J., Sturmey, R. G., Baumann, C. G., & McEvoy, T. G. (2007). Embryo viability and metabolism: obeying the quiet rules. Human Reproduction, 22(12), 3047-3050.
[73] Leese, H. J. (2002). Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. Bioessays, 24(9), 845-849.
[74] Sturmey, R. G., Bermejo-Alvarez, P., Gutierrez-Adan, A., Rizos, D., Leese, H. J., & Lonergan, P. (2010). Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Molecular Reproduction and Development: Incorporating Gamete Research, 77(3), 285-296.
[75] Baumann, C. G., Morris, D. G., Sreenan, J. M., & Leese, H. J. (2007). The quiet embryo hypothesis: molecular characteristics favoring viability. Molecular reproduction and development, 74(10), 1345-1353.
[76] Calarco, P. G. (1995). Polarization of mitochondria in the unfertilized mouse oocyte. Developmental genetics, 16(1), 36-43.
[77] Quinn, P., & Wales, R. G. (1973). The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. Reproduction, 35(2), 301-309.
[78] Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P. B., & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biology of reproduction, 64(3), 904-909.

[79] Van Blerkom, J., Davis, P. W., & Lee, J. (1995). Fertilization and early embryolgoy: ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Human reproduction, 10(2), 415-424.
[80] Zhao, J., & Li, Y. (2012). Adenosine triphosphate content in human unfertilized oocytes, undivided zygotes and embryos unsuitable for transfer or cryopreservation. Journal of International Medical Research, 40(2), 734-739.
[81] Brevini, T. A., Vassena, R., Francisci, C., & Gandolfi, F. (2005). Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. Biology of reproduction, 72(5), 1218-1223.
[82] Brad, A. M., Bormann, C. L., Swain, J. E., Durkin, R. E., Johnson, A. E., Clifford, A. L., & Krisher, R. L. (2003). Glutathione and adenosine triphosphate content of in vivo and in vitro matured porcine oocytes. Molecular Reproduction and Development: Incorporating Gamete Research, 64(4), 492-498.
[83] Sturmey, R. G., Brison, D. R., & Leese, H. J. (2008). Assessing embryo viability by measurement of amino acid turnover. Reproductive biomedicine online, 17(4), 486-496.
[84] Lafleur, M. V. M., Hoorweg, J. J., Joenje, H., Westmijze, E. J., & Retèl, J. (1994). The ambivalent role of glutathione in the protection of DNA against singlet oxygen. Free radical research, 21(1), 9-17.
[85] Lim, J. M., Liou, S. S., & Hansel, W. (1996). Intracytoplasmic glutathione concentration and the role of β-mercaptoethanol in preimplantation development of bovine embryos. Theriogenology, 46(3), 429-439.
[86] Hansen, J. M., & Harris, C. (2015). Glutathione during embryonic development. Biochimica et Biophysica Acta (BBA)-General Subjects, 1850(8), 1527-1542.
[87] Ali, I., Liu, H. X., Zhong-Shu, L., Dong-Xue, M., Xu, L., Shah, S. Z. A., ... & Nan-Zhu, F. (2017). Reduced glutathione alleviates tunicamycin-induced endoplasmic reticulum stress in mouse preimplantation embryos. Journal of Reproduction and Development.
[88] Perreault, S. D., Barbee, R. R., & Slott, V. L. (1988). Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Developmental biology, 125(1), 181-186.

[89] Sutovsky, P., & Schatten, G. (1997). Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biology of reproduction, 56(6), 1503-1512.
[90] Ozawa, M., Nagai, T., Somfai, T., Nakai, M., Maedomari, N., Miyazaki, H., ... & Kikuchi, K. (2010). Cumulus cell-enclosed oocytes acquire a capacity to synthesize GSH by FSH stimulation during in vitro maturation in pigs. Journal of Cellular Physiology, 222(2), 294-301.
[91] Rodríguez-González, E., López-Bejar, M., Mertens, M. J., & Paramio, M. T. (2003). Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. Molecular Reproduction and Development: Incorporating Gamete Research, 65(4), 446-453.
[92] De Matos, D. G., Furnus, C. C., Moses, D. F., Martinez, A. G., & Matkovic, M. (1996). Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. Molecular Reproduction and Development: Incorporating Gamete Research, 45(4), 451-457.
[93] Jiao, G. Z., Cao, X. Y., Cui, W., Lian, H. Y., Miao, Y. L., Wu, X. F., ... & Tan, J. H. (2013). Developmental potential of prepubertal mouse oocytes is compromised due mainly to their impaired synthesis of glutathione. PloS one, 8(3), e58018.
[94] Gasparrini, B., Boccia, L., Marchandise, J., Di Palo, R., George, F., Donnay, I., & Zicarelli, L. (2006). Enrichment of in vitro maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenology, 65(2), 275-287.
[95] Gasparrini, B., Sayoud, H., Neglia, G., de Matos, D. G., Donnay, I., & Zicarelli, L. (2003). Glutathione synthesis during in vitro maturation of buffalo (Bubalus bubalis) oocytes: effects of cysteamine on embryo development. Theriogenology, 60(5), 943-952.
[96] Perreault, S. D., Barbee, R. R., & Slott, V. L. (1988). Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Developmental biology, 125(1), 181-186.
[97] Telford, N. A., Watson, A. J., & Schultz, G. A. (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Molecular reproduction and development, 26(1), 90-100.

[98] Furnus, C. C., & De Matos, D. G. (1999). The availability of cysteine in culture medium appears to be the limiting factor for glutathione synthesis in mammalian oocytes. Theriogenology, 51(1), 373.
[99] de Matos, D. G., Furnus, C. C., & Moses, D. F. (1997). Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. Biology of reproduction, 57(6), 1420-1425.
[100] Uhde, K., van Tol, H. T., Stout, T. A., & Roelen, B. A. (2018). Metabolomic profiles of bovine cumulus cells and cumulus-oocyte-complex-conditioned medium during maturation in vitro. Scientific reports, 8(1), 1-14.
[101] Maddocks, O. D., Berkers, C. R., Mason, S. M., Zheng, L., Blyth, K., Gottlieb, E., & Vousden, K. H. (2013). Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. Nature, 493(7433), 542-546.
[102] Eppig, J. J. (1996). Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reproduction, Fertility and Development, 8(4), 485-489.
[103] Jeong, W. J., Cho, S. J., Lee, H. S., Deb, G. K., Lee, Y. S., Kwon, T. H., & Kong, I. K. (2009). Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. Theriogenology, 72(4), 584-589.
[104] Zicarelli, L., Donnay, I., De Rosa, A., Boccia, L., Monaco, E., Attanasio, L., & Gasparrini, B. (2005). Use of thiol compounds during in vitro maturation of buffalo oocytes: effects on embryo development. Italian Journal of Animal Science, 4(sup2), 304-306.
[105] Gasparrini, B., Boccia, L., Marchandise, J., Di Palo, R., George, F., Donnay, I., & Zicarelli, L. (2006). Enrichment of in vitro maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenology, 65(2), 275-287.
[106] Deneke, S. M., & Fanburg, B. L. (1989). Regulation of cellular glutathione. American Journal of Physiology-Lung Cellular and Molecular Physiology, 257(4), L163-L173.
[107] Meister, A. (1985). Methods for the selective modification of glutathione metabolism and study of glutathione transport. Methods in enzymology, 113, 571-585.
[108] Curnow, E. C., Ryan, J. P., Saunders, D. M., & Hayes, E. S. (2010). Developmental potential of bovine oocytes following IVM in the presence

of glutathione ethyl ester. Reproduction, Fertility and Development, 22(4), 597-605.
[109] Gardiner, C. S., & Reed, D. J. (1994). Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. Biology of reproduction, 51(6), 1307-1314.
[110] Lee, C. S., Koo, D. B., Fang, N., Lee, Y., Shin, S. T., Park, C. S., & Lee, K. K. (2000). Potent and stage-specific action of glutathione on the development of goat early embryos in vitro. Molecular Reproduction and Development: Incorporating Gamete Research, 57(1), 48-54.
[111] Sinclair, K. D., Lunn, L. A., Kwong, W. Y., Wonnacott, K., Linforth, R. S. T., & Craigon, J. (2008). Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. Reproductive biomedicine online, 16(6), 859-868.
[112] Matoba, S., Bender, K., Fahey, A. G., Mamo, S., Brennan, L., Lonergan, P., & Fair, T. (2014). Predictive value of bovine follicular components as markers of oocyte developmental potential. Reproduction, Fertility and Development, 26(2), 337-345.
[113] Hemmings, K. E., Leese, H. J., & Picton, H. M. (2012). Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. Biology of reproduction, 86(5), 165-1.
[114] Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod 2002; 17:999–1005.
[115] Gunter, C., Frank, M., Tian, Y., Murti, K. G., Rehg, J. E., & Jackowski, S. (2007). Probucol therapy overcomes the reproductive defect in CTP: phosphocholine cytidylyltransferase β2 knockout mice. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1771(7), 845-852.
[116] Prates, E. G., Nunes, J. T., & Pereira, R. M. (2014). A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. Mediators of inflammation, 2014.
[117] Welte, M. A., & Gould, A. P. (2017). Lipid droplet functions beyond energy storage. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1862(10), 1260-1272.
[118] Nuttinck, F., Guienne, B. M. L., Clément, L., Reinaud, P., Charpigny, G., & Grimard, B. (2008). Expression of genes involved in prostaglandin E2 and progesterone production in bovine cumulus-oocyte

complexes during in vitro maturation and fertilization. Reproduction, 135(5), 593-604.
[119] Shehadeh, A., Bruck-Haimson, R., Saidemberg, D., Zacharia, A., Herzberg, S., Ben-Meir, A., & Moussaieff, A. (2019). A shift in follicular fluid from triacylglycerols to membrane lipids is associated with positive pregnancy outcome. The FASEB Journal, 33(9), 10291-10299.
[120] Gonzalez, M. B., Lane, M., Knight, E. J., & Robker, R. L. (2018). Inflammatory markers in human follicular fluid correlate with lipid levels and Body Mass Index. Journal of reproductive immunology, 130, 25-29.
[121] Genicot, G., Leroy, J. L. M. R., Van Soom, A., & Donnay, I. (2005). The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology, 63(4), 1181-1194.
[122] Ambruosi, B., Lacalandra, G. M., Iorga, A. I., De Santis, T., Mugnier, S., Matarrese, R., ... & Dell'Aquila, M. E. (2009). Cytoplasmic lipid droplets and mitochondrial distribution in equine oocytes: implications on oocyte maturation, fertilization and developmental competence after ICSI. Theriogenology, 71(7), 1093-1104.
[123] Boni, R., Santella, L., Dale, B., Di Palo, R., & Barbieri, V. (1992). Maturazione in vitro di oociti bufalini: indagine ultrastrutturale.
[124] Mondadori, R. G., Santin, T. R., Fidelis, A. A. G., Porfírio, E. P., & Báo, S. N. (2010). Buffalo (Bubalus bubalis) pre-antral follicle population and ultrastructural characterization of antral follicle oocyte. Reproduction in domestic animals, 45(1), 33-37.
[125] Mondadori, R. G., Santin, T. R., Fidelis, A. A. G., da Silva, J. S., Rumpf, R., & Báo, S. N. (2010). Ultrastructure of in vitro oocyte maturation in buffalo (Bubalus bubalis). Zygote, 18(4), 309-314.
[126] Marin, D. F. D., da Costa, N. N., Santana, P. D. P. B., de Souza, E. B., & Ohashi, O. M. (2019). Importance of lipid metabolism on oocyte maturation and early embryo development: Can we apply what we know to buffalo?. Animal reproduction science, 211, 106220.
[127] Zhuang, X. J., Huang, Y., Duan, Y. P., Zhang, M., Lu, Y. Q., & Lu, K. H. (2012). Translocation of active mitochondria during buffalo (Bubalus bubalis) oocytes in vitro maturation, fertilization and preimplantation embryo development. Reproduction in domestic animals, 47(3), 443-448.
[128] Nagano, M., Katagiri, S., & Takahashi, Y. (2006). Relationship between bovine oocyte morphology and in vitro developmental potential. Zygote, 14(1), 53-61.

[129] Kruip, T. A., Cran, D. G., Van Beneden, T. H., & Dieleman, S. J. (1983). Structural changes in bovine oocytes during final maturation in vivo. Gamete Research, 8(1), 29-47.
[130] Dunning-Davies, B. M., Fry, C. H., Mansour, D., & Ferguson, D. R. (2012). The regulation of ATP release from the urothelium by adenosine and transepithelial potential. BJU international, 111(3), 505-513.
[131] Ferguson, E. M., & Leese, H. J. (2006). A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Molecular reproduction and development, 73(9), 1195-1201.
[132] McEvoy, T. G., Coull, G. D., Broadbent, P. J., Hutchinson, J. S., & Speake, B. K. (2000). Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. Journal of reproduction and fertility, 118(1), 163-170.
[133] Sturmey, R. G., O'toole, P. J., & Leese, H. J. (2006). Fluorescence resonance energy transfer analysis of mitochondrial: lipid association in the porcine oocyte. Reproduction, 132(6), 829-837.
[134] Assis Neto, A. C., Pereira, F. T. V., Santos, T. C., Ambrosio, C. E., Leiser, R., & Miglino, M. A. (2010). Morpho-physical recording of bovine conceptus (Bos indicus) and placenta from days 20 to 70 of pregnancy. Reproduction in domestic animals, 45(5), 760-772.
[135] Strazzullo, M., Gasparrini, B., Neglia, G., Balestrieri, M. L., Francioso, R., Rossetti, C., ... & Campanile, G. (2014). Global transcriptome profiles of Italian Mediterranean buffalo embryos with normal and retarded growth. Plos one, 9(2), e90027.
[136] Seidel Jr, G. E. (2006). Modifying oocytes and embryos to improve their cryopreservation. Theriogenology, 65(1), 228-235.
[137] Kim, J. Y., Kinoshita, M., Ohnishi, M., & Fukui, Y. (2001). Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction, 122(1), 131-138.
[138] Aardema, H., Vos, P. L., Lolicato, F., Roelen, B. A., Knijn, H. M., Vaandrager, A. B., ... & Gadella, B. M. (2011). Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biology of reproduction, 85(1), 62-69.
[139]
[140] Da Broi, M. G., Giorgi, V. S. I., Wang, F., Keefe, D. L., Albertini, D., & Navarro, P. A. (2018). Influence of follicular fluid and cumulus cells on oocyte quality: clinical implications. Journal of assisted reproduction and genetics, 35(5), 735-751.

[141] Auclair, S., Uzbekov, R., Elis, S., Sanchez, L., Kireev, I., Lardic, L., ... & Uzbekova, S. (2013). Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. American Journal of Physiology-Endocrinology and Metabolism, 304(6), E599-E613.
[142] Yesilaltay, A., Dokshin, G. A., Busso, D., Wang, L., Galiani, D., Chavarria, T., ... & Krieger, M. (2014). Excess cholesterol induces mouse egg activation and may cause female infertility. Proceedings of the National Academy of Sciences, 111(46), E4972-E4980.
[143] Buschiazzo, J., Ialy-Radio, C., Auer, J., Wolf, J. P., Serres, C., Lefèvre, B., & Ziyyat, A. (2013). Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization. PloS one, 8(4), e62919.
[144] Hu, Z., Shen, W. J., Cortez, Y., Tang, X., Liu, L. F., Kraemer, F. B., & Azhar, S. (2013). Hormonal regulation of microRNA expression in steroid producing cells of the ovary, testis and adrenal gland. PloS one, 8(10), e78040.
[145] Azhar, S., Tsai, L., Medicherla, S., Chandrasekher, Y., Giudice, L., & Reaven, E. (1998). Human granulosa cells use high density lipoprotein cholesterol for steroidogenesis. The Journal of Clinical Endocrinology & Metabolism, 83(3), 983-991.
[146] Yamashita, Y., Shimada, M., Okazaki, T., Maeda, T., & Terada, T. (2003). Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. Biology of Reproduction, 68(4), 1193-1198.
[147] Su, Y. Q., Sugiura, K., Wigglesworth, K., O'Brien, M. J., Affourtit, J. P., Pangas, S. A., ... & Eppig, J. J. (2008). Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells.
[148] Auclair, S., Uzbekov, R., Elis, S., Sanchez, L., Kireev, I., Lardic, L., ... & Uzbekova, S. (2013). Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. American Journal of Physiology-Endocrinology and Metabolism, 304(6), E599-E613.
[149] Aardema, H., Vos, P. L., Lolicato, F., Roelen, B. A., Knijn, H. M., Vaandrager, A. B., ... & Gadella, B. M. (2011). Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. Biology of reproduction, 85(1), 62-69.
[150] Carro, M., Buschiazzo, J., Rios, G. L., Oresti, G. M., & Alberio, R. H. (2013). Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes. Theriogenology, 79(4), 687-694.

[151] Jaspard, B., Fournier, N., Vieitez, G., Atger, V., Barbaras, R., Vieu, C., ... & Collet, X. (1997). Structural and functional comparison of HDL from homologous human plasma and follicular fluid: a model for extravascular fluid. Arteriosclerosis, thrombosis, and vascular biology, 17(8), 1605-1613.
[152] von Otte, S., Paletta, J. R., Becker, S., König, S., Fobker, M., Greb, R. R., ... & Nofer, J. R. (2006). Follicular fluid high density lipoprotein-associated sphingosine 1-phosphate is a novel mediator of ovarian angiogenesis. Journal of Biological Chemistry, 281(9), 5398-5405.
[153] Aardema, H., Lolicato, F., van de Lest, C. H., Brouwers, J. F., Vaandrager, A. B., van Tol, H. T., ... & Gadella, B. M. (2013). Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biology of reproduction, 88(6), 164-1.
[154] Bender, K., Walsh, S., Evans, A. C. O., Fair, T., & Brennan, L. (2010). Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction, 139(6), 1047-1055.
[155] Marei, W. F., Wathes, D. C., & Fouladi-Nashta, A. A. (2010). Impact of linoleic acid on bovine oocyte maturation and embryo development. Reproduction, 139(6), 979.
[156] Homa, S. T., & Brown, C. A. (1992). Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes. Reproduction, 94(1), 153-160.
[157] Zhang, N., Wang, L., Luo, G., Tang, X., Ma, L., Zheng, Y., ... & Jiang, Z. (2019). Arachidonic acid regulation of intracellular signaling pathways and target gene expression in bovine ovarian granulosa cells. Animals, 9(6), 374.
[158] Pompeia, C., Lima, T., & Curi, R. (2003). Arachidonic acid cytotoxicity: can arachidonic acid be a physiological mediator of cell death?. Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease, 21(2), 97-104.
[159] Ciepiela, P., Bączkowski, T., Drozd, A., Kazienko, A., Stachowska, E., & Kurzawa, R. (2015). Arachidonic and linoleic acid derivatives impact oocyte ICSI fertilization–a prospective analysis of follicular fluid and a matched oocyte in a ‘one follicle–one retrieved oocyte–one resulting embryo’investigational setting. PLoS One, 10(3), e0119087.
[160] Marandykina, A., Palacios-Prado, N., Rimkutė, L., Skeberdis, V. A., & Bukauskas, F. F. (2013). Regulation of connexin36 gap junction

channels by n-alkanols and arachidonic acid. The Journal of physiology, 591(8), 2087-2101.
[161] Khan, R., Jiang, X., Hameed, U., & Shi, Q. (2021). Role of Lipid Metabolism and Signaling in Mammalian Oocyte Maturation, Quality, and Acquisition of Competence. Frontiers in cell and developmental biology, 9, 384.

This thesis demonstrated that the reduced oocyte competence recorded during the NBS in buffalo is associated to several alterations of miRNA profiling and gene expression at the different levels of the follicle, reflected in metabolic variations.

The results of Experiment 1 showed a seasonal variation in both oocytes and follicular cells in the expression of miRNA, known to be involved in follicular maturation and development regulation. The most intriguing differentially expressed (DE) miRNA in oocytes are miR-143, miR-25, miR-222 and miR-199a, involved in steroidogenesis and regulation of the follicular growth in other species. Some of the DE expressed miRNAs identified between the NBS and BS in buffalo FCs (miR-184, miR-2411 and miR-34c) also show cyclic variations in cattle. In addition, some of the DE-miRNAs identified in this study, such as mir-141, miR-199a, miR-184 and miR-486-5p, have been reported to show variations in several human ovarian disorders.The target gene analysis revealed that DE-miRNAs identified in oocytes collected from the NBS and BS regulate genes involved in triglyceride and sterol biosynthesis, essential for lipid metabolism, providing a potent energy source during oocyte maturation. In FCs, the DE-miRNA target genes were related to pathways involved in transformation of growth factor β (TGFβ) and circadian clock photoperiod. Between the two seasons, only 2 differentially expressed genes (DEGs) were found in FCs whereas 22 DEGs were observed in the oocytes, most of which are known to be related to competence, such as secreted phosphoprotein 1 (SPP1), RUNX family transcription factor 2 (RUNX2), Cathepsin K (CTSK), heat shock protein family A (Hsp70) and Insulin like growth factor 2 (IGF2). Other interesting DEGs influenced by season were interleukin-1 beta (IL-1β) and Apolipoprotein E (APOE) that regulate hormone synthesis and metabolism, as well as the folate receptor beta (FOLR2), involved in folate transport in oocytes during follicular development.

The Experiment 2 demonstrated that specific miRNAs contained in EVs isolated from FF are involved in regulating follicular development and modulating seasonal effects on oocyte competence. In particular, a differential expression of miRNAs was observed in relation to follicular growth, with 413 DE-miRNAs identified between antral and pre-ovulatory follicles. Among these, the most significant were found to target genes involved in lipid and steroid metabolism, response to glucocorticoid and estradiol stimulus. Interestingly, differences in miRNAs cargo of EVS were also shown between seasons, with 14 and 12 DE-miRNAs in antral

and pre-ovulatory follicles, respectively. The GO analysis showed that season affects the expression of miRNAs in antral follicles that target genes related to interleukin-6 (IL-6) production and secretion. This is an interesting finding due to the known role of cytokines in regulation of physiological reproductive events, like follicular development and ovulation. Furthermore, IL-6, considered both pro-inflammatory and anti-inflammatory agent, plays a pivotal role in modulating ovarian function, by regulating steroidogenesis, angiogenesis, as well as granulosa cell function and oocyte competence in other species. Therefore, we speculate that the altered IL-6 secretion may be one of the factors interfering with the acquisition of oocyte competence during the NBS in buffalo. With regard to pre-ovulatory follicles the GO analysis revealed that the seasonal variations are referred to miRNAS targeting genes involved in regulating focal adhesion assembly and cell-substrate junction organization, fundamental pathways for the appropriate bidirectional communication between the oocyte and somatic cells, necessary for correct oocyte growth and maturation. The results of Experiment 3 demonstrated substantial differences in metabolites content in all the follicular components between seasons in buffalo, suggesting that the reduced oocyte competence during the NBS is due to alterations of several metabolic pathways, such as glutathione metabolism, energy generating metabolism, phospholipid biosynthesis, amino acid metabolism. The lipid composition of the follicular environment also varied significantly in relation to season. The findings suggest that during NBS metabolic changes take place, likely related to a condition of NEB, influencing the follicular environment and are in part reflected at the somatic cells and the oocyte in the attempt to counteract suboptimal conditions. This experiment also permitted to identify potential markers of oocyte competence in the FF in buffalo. Markers that were positively associated to competence (BS) are glutathione, glutamate, lactate and cholines, whereas a negative association with competence was found for leucine, isoleucine and β-hydroxybutyrate. Taken together, these results pay the way to develop corrective strategies to modulate the follicular environment or, more easily the IVM medium to improve the competence of oocytes retrieved during the NBS. However, the amount of data produced confirm the complexity of the phenomenon of seasonality and undoubtedly suggest to carry out further studies to better interpret some of the changes observed. In particular, the absolute quantification of targeted metabolites, as well as the identification of fatty acids showing greatest variations and the characterization of oxidative

status would help to interpret the oocyte response and consequently find a way to manipulate metabolic profile and improve competence during unfavorable season.

In perspective further studies are also needed to evaluate whether the addition to the IVM system of specific miRNas up-regulated during the BS and/or EVs collected during the BS would improve the competence of oocytes recovered during the NBS.