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Physiology of Maturation of the Cumulus-Oocyte Complex in the Horse

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Physiology of Maturation of the Cumulus-Oocyte Complex in the Horse.

By

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DISSERTATION

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DISSERTATION ABSTRACT

Assisted reproductive technologies (ART) like *in vitro* production (IVP) of embryos in the horse is an important tool in the equine industry. In particular, the aspiration of antral follicles and the collection of immature oocytes is the most common and widely practiced procedure to obtain female gametes for IVP. However, *in vitro* maturation (IVM) of these immature oocytes remains a significant limitation for efficient IVP. This is mainly due to suboptimal maturation media formulations and overall suboptimal maturation culture conditions. Maturation of the oocyte is a critical and delicate phase where the oocyte acquires its capacity to be fertilized by one spermatozoon and then undergoes embryogenesis. In Chapter 1, a review of the pertinent literature regarding oocyte maturation and associated processes is presented.

In the studies presented in Chapter 2 of this dissertation, it is described for the first time, that the gene expression profile of oocytes and surrounding cumulus cells (CC) and the associated changes that occur during maturation. Oocytes expressed 13,918 genes, with 68.46% being protein-coding genes. 749 differentially expressed genes (DEG) were observed between the immature (GV) and Mature (MII) groups. Within these DEG, 330 presented a higher expression in the OC-MII group and were associated with pathways of the cell cycle and chromosome segregation. 419 genes showed a lower expression in the OC-MII group and were associated with ribonucleoprotein complex biogenesis and nucleolus. In CC samples, it was established 927 DEG with 609 upregulated and 318 downregulated genes. It was determined that many pathways related to GTPase activity were part of the upregulated genes. Downregulated genes were mainly related to the extracellular matrix organization, collagen trimer, and collagen fibril. A weighted gene co-

expression network analysis (WGCNA) of maturation revealed that important pathways are associated with nuclear maturation and the meiotic cycle. Moreover, the expression of the follicle-stimulating hormone receptor (*FSHR*) increased while the luteinizing hormone receptor (*LHCGR*) decreased during maturation.

The studies presented in Chapter 3 of this dissertation were designed to compare IVM to *in vivo* maturation (IVV) by evaluating the gene expression profile of the CC. There were over six thousand DEG between the two groups with 2180 genes upregulated and 4481 downregulated in the IVM group. Genes associated with extracellular matrix and cumulus expansion were downregulated in the IVM group. Also, the expression of *FSHR* was higher, while *LHCGR* expression was lower in the IVM compared with the IVV group. Additionally, cumulus expansion during IVM exhibited an increasing linear rate of expansion. The results from Chapters 2 and 3 combined suggested that gonadotropins (FSH and LH) might have an important role during oocyte maturation in the horse, thus a re-evaluation of the maturation media formulations, like the concentration of FSH used in the formulation, could result in the improvement of maturation outcomes. Additionally, the reduced activation of genes and pathways associated with cumulus expansion is another important factor that should be addressed and investigated in future studies.

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INTRODUCTION TO THE DISSERTATION

Assisted reproductive technologies (ART) have become an important tool in the last few years for the reproductive success of horses and represent an early acceptance of new technology by the horse breeding industry worldwide. This means that techniques like retrieval of immature oocytes, *in vitro* maturation (IVM), and intracytoplasmic sperm injection (ICSI) still have room for improvement. The objective of the studies presented in this dissertation was to expand the current knowledge regarding equine oocyte maturation and to determine key genes and physiological mechanisms associated with maturation, as well as to create new knowledge for the optimization of the maturation culture conditions. In Chapter 1, the presented literature review describes the current information about the physiology of maturation of oocytes in the horse, highlighting what is known in the horse and other species as well as what knowledge remains missing.

Chapter 2 will describe the experiments performed and the respective findings related to the dynamics in transcriptomics of oocytes and CC during IVM. This includes the main genes that are differentially expressed between immature and matured samples as well as those biological pathways relevant to maturation physiology. Additionally, the concentration of AMH and inhibin A in follicular fluid from aspirated follicles of various diameters was investigated.

Finally, Chapter 3 of this dissertation will describe the experiments comparing the gene expression profiles in CC between *in vitro* matured (IVM) and *in vivo* matured (IVV) cumulus-oocyte complexes (COC) and the changes in cumulus expansion of *in vitro* matured COCs.

The Conclusion Chapter will summarize the research findings included in this dissertation and emphasize that this work is critical and furthers the field regarding the 1) lack of knowledge of physiological changes occurring in the oocyte and in the cumulus, but also as a dynamic and interconnected complex, and 2) the main differences observed on IVM oocytes compared to those matured *in vivo*. Note that figures are presented in continuous numerical order, starting with the chapter number and then figure number (e.g. Figure 2.1, which corresponds to figure 1 of chapter 2.

CHAPTER 1

Literature Review

INTRODUCTION

Assisted reproductive technologies (ART) have become an important tool for the reproductive success of many species, including horses. Intracytoplasmic sperm injection (ICSI) is an example of a valuable reproductive technology that is useful for mares that cannot conceive by other means but also for mares in athletic or show performance that compete all year round and are not accessible for breeding opportunities. Moreover, ICSI is ideal to optimize the use of semen from stallions with reduced availability and those with poor fertilization capacity during traditional reproductive methods. During a normal estrous cycle, most mares usually develop only a single dominant follicle (most of the time) that completes its development and ovulates an *in vivo* matured (IVV) oocyte. Induced maturation of these oocytes has been a particularly useful method to obtain mature oocytes with great developmental potential, by hormonally stimulating the mare using systemic gonadotropins when there is at least one dominant follicle (33 mm or bigger) present in at least one of the ovaries. However, only one to two of these dominant follicles typically responds to maturation induction[1]. Aspiration of induced matured oocytes carries the constraint of a time-sensitive follicle aspiration 36 h after induction along with the recovery of only one oocyte on each induction. Under this scenario, an alternative has been established where multiple immature oocytes (germinal vesicle stage - GV) are aspirated from small-to-medium sized antral follicles that are present in the ovaries during each aspiration procedure. These immature oocytes are then matured using *in vitro* culture methods (IVM). This technique increases the number of potentially

fertilizable eggs due to the numerous antral follicles present in both ovaries, which in turn, increases the chance of obtaining an embryo after each aspiration procedure. Nevertheless, IVM is less efficient than IVV as only one-half of the collected oocytes will mature, having a lower ability to produce pregnancy compared to oocytes matured *in vivo* [2]. Hence, understanding the underlying mechanisms occurring during equine oocyte maturation could help understand the relatively low developmental competence observed in IVM oocytes. Moreover, this information will lead to innovative enhancements of IVM culture protocols, which will help improve the number of fertilizable oocytes that may become embryos. The goal of this review is to highlight existing knowledge about IVM of equine oocytes that will serve as a background for the following studies described in this dissertation.

TRANSVAGINAL OOCYTE ASPIRATION AND INTRACYTOPLASMIC SPERM INJECTION (ICSI), THE SURGING ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) IN HORSES

For *in vitro* embryo production (IVP) in the horse, intracytoplasmic sperm injection (ICSI) is the most commonly used and efficient method since *in vitro* fertilization has not been successful in horses [2]. This technique allows us to obtain foals from older mares with low success from more traditional assisted reproductive techniques such as artificial insemination (AI) and embryo transfer (ET). Early attempts to adapt the ICSI technique focused on collecting oocytes post-mortem from mares euthanized or from abattoirs. The ovaries can be dissected, and follicle walls scrapped while flushing the contents. However, the collection of oocytes in the case of an unexpected death could signify a

delay between the time of ovariectomy and the time when the retrieved oocyte is placed in an appropriate culture medium [3]. Later, investigators utilized a flank laparotomy approach in live standing-sedated mares in order to reach ovaries and collect the oocytes. This technique has been replaced by ultrasound-guided transvaginal aspiration (TVA) also referred as *ovum* pickup (OPU). TVA is performed in sedated standing mares and is currently the most common practice to collect oocytes from donor mares. Because of the close and broad-based attachment of the equine oocyte to the follicle wall [4], scraping the follicle is the most effective method for oocyte recovery [5], but also makes the TVA a more challenging procedure compared to methods used to retrieve oocytes in other species. Two types of oocytes can be obtained during a TVA procedure depending what type of follicle is aspirated. One of them correspond to secondary oocytes obtained from preovulatory follicles. These IVV oocytes can be obtained from a dominant pre-ovulatory follicle (>33 mm diameter) after the mare has been induced to ovulate by administering gonadotropins, resulting in the retrieval of one *in vivo* matured COC per procedure [1]. The other type corresponds to immature (GV) primary oocytes that are aspirated from antral follicles (5-30 mm). Immature oocytes need to be matured *in vitro* to reach the MII stage and become fertilizable by ICSI. IVV oocytes present better maturation rates and superior development competence (ability to become a viable blastocyst) [1]. However, these oocytes require strict monitoring of follicle dynamics and growth and precise timing for induction, considering that oocytes need to be fertilized timely after aspiration. Additionally, the main disadvantage of this method is the yield of only one IVV oocyte. Instead, aspiration of small, or non-dominant antral follicles presents several advantages: I) allows for the retrieval of a bigger pool of immature oocytes, II) aspiration can be

performed with smaller time constraints, III) monitoring of ovarian activity is not critical although recommended in order to aspirate when an adequate number of follicles are present (8 – 10) [6]. More importantly, IV) this can be performed year-round for many mares, not having significant adverse effects over maturation and blastocyst rates during the non-breeding season [7]. Regardless of all the benefits mentioned, culture of oocytes for IVM has not been optimized and remains an important constraint and main bottleneck during IVP in the horse. Thus, the studies presented in this dissertation aim to contribute more knowledge about the physiology of maturation of the horse oocyte.

FOLLICULOGENESIS, OOGENESIS, AND MATURATION

In most mammalian species, meiosis is initiated during fetal or early postnatal life. During the gestation period in horses, by days 70-80, oogonia populate the fetal ovaries followed by a massive germ-cell degeneration event between fetal days 100-120 [8]. This degeneration progressively declines up to day 150, which coincides with the appearance of a finite population of primordial follicles (oocytes surrounded by a single, squamous layer of somatic cells) that will form the ovarian reserve and by days 180-200, primary oocytes are arrested in the diplotene stage of meiosis I (MI) [8]. Activation of primordial follicles is accomplished by a change in granulosa cell morphology from flat to cuboidal, and the oocyte begins to grow in diameter. These follicles are now called primary follicles, initiating follicular development, and thus determining the continuity of folliculogenesis through the reproductive lifespan of mammals [9], [10]. During its entire development, the oocyte regulates its own microenvironment within the follicle by communicating with the surrounding somatic cells of the follicle, which allows the oocyte to have partial control of

its own development [11]. Throughout folliculogenesis, communication between the oocyte and surrounding somatic cells happens through paracrine signals and gap junctions [12] although this is poorly understood in the horse. This bidirectional continuous signaling synchronizes follicle development with oocyte growth and eventually its maturation as well [13] and is a key process in developing the somatic-cell/germinal-cell niche. Oocyte-derived signals regulate the development and function of surrounding granulosa cells, and appropriately developed granulosa cells produce their own paracrine factors to coordinate the growth and development of a fertilizable oocyte with full competence to undergo embryo development during and beyond the critical preimplantation period [9], [14]. Hence, the function and development of COCs depend on delicate coordination by the oocyte-cumulus/granulosa cell regulatory loop [15] that is continuously changing throughout the development of the follicle. For almost the entire duration of folliculogenesis, the oocyte remains arrested in meiosis I, and is therefore classified as a primary oocyte [16].

Oocyte maturation is the final process where the oocyte transitions between the primary and secondary stages of development and acquire the competence to be fertilized and undergo subsequent embryogenesis [17]. This process can be divided into two parts, nuclear maturation, and cytoplasmic maturation referring to specific changes occurring either in the nucleus or ooplasm, respectively. During nuclear maturation, the oocyte resumes meiosis starting from the arrested dictyate stage of meiotic prophase I which is characterized by the presence of an intact GV which corresponds to the chromatin and nucleolus delimited by the nuclear envelope. The progression of meiosis results in the GV breakdown (GVBD) where the nuclear envelope breaks, concomitantly

with the complete condensation of chromosomes, and the formation of the critically important meiotic spindle. At the metaphase of MI, the chromosomes are aligned in the spindle, and the first meiotic division occurs [18]. After the oocyte completes the first meiotic division, it will arrest at Metaphase II (MII) of the second meiotic division until fertilization occurs [19]–[21]. At this point, the oocyte can be termed as “egg” [18]. The meiotic division leads to the extrusion of the first polar body (PB) into the perivitelline space which is an observable morphological manifestation of the resumption of meiosis and achievement of MII stage [15]. Thus, this event has an important and useful application for the assessment of oocyte maturation in a clinical setting and is the sole indicator of maturity status when selecting oocytes for intracytoplasmic sperm injection (ICSI). Cytoplasmic maturation involves the changes occurring in the ooplasm of the oocyte that ultimately prepare the oocyte for monospermic fertilization and subsequent early embryo development. These changes include organelle redistribution and reorganization of the ooplasm, including the movement of vesicles, Golgi apparatus, and endoplasmic reticulum [22]. Mitochondria arrange around lipid droplets, and they take an even distribution in the center of the ooplasm, preparing the oocyte with an energy source for development [23]. Also, cortical granules (CG) migrate to the periphery towards the oolemma [3] in preparation for the prevention of polyspermy. Another important concomitantly occurring event is the process of mucification and hyaluronic acid synthesis which enables the CC mass to expand [24]. In the ovary, the ovulation of an oocyte from the dominant ovulatory follicle would depend upon CC expansion [25]. The importance of the assessment of expansion relies on the fact that the quality of the cumulus is often used as a major criterion when selecting oocytes with an emphasis on the degree of

expansion after maturation. Furthermore, there is a positive correlation between *in vitro* CC expansion and improved blastocyst rates in bovine oocytes [26]. Moreover, Cheng et al. [25] reported a positive correlation between the degree of cumulus expansion and the rate of development to the 2-cell stage in mouse embryos. As the oocyte matures, it secretes cumulus expansion-enabling factors that promote CC expansion and oocyte maturation but is not necessarily dependent on gonadotropins [27]. However, in early studies, it has been shown that FSH (and not LH) can significantly stimulate hyaluronic acid synthesis in murine cumulus-oocyte complexes [28]. In CC, hyaluronic acid synthase 2 is the main enzyme responsible to produce the hyaluronic acid secreted to the extracellular space, which in contact with water creates a mucinous matrix, that expands the intercellular space between CC [27]. It is important to mention that the time needed for expansion during IVM is longer than that required for IVV and is highly dependent on substrates in the medium [25].

Another effect of maturation and cumulus expansion is the disruption of transzonal projections (TZPs). TZPs are thin actin- and microtubule-rich cytoplasmic projections from the CC that stretch and project through the zona pellucida (ZP) and reach the surface of the oocyte with gap junctions [29]. This communication portal allows paracrine crosstalk between oocyte and CC exerting a regulatory loop [12], [30]. The cumulus provides a metabolic, protective, and regulatory role over the oocyte and plays an essential part in the nuclear and cytoplasmic maturation events, overall, contributing to the acquisition of developmental competence from the oocyte and its ability to become a healthy embryo [1], [31].

The actual transcriptomic dynamics of the oocyte and CC during oocyte maturation have not been described in the horse. The changes in gene expression during maturation are likely to hold key information regarding critical biological pathways necessary for maturation and developmental competence acquisition. In bovine COCs, transcription of key genes associated with cumulus expansion like the tumor necrosis factor-inducible gene 6 (*TNFAIP6*) and oocyte maturation like inhibin subunit β A (*INHBA*) and Follistatin (*FST*) has been found to be upregulated in CC from IVV COCs, while in CC from IVM COCs, transcription of genes related to stress is being increased [32]. These cellular stress-related transcripts are also upregulated in CC associated with chromosomally abnormal oocytes [33]. Genes such as *ERG*, *AREG* and *PTX3*, are associated with cumulus expansion and maturation and have been found to be downregulated in CC from IVM COCs in humans [34]. Other studies in humans have found a significant association between differentially expressed genes and embryo morphology and pregnancy outcomes, these correspond to the upregulation of *BCL2L11* (related to apoptosis) and *PCK1* (related to gluconeogenesis) and the downregulation of *NFIB* (transcription factor) proposing them as biomarkers for pregnancy outcome [35].

CULTURE MEDIA FORMULATIONS FOR IVM OF EQUINE OOCYTES

Different media types used for oocyte IVM are fundamentally formulations designed for the culture of somatic cells and tissues [36]. Each laboratory has developed different maturation media protocols that aim to satisfy metabolic requirements from both

the oocyte and surrounding somatic cells (cumulus/granulosa) based on each particular species. Examples of maturation media and their principal components described in the literature for equine oocytes are: Donkey: M199 with 20% fetal calf serum (FCS) and 50ng/ml Epidermal Growth Factor (EGF) [37]; Horse: M199, 9.5 µg/ml equine FSH, 15 µg/ml equine LH, 1 µg/ml estradiol-17β, 2.2 g/L NaHCO3, 100 IU/ml penicillin, 100 IU/ml streptomycin. [3]. Horse: DMEM/F12 supplemented with follicular fluid (gonadotropin-induced pre-ovulatory follicle), FBS, ovine FSH, and gentamycin [38]. There are also commercially available formulations (i.e., IVF-Bioscience, Stroebechmedia). Still, the average IVM rate success (based on nuclear maturation assessment) reported from horse IVP laboratories ranges from 50 - 80% [6], [38]–[42].

SUMMARY

Equine oocyte maturation is a complex process leading to the development of a competent oocyte. The current lack of knowledge of the specific mechanisms involved during maturation has limited the formulation of optimized maturation media resulting in sub-optimal outcomes that could be improved. The information presented in Chapter 2 of this dissertation will describe detailed mechanisms that seem to be relevant during the oocyte maturation of horses. Chapter 3 will provide a systematic characterization of differences between the in vitro conditions versus *in vivo* conditions. Moreover, it presents biological pathways that could be altered in *in vitro* culture that might be the cause for the reduced rates of achieving developmental competence which is observed in IVM oocytes compared to their IVV counterparts.

The main goal of this dissertation is to present the findings acquired during the progress of my Ph. D. degree and to provide a starting point for further research into specific aspects of maturation that require more attention. With this information, precise and targeted modifications to the maturation media formulation and culture conditions will be made aiming for improved outcomes of horse embryo *in vitro* production.

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CHAPTER 2

Transcriptomic dynamics of *in vitro* equine oocyte maturation

ABSTRACT

In vitro production of embryos (IVP) has become one of the important assisted reproductive techniques (ART) for the successful breeding of horses. The most common practice for equine IVP is the retrieval of immature oocytes using ultrasound-guided transvaginal aspiration (TVA) followed by IVM (In Vitro Maturation), intracytoplasmic sperm injection (ICSI), and *in vitro* culture of zygotes to the blastocyst stage. For equine IVP, IVM remains one of the main limitations with only about half of immature oocytes advancing to metaphase II (MII) and acquiring developmental competence to be fertilized and become an embryo. Currently, there is a lack of understanding about the physiological events occurring during oocyte maturation, including the potential crosstalk between the oocyte and surrounding CC. This information is key for the optimization of IVM culture conditions and subsequent IVP outcomes. Thus, the aim of this study was to elucidate the gene expression dynamics during oocyte maturation by comparing the transcriptomics of immature (GV) and *in vitro* matured (MII) oocytes (OC) (OC-GV and OC-MII, respectively) and the transcriptome of CC surrounding OC-GV and OC-MII (CC-GV and CC-MII, respectively). Additionally, this study aimed to determine the concentrations of Anti-Müllerian hormone (AMH) and inhibin A in follicular fluid from antral follicles during TVA procedures.

A total of 13,918 genes were expressed in OC, with 749 differentially expressed genes (DEG) OC-GV and OC-MII. Of these, 330 were highly and 419 lowly expressed in OC-MII relative to OC-GV samples. In CC, there were 13,104 expressed genes with 927 DEG (609 highly and 318 lowly expressed in CC-MII relative to CC-GV samples). It was also found the expression of the glycoprotein hormone α chain (*CGA*) in OC samples but no expression of the β subunits for gonadotropins was detected. Additionally, *FSHR* was highly expressed in CC-MII while the *LHCGR* presented a lower expression in CC-MII compared to CC-GV samples. It was reported that the expression of the Anti-Mullerian hormone gene (*AMH*) showed a higher expression in CC-MII. The AMH receptor 2 gene (*AMHR2*) was found to be expressed in OC and CC samples, without significant differences between GV and MII samples. The concentration of AMH analyzed in follicular fluid showed that concentration peaked in 15 mm follicle size. The inhibin α subunit gene (*INHA*) was also detected in CC samples, with a higher expression in CC-MII samples, while the inhibin subunit β A gene (*INHBA*) was similarly expressed in CC-GV and CC-MII. The follicular fluid concentration of inhibin A in follicular fluid displayed an increase proportional to the size of the follicle. Overall, these findings suggest that AMH and inhibin A could be part of the paracrine communication between the CC and the OC in the horse. These results serve as a rational starting point to further investigate the biological pathways relevant to oocyte maturation in the horse and for improvements in equine ART.

INTRODUCTION

In vitro embryo production (IVP) is an expanding field in the equine breeding industry, increasing the efficiency of breeding programs and contributing to the production of live foals from valuable mares and stallions which could not otherwise produce progeny using more traditional breeding methods. Retrieval of immature oocytes from antral follicles of varying sizes in mares is an important source of female gametes for IVP. In comparison to dominant follicle aspiration, the collection of immature oocytes presents important advantages such as flexible timing for collection, retrieval of numerous oocytes during each oocyte aspiration procedure [1], the possibility of oocyte shipping, and is the only option to obtain oocytes from a recently deceased mare. Immature oocytes and surrounding CC, also known as cumulus-oocyte complexes (COC), need to be matured *in vitro* (IVM) to promote the resumption of meiosis and ultimately fertilization potential and developmental competence before they can be fertilized by intracytoplasmic sperm injection (ICSI). Currently, IVM presents a bottleneck in the equine IVP procedure, with variable and suboptimal outcomes. Reported maturation rates range from 50 – 88% [6], [38], [40]–[43]. More importantly, suboptimal oocyte maturation results in significantly lower embryo development rates compared to *in vivo* matured oocytes [31].

Oocyte maturation is a critical and complex process resulting in profound changes in COCs that prepare the oocyte to undergo eventual embryogenesis once fertilized [16]. This process can be divided into nuclear and cytoplasmic maturation. Nuclear maturation refers to the resumption and completion of the first meiotic division, which starts at the GV stage (Diplotene of Prophase I) and ends in the Metaphase II of the second meiotic division, resulting in the extrusion of the first polar body [10]–[12]. Nuclear maturation

corresponds to the most common assessment to classify an oocyte as “matured”, by observing the presence of a polar body. Cytoplasmic maturation involves changes occurring in the cytoplasm and includes organelle redistribution and reorganization, as well as the movement of vesicles, mitochondria, Golgi apparatus, and endoplasmic reticulum [13]. Additionally, cortical granules (CG) migrate to a peripheral subcortical location [14]. These cytoplasmic modifications are considered key events in the preparation of eggs for fertilization, thus determining the competence of the oocyte to become an embryo [10], [13], [15]–[17]. The changes occurring in the nucleus and the ooplasm are the result of several signaling pathways within a COC with an orchestrated regulatory loop between the CC and the OC [18]. In this crosstalk, CC strictly control the oocyte through its development by maintaining meiotic arrest, inducing meiotic resumption, and regulating changes in the ooplasm for the developmental competence of the embryo after fertilization [19]–[24]. This fine and dynamic control of CC over the oocyte suggest that the transcriptome of CC dynamically changes throughout maturation, affecting the oocyte and presenting unique molecular signatures during the maturation process. It has been shown that human CC present distinct gene expression profiles depending on the maturity status or stage of the OC with which they are associated, and that there are distinct differences among the GV stage, MI stages, and MII stage [25]. The exact molecular signature of equine oocytes and CC during maturation remains unknown, and the same is true for the crosstalk between oocytes and CC. A comprehensive understanding of this dynamic communication between the OC and surrounding CC is a prerequisite for the optimization of IVM culture conditions and consequently better IVP outcomes. Hence, the objective of this study was to characterize molecular changes

during equine oocyte IVM by investigating the transcriptome profile of immature and *in vitro* matured OC and their surrounding CC.

MATERIAL AND METHODS

Animals

Reproductively healthy warmblood-mixed and light breed mares (*Equus caballus; n =10*), with an age range between 5-12 years old were used in this study. All the mares were owned by Rood & Riddle Equine Hospital, KY, USA, and were kept as part of the embryo recipient herd. Mares were housed in paddocks with access to fresh water and hay *ad libitum*. Mares were used for only one aspiration session during this study.

Cumulus-oocyte complex collection

Transvaginal aspiration of oocytes (TVA) was performed as described elsewhere [4], [7] with some modifications. Briefly, mares were restrained in stocks, the rectum was emptied, and the perineal area was aseptically cleaned. The sedation protocol included 0.01- 0.02 mg /Kg i.v., butorphanol (Torbugesic; Zoetis) and 0.01–0.04 mg/kg, i.v., detomidine (Dormosedan; Zoetis). To facilitate manipulation and decrease the risk of rectal tears, 0.16–0.24 mg/kg, i.v., N-butylscopolamine bromide (Buscopan; Boehringer Ingelheim Vetmedica) was administered to relax the smooth muscles of the rectum. Each mare was treated with 1mg/kg, i.v., flunixin meglumine (Banamine; Schering-Plough) and 6.6 mg/Kg i.m. ceftiofur crystalline free acid (Excede; Zoetis) immediately following each

TVA procedure. Once the mares were sedated, a customized vaginal extension with a microconvex transducer (Exapad, IMV) with needle guide was inserted into the vagina and antral follicles ranging from 5-25 mm of diameter were aspirated using a 60 cm, 12-gauge double lumen needle (MILA, Inc) connected to an aspiration pump (Pioneer Pro-Pump®) set at 200mmHg. Each follicle was emptied and flushed 8 times using Emcare Complete Ultra Flush Medium (ICP Bio Reproduction™) supplemented with heparin (5 IU/mL) while rotating the aspiration needle to detach the COC from the follicle wall. After all the follicles were aspirated, the collected content was filtered using a low volume E.T filter (MAI™; 04137) and the COCs were located using a stereomicroscope (Olympus) and isolated at room temperature. The recovered COCs were placed in an oocyte-holding medium (Syngro, Vetoquinol) at room temperature (22-24 °C) until the search for all COCs was finished. COCs collected from each mare were randomly divided into two experimental groups: immature (GV) and *in vitro* matured (MII).

Oocyte maturation and cumulus cells denuding

COCs assigned to the immature group (GV; n = 42) were placed into droplets of hyaluronidase (EQ-STRIP™ - IVF Bioscience) and CC were mechanically stripped from the oocytes by pipetting up & down several times using a micropipette until all CC were detached and a denuded zona pellucida (ZP) could be observed. For the *in vitro* matured group (MII), assigned COCs (n = 48) were rinsed and transferred to a well of CO_2 equilibrated maturation medium (EQ-IVM™ - IVF Bioscience) and were incubated in a humidified atmospheric air (21% O_2) with 6% CO_2 at 38.2 °C for 24-26 h. After maturation, CC were removed from oocytes as described above. The maturity status was assessed

on denuded oocytes on a stereomicroscope by determining the presence of a polar body [6]. Denuded oocytes and CC from immature and mature COCs were immediately transferred to individually labeled RNAase-free tubes and 100 uL of RNA*Later*® were added, tubes kept at room temperature for 20 min before being stored at -80°C until the next step.

RNA extraction and assessment

In a preliminary study, RNA was extracted from a single oocyte and also from all its surrounding CC using the technique described below, however, the RNA yield was not enough for downstream analysis (data not shown). Therefore, to obtain enough RNA material for sequencing, six oocytes or all their surrounding CC were pooled into a single biological sample (n). This resulted in 6 CC sample units: (matured CC (CC-MII, n=3), immature CC (CC-GV, n=3)), and 8 OC samples: (matured OC (OC-MII, n=4) and immature OC (OC-GV, n=4)) used in this study. To decrease the individual mare effect, pools of OC or CC were formed from COCs aspirated from different mares in order to generate each study unit. RNA extraction was performed using the RNeasy Mini Kit (#74104, Qiagen) followed by DNA digestion performed on-column using RNase-free DNase I (#79254: Qiagen) according to the manufacturer's guidelines. After extraction, RNA was quantified and assessed for integrity using the RNA Nano 6000 Assay Kit of Agilent 2200 TapeStation (Agilent Technologies, USA).

Library preparation, next-generation RNA sequencing, and bioinformatics analysis

Extracted RNA was used for cDNA synthesis and mRNA library preparation using SMART-Seq® v4 Ultra® Low Input RNA Kit (#634894, Takara Bio) and Nextra XT DNA library preparation Kit (#FC-131-1024, Illumina, San Diego, CA, USA). RNA sequencing was conducted on the illumina NovaSeq 6000 (Illumina, San Diego, CA, USA), generating on average 12.2 ± 1.05 Gb (Mean ± STDEV) of raw 150 bp paired-end reads, averaging 80 million reads per sample (Supplementary Table 2.1). Generated reads and adapters were trimmed with TrimGalore 0.4.0 for quality and then mapped to EquCab3.0 using STAR 2.7.2a. [26]–[28]. The mapped reads were quantified using FeatureCount of the package Subreads v2.0.0 [29]. Additionally, read counts were normalized for clustering and principal component analysis (PCA) using the EdgeR function to calculate the minimal count per million (CMP) using the log2(CPM+c) transformation [30]. A minimum of 0.5 CPM was set and the 1500 most variable genes were selected to construct the heatmaps. Differentially expressed genes (DEGs) analysis was performed using DESeq2 with a false discovery rate (FDR) adjusted p-value of 0.1 and a minimum fold change (FC) of 1.5 [31]. The results of the expression difference are reported in the results including the log2 of fold change (log2FC) and adjusted p-value (adj-p) and also it is reported the normalized gene expression (log2CPM) value averaged from each group (IVM and IVV) of the genes described. Gene ontology analysis for DEGs was performed using DAVID Bioinformatics Resources version 6.8[32] along with the PANTHER classification system version 13.0 [33]. Additionally, the graphical gene-set enrichment tool ShinyGO platform [34] was used for the gene ontology analysis of DEGs.

The potential for crosstalk between OC and CC was investigated by mapping the DEGs in OC & CC to the available ligand-receptor pairs in the FANTOM5 database for protein-coding genes [35]. A Weighted Gene Co-Expression Network Analysis (WGCNA) was performed using the normalized expression values fragments per kilobase of transcript per million mapped reads (FPKM) [36]–[38]. A graphic representation of the methodology pipeline followed in this study is presented in Supplementary Figure 2.1.

Follicular Fluid Collection and Hormone Measurements

To identify the concentration of AMH and inhibin A in the follicular fluid, a second group of mixed and light breed mares (n = 13) of ages ranging from 6 to 15 years was enrolled in the study. Mares resided at the UC Davis Center for Equine Health and were maintained as stipulated by the Institutional Animal Care and Use Committee protocols at the University of California.

Follicular fluid (FF) was aspirated as described above from antral follicles of diameters ranging from 5 to 30 mm using a double lumen (injection/aspiration) system composed of a 60-cm long 12-gauge needle and 16-gauge inner cannula. The cannula was connected through a 180-cm sterile transparent tubing to the aspiration pump (Minitube, USA) with a vacuum setting of 150 mmHg. Along the transparent tubing, a 3-way valve was attached which allowed the diversion of the collection into a 0.5-ml syringe. As each individual follicle was targeted and measured, the needle was inserted through the vaginal and follicle wall and the pump was turned on. As the characteristic bright yellow FF was returning through the tubing, approximately 300 µL were obtained in the

sterile 0.5-ml syringe using the 3-way valve. The fluid was transferred to a labeled tube and placed on ice. At the end of the procedure, samples on ice were transported to the laboratory where tubes were centrifuged for 20 min at 400 x G as described previously [39]. The supernatant was aliquoted (20 µL per tube) and frozen at -20 °C until further hormone analysis. Concentrations of AMH and inhibin A in follicular fluid were quantitatively determined using a sandwich-type immunoassay with monoclonal antibodies validated in horses by the Clinical Endocrinology Laboratory at UC Davis. The degree of enzymatic turnover of the substrate by the enzyme conjugate was measured by colorimetric detection by dual-wavelength absorbance measured at 450 nm as the primary filter and 630 nm as the reference filter as described previously [40], [41]. AMH and inhibin A concentrations were determined using the Equine AMH ELISA assay (AI-115, Ansh Labs LLC) and the Equine Inhibin A ELISA assay (AI-161, Ansh Labs LLC) respectively, following manufacturer protocols. It was previously determined (data not published) that the AMH concentration in follicular fluid is at least 1000-fold higher than in serum in the mare. Follicular fluid frozen aliquots were thawed and then diluted (1:1000) in Diluent A (provided in the assay kit) immediately before each analysis. Inhibin A on the other hand required three different dilution factors based on the follicle size, in order for sample concentrations to be within the range of the standard curve of the assay. The dilution factors were as follows: Follicle size 5-10mm=1:300, 15mm=1:400; >20mm=1:500. A pooled FF sample was included on every plate as an internal control to determine intra- and inter-assay coefficients of variations. All samples were measured in duplicate, and plates were analyzed in a Molecular Devices VersaMax ELISA plate reader

(Sunnyvale, CA, USA). Differences in concentration between follicle sizes were examined using a mixed model and contrast analysis (Kenward-roger method).

RESULTS

On average, 80 million raw reads were generated from each sample with >90% having quality over Q30 Phred score (Supplementary Table 2.1). There was no difference between the amount of generated data, quality, and percentage of GC content among the groups (P >0.05). Data were mapped to the equine reference genome (EquCab3.0) with an average of 95% mapping rate (range: 93.4 - 95.9%; Supplementary Table 2.1).

Transcriptome profile of equine oocytes and cumulus cells

A total of 13,918 genes were expressed in OC samples. Among these genes, 68.46% were categorized as protein-coding genes. The heat map of the OC samples (Figure 2.1A) shows a general pattern of expression among samples using the 1500 most variable genes between the two groups based on the z-score (-4 - +4) gene expression represented in a colorimetric scale (blue: -4 to red +4) representing similarities and differences between samples. Based on this graph, sample D of the MII group present a gene expression pattern more similar to that observed in the GV group, while sample G of the GV group shows a pattern similar to those in the MII group. Additionally, the principal component analysis plot (Figure 2.2A) shows how the samples are distributed in the plot based on their gene expression similarities or differences. Although not

clustered close together within groups, there is a separation between the GV and MII groups.

A total of 13,104 genes were detected in CC samples. Among these genes, 68.47% were categorized as protein-coding genes. The heat map (Figure 2.1B) showed the general pattern of gene expression among CC samples using the 1500 most variable genes based on their Z-score as explained above. The PCA plot (Figure 2.2B) in this case CC-GV samples clustered very close together while CC-MII samples were more scattered, still, both groups appeared apart from each other.

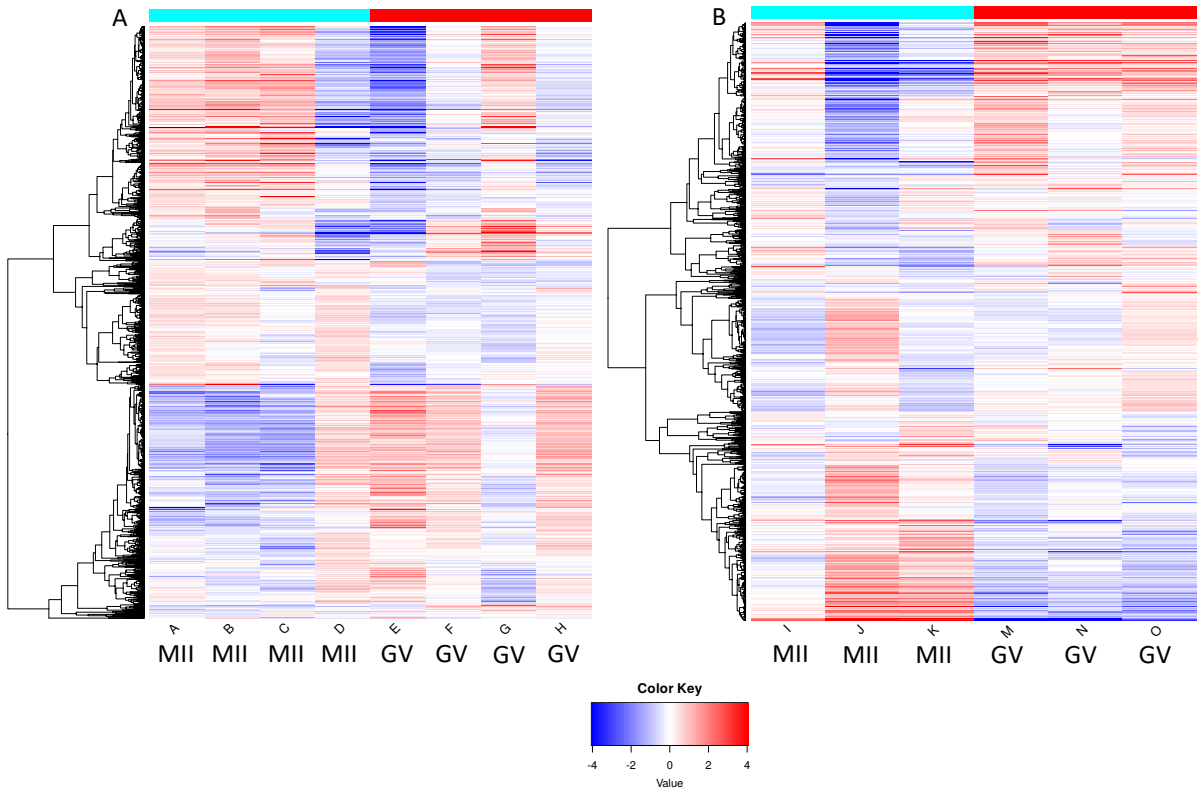

Figure 2.1. Heat map of the 1500 most variable genes comparing the immature group (GV) to the in vitro matured group (MII) in oocytes (A) and CC (B) samples. This heat map was created using the read count values and data was normalized using the EdgeR: log2(CPM+c) transformation. Normalized gene expression is indicated in a color scale with red indicating high expression and blue low expression.

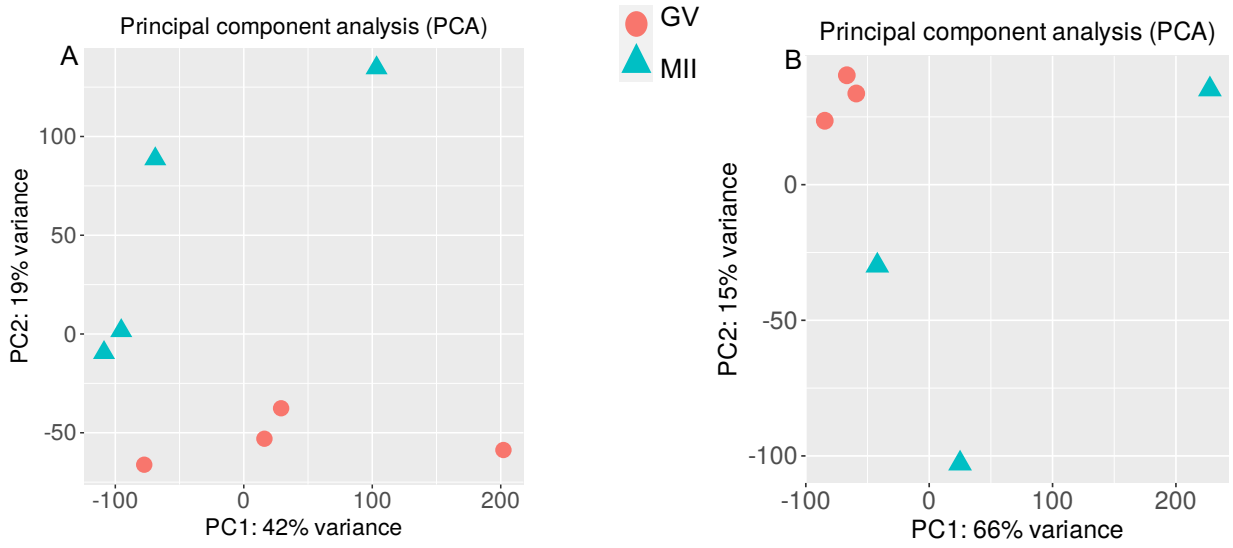

Figure 2.2. Principal Component Analysis plots of oocyte samples (A) and CC samples (B). Read counts were normalized using the log2(CMP+c) transformation and defined a minimal count per million (CMP) of 0.5.

Dynamics of gene expression during oocyte maturation

When comparing the gene expression in immature (GV) and *in vitro* matured oocytes (MII), there were 749 DEGs (Fold change >1.5, FDR <0.1) of which 330 showed a higher expression and 419 a lower expression in the OC-MII group relative to the OC-GV group. DEGs are presented in a volcano plot (Figure 2.3A) and the GO analysis revealed that pathways associated with highly expressed genes are related to aspects of nuclear maturation like chromosome organization, DNA packaging complex and centrosome, while lowly expressed genes were related to nucleolus and ribonucleoprotein complex biogenesis. These are presented in Figure 2.3C and Figure 2.3E, respectively.

In CC, there were 927 DEGs (Fold change >1.5, FDR <0.1), with 609 showing a higher and 318 a lower expression in the CC-MII group relative to the CC-GV group. These are represented in the volcano plot (Figure 2.3B). Based on the GO analysis of DEGs, it was determined that the most relevant pathways associated with upregulated genes are GTPase activity (Figure 2.3D), while pathways associated with downregulated genes are mostly related to the extracellular space, extracellular matrix organization, and collagen trimer (Figure 2.3F).

Evaluating specific genes of the DEG list, it was observed that the LH receptor (*LHCGR*) showed a lower expression (log2FC: -2.54, adj-P:0.00979) in CC-MII (log2CPM: 6.8) compared to CC-GV (log2CPM: 9.3) while the FSH receptor (*FSHR*) showed a higher expression (log2FC: 3.3 adj-P: 0.00123) in the CC-MII samples (log2CPM: 14.5) compared to CC-GV (log2CPM: 11.4). For genes associated to extracellular space and collagen trimer it was determined that *COL1A1* (log2FC: -3.51; adj-P: 0.0644), *COL1A2* (log2FC: -3.36; adj-P: 0.00052), *COL3A1* (log2FC: -3.99; adj-P: 0.00318), *COL5A1* (log2FC: -2.64; adj-P: 0.00702), *COL6A1* (log2FC: -4.07; adj-P: 0.0306), *COL14A1* (log2FC: -4.44; adj-P: 0.00447), and *COL16A1* (log2FC: -2.21; adj-P: 0.0257) reduced their expression in CC-MII samples relative to CC-GV, while only *COL11A1* (log2FC: 2.1; adj-P: 0.0178) showed a higher expression in CC-MII samples relative to CC-GV.

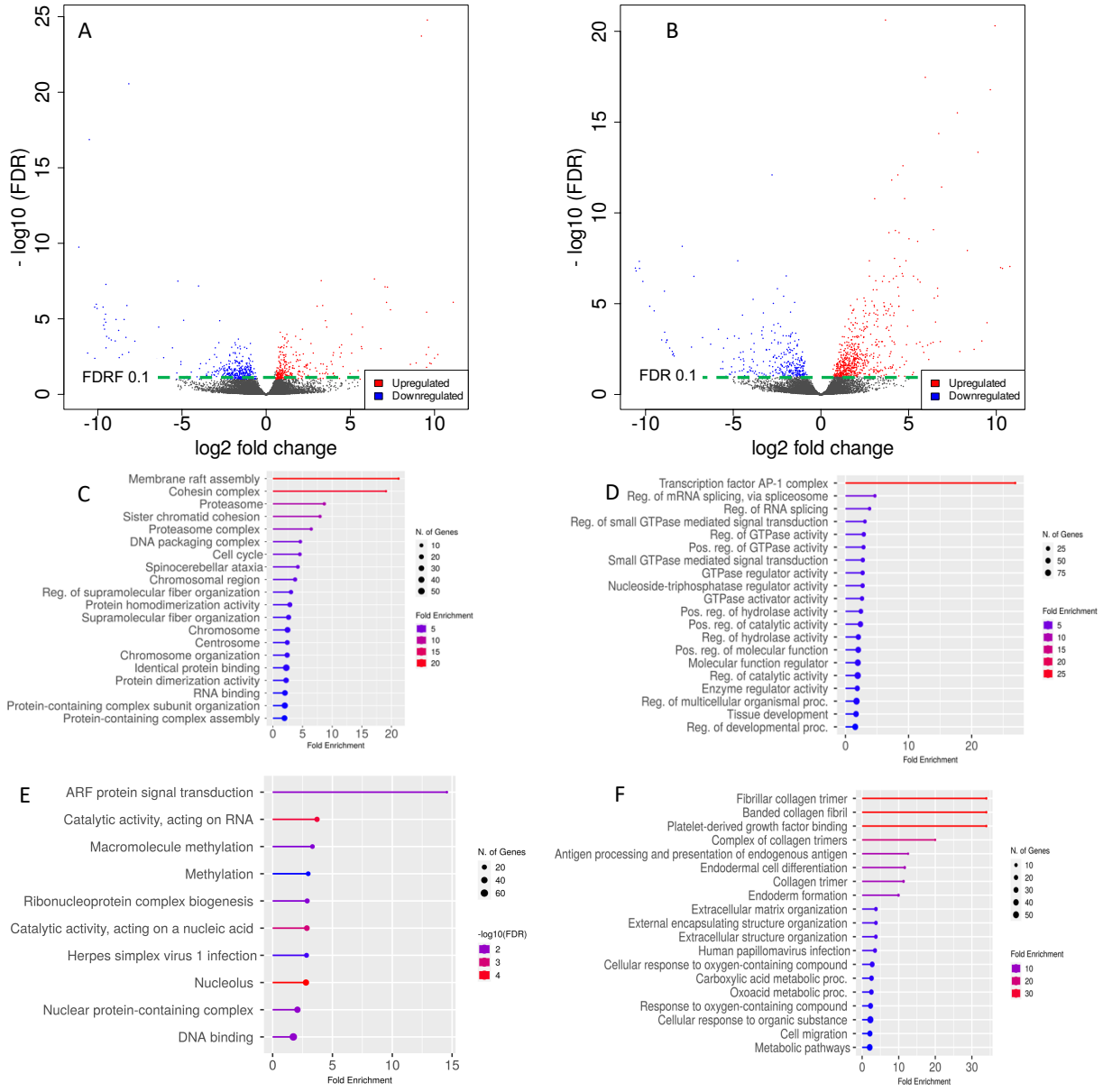

Figure 2.3. Analysis of differentially expressed genes (DEG) represented in a volcano plot for oocyte samples (A) and CC samples (B) denoting the downregulated genes (blue) on the left compared to upregulated genes (red) on the right using a fold change cutoff of 1.5. The dotted green line indicates the FDR cutoff of 0.1. GO analysis in the ShinyGO v0.61 web platform of enriched pathways on genes highly expressed on OC-MII (C) and lowly expressed on OC-MII (E) relative to OC-GV, and those highly expressed in CC-MII (D) and those lowly expressed on CC-MII (F) relative to CC-GV.

Weighted Gene Co-Expression Network Analysis (WGCNA)

A WGCNA was performed to identify the gene co-expression patterns associated with maturation and determined hub genes with high interactions with other genes among OC and CC samples. Three modules were identified in OC samples with a significant p-value ($P<0.05$): module 1 ($P= 0.03$), Module 2 ($P= 0.008$), and Module 3 ($P= 0.05$). Then, a GO analysis of the genes associated with each module was performed. The genes in module 1 were associated with protein tyrosine phosphatase activity while the genes in module 2 were related to nuclear maturation and the meiotic cell cycle. Module 3 did not present any significant pathways in the GO analysis. In CC samples, only one module was established as being significantly represented, including genes related to DNA ligation and sphingolipid metabolism.

Ligand-receptor interaction analysis for assessment of oocyte and cumulus cells crosstalk.

At first, only genes represented in the DEG list were used to identify ligand-receptor pairs described in the FANTOM database by looking at potential ligands and receptors on both ends (OC and CC). When a ligand-receptor combination was observed, the gene expression of any relative gene involved was analyzed as well. Among these potential communication paths, the Anti-Mullerian hormone gene (*AMH*) showed a higher expression (log2FC: 3.02; adj-P: 0.0324) in CC-MII (log2CPM: 12.3) compared to CC-GV (FPKM: 8.3). The AMH receptor 2 gene (*AMHR2*) was expressed without significant difference between the OC-GV and OC-MII groups or between the CC-GV and CC-MII. The inhibin α subunit gene (*INHA*) presented a higher expression (log2FC: 2.1; adj-P:

0.0168) in the CC-MII group (log2CPM: 18.5) compared to the CC-GV (log2CPM: 15.1), however, although expressed, there was no difference in the expression level of the inhibin subunit β A gene (*INHBA*) between CC-GV and CC-MII. The Activin A receptor type I (*ACVR1*) showed a high expression (log2FC: 1.2; adj-P: 0.0865) in OC-MII (log2CPM: 10.2) relative to OC-GV (log2CPM: 8.9).

Additionally, glycoprotein hormone α polypeptide (*CGA*; the α chain segment of gonadotropin dimers) showed a higher expression (log2FC: 4.39; adj-P: 0.00341) in OC-MII (log2CPM: 9.1) compared to OC-GV (log2CPM: 4.4). However, the expression of Lutropin/choriogonadotropin subunit β (*LHB*) or the Follitropin subunit β (*FSHB*) was not detected in this study. Nevertheless, a high expression of *FSHR* and a low expression of the *LHCGR* were observed in CC-MII as mentioned above. A graphical representation of this suggested ligand-receptor interactions is presented in Figure 2.4.

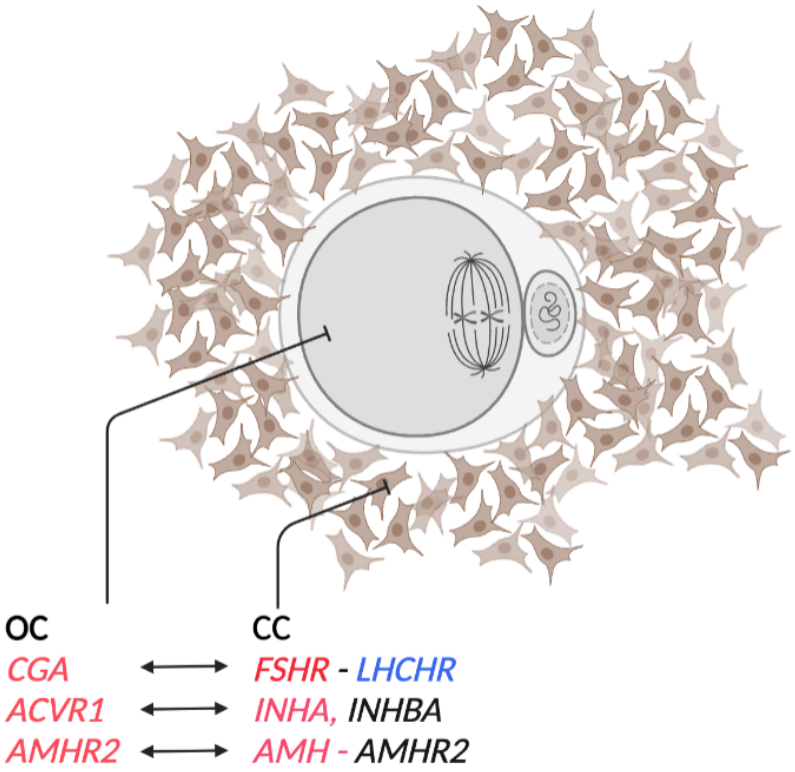

Figure 2.4. Schematic representation of the suggested crosstalk between oocyte and CC. Genes are colored based on the expression: red represents high expression and blue represents a low expression of genes in MII samples relative to GV samples, in black are those that did not present a significant difference in expression between GV and MII. The illustration was made in Bio render.

Hormone Concentration in Follicular Fluid

The follicular fluid concentration of AMH reached the highest level ($p<0.05$) in follicles of size 15 mm with a mean of 2,107.1 ng/mL. Also, it was observed a decrease in the concentration as the follicle increased in size (>15 mm) (Figure 2.5). Inhibin A concentrations in FF exhibited a progressive increase as the follicle increased in size starting at 23,313.5 pg/mL in size 5mm follicles to 420,107.5 pg/mL in size 40mm follicles (Figure 2.5).

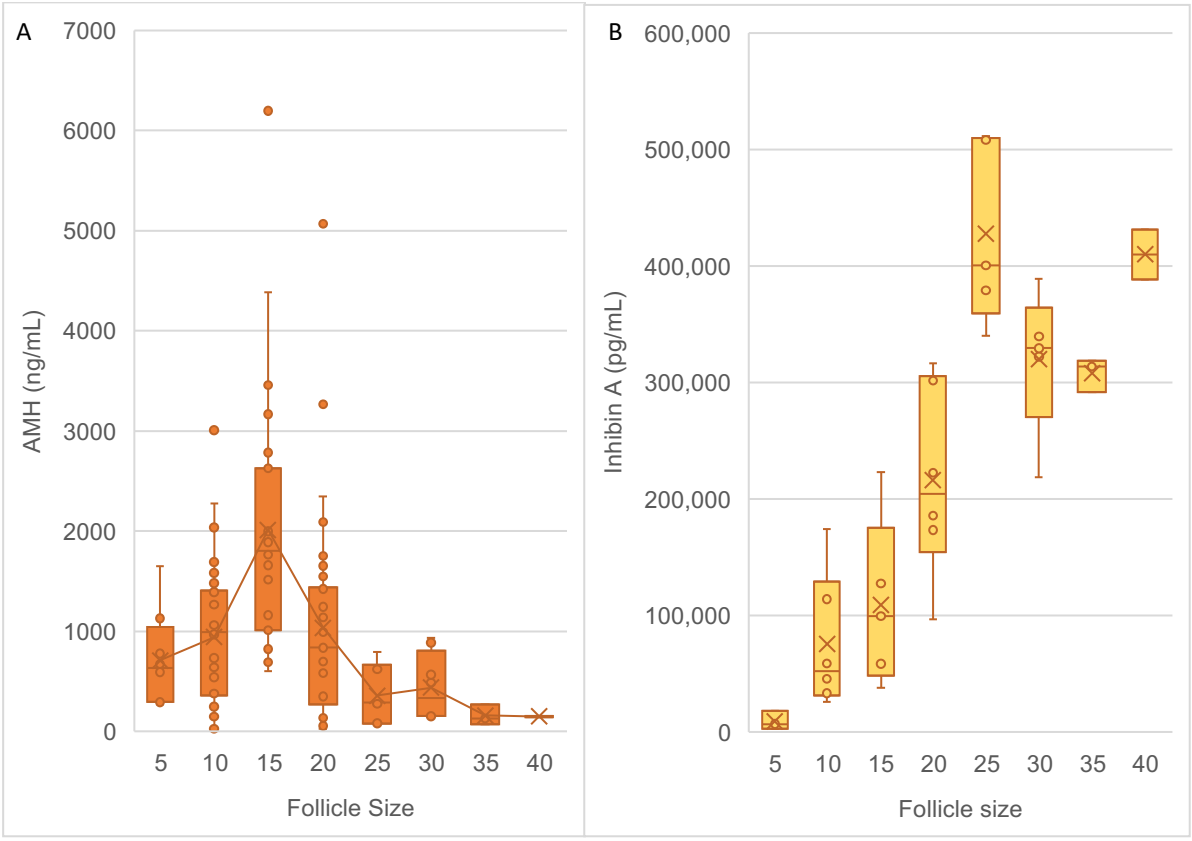

Figure 2.5. Box plots of follicular fluid concentration of AMH (A) and inhibin A (B). Hormone levels were measured using the Equine AMH ELISA assay and Equine inhibin A ELISA kit, respectively.

DISCUSSION

During the final stages of oogenesis, the oocyte acquires meiotic and developmental competence in a process known as oocyte maturation [42]. Oocyte maturation is a strictly regulated process occurring within the follicle in normal circumstances. To the best of the author's knowledge, this study is the first transcriptome profile characterization of immature (GV) and *in vitro* matured (MII) oocytes and CC in horses.

These results show that a similar number of gene transcripts were detected in oocytes (13,918) and CC (13,104) of the 20,322 protein-coding genes found in the horse genome [43]. Based on the constructed heatmaps and PCA plots, a divergent gene expression pattern was observed among OC and CC samples with not a very clear signature defining the maturation stages (GV or MII). OC-GV and OC-MII samples appeared to be scattered but both groups were separated from each other. CC-GV samples on the other hand formed a defined cluster while the CC-MII samples were scattered. It is important to mention that oocytes in this study were identified as matured (MII) based on the presence of a polar body, which is the assessment of nuclear maturation achievement. Therefore, the lack of uniformity of the gene expression observed in this study could suggest that, at least in part, might be due to different cytoplasmic maturity levels among OC-MII samples at the time of nuclear maturation assessment. It is worth mentioning that the number of samples in this study is low, which could also be a factor to the observed results. Nonetheless, developing a non-invasive assay that would evaluate the oocyte cytoplasmic maturation in addition to nuclear maturation status, could potentially better predict the developmental capacity of the oocyte.

Following the analysis of enriched pathways among the DEGs in CC samples, it was determined that genes with higher expression in OC-MII samples were associated with aspects of nuclear maturation, including pathways of the chromosomal region, centrosome, chromosome organization, and sister chromatid cohesion. Additionally, the significantly represented module 2 of the WGCNA presented pathways associated with the meiotic cycle and RNA processing. An important event occurring in the oocyte during

maturation is the resumption of the meiotic cycle from prophase I and advancement to the metaphase II stage. The results of the GO analysis of DEGs and the WGCNA showed that genes related to these pathways are highly expressed in OC-MII. However, the specific mechanism regulating the control of meiosis in equine oocytes has yet to be described. One of the modules related to nuclear maturation presented genes associated with protein tyrosine phosphatase activity. It is known that cyclin-dependent kinase 1 (*CDK1*) drives oocyte maturation while the tyrosine-protein phosphatase *CDC14* is a dual-specificity phosphatase that reduces CDK1 activity and reverses the actions of CDK1 during mitosis [44]. *CDC14A* was not differentially expressed on OC samples and showed a similar expression between OC-GV and OC-MII. *CDC14B* was also not part of the DEG list and showed a similar expression in OC-GV and OC-MII. The extent of these observations is insufficient to determine the exact cell cycle regulation mechanism in the oocyte.

In the case of CC samples, highly expressed genes in CC-MII were associated with pathways related to GTPase activity. GTPases act independently by hydrolyzing GTP to GDP in the cytoplasm. One of the main functions of the GTPase is cell organization and movement by regulating actin and microtubule filaments [45]. During maturation, the oocyte goes through changes in the cytoplasm with the reorganization of organelles and the formation of the meiotic spindle for chromosome segregation, all these activities require the assembly of the cytoskeleton, including microfilaments and microtubules that facilitate these events. In porcine oocytes, the small Rho GTPase RhoA has a key role in regulating the organization of the cytoskeleton, including cell migration, polarity, and division [11]. In this study, it was found that RHOB had a lower expression

(log2FC: -1.91; adj-P: 0.0235) in OC-MII samples (log2CPM: 8.3) compared to OC-GV (log2CPM: 10.3), while the opposite was observed in CC samples (log2FC: 2.6; adj-P: 0.000195), where CC-MII presented a higher expression (log2CPM 11.8) than that observed in CC-GV (log2CPM: 9.2). Based on the literature reviewed, the author could not find reports about the exact mechanisms regulating the cytoskeleton organization in horse COCs.

It was also found that the *LHCGR* presented a low expression while the *FSHR* presented a high expression in CC-MII samples relative to CC-GV. It has been reported that the expression of *LHCGR* on porcine CC depends on oocyte factors as well as the levels of FSH available in the culture media [46]. The majority of oocyte maturation media formulations include varying concentrations of FSH and some commercially available options also include LH. Unfortunately, a commercially available maturation medium was used in this study, and the actual concentration of FSH or LH was not disclosed. However, based on the information obtained from another study that aimed to compare the effect of different maturation media on the gene expression of CC (de la Fuente et al., unpublished data), it was further confirmed that the expression of *LHCGR* in CC among all tested media is comparatively low. The next step in this investigation is to determine the effect of different FSH concentrations on CC gene expression and then evaluate those concentrations and their effect on oocyte maturation and embryo development. The current study also reported the expression of *CGA* on OC, along with a significantly higher expression in CC-MII samples compared to CC-GV. Based on the reviewed literature, there are no reports of the synthesis of CGA from the COC nor the oocyte. Moreover, it is only described that *CGA* is expressed in pituitary gonadotropic and thyrotropic cells in

all vertebrates as well as in chorionic syncytiotrophoblast cells of *Equidae* [47]. These findings present a particular paradox as glycoprotein hormones like LH and FSH exert their biological functions as a heterodimeric molecule, composed of an α and β subunit, and the Lutropin/choriogonadotropin subunit β (*LHB*) or the Follitropin subunit β (*FSHB*) were not detected in this study. Thus, the physiological relevance of *CGA* expression, by itself, is difficult to assess as there is not literature describing it, even though it is listed as a ligand in the FANTOM5 database for protein-coding genes [35]. Nonetheless, this is the first report of the expression of *CGA* on equine oocytes, and even when the data available is insufficient to suggest any biological function at this moment, this finding expand the reason to investigate in more detail the effect of gonadotropins during oocyte maturation in the horse.

Another group of signaling molecules in the ovary corresponds to the transforming growth factor β (TGF-β) superfamily. It is well documented that these molecules are expressed by the oocyte and CC in numerous species and act as key intraovarian regulatory signals during the development of the follicle and oocyte [48]. Anti-Müllerian hormone (*AMH*) was expressed in CC samples and its relative expression was higher in CC-MII samples compared to CC-GV. It was also found that the AMH receptor 2 gene (*AMHR2*) was expressed in OC and CC samples without major differences in expression between GV and MII groups. These results suggest that AMH could have a paracrine role during equine oocyte maturation. A similar *AMH* expression pattern has been found in human CC where *AMH* expression is positively associated with the developmental stage of the follicle, showing higher *AMH* expression in CC from pre-ovulatory follicles of IVF patients [49]. In mares, it has been shown that the circulating concentration of AMH is

positively associated with the number of antral follicles between 6-20 mm present in the ovaries. However, the concentration of AMH in follicular fluid peaks in follicles size 15 mm and then decreases as the follicle grows as was determined in this study. One reason for this reduction in AMH concentration in follicular fluid is that *AMH* is expressed by granulosa cells in early follicle development, but this expression progressively changes to the CC as the follicle develops [50]–[52] due to the influence of paracrine factors secreted from the oocyte in a stage-specific type of regulation [53], [54]. This supports the hypothesis, at least in part, that AMH participates in the crosstalk between oocyte and cumulus cells during maturation in horses. This idea can also be supported by finding during mice oocyte maturation, which showed how a maturation medium supplemented with recombinant AMH increased blastocyst formation [55]. However, the exact signaling pathway activated by AMH and its receptors is beyond the scope of this study.

The results also showed that the inhibin subunit α gene (*INHA*) is expressed in CC samples along with the inhibin subunit β A (*INHBA*). However, only *INHA* showed an increase in its expression in CC-MII samples (fold change= 2.08; p= 0.0168). Inhibin is another TGF-β molecule that plays an endocrine role in the pituitary gland and a paracrine and autocrine role within the ovary and the follicle. Inhibin follows a physiological cyclic activity, showing a peak in the circulating concentration at ovulation and the lowest concentration at mid-cycle. It is also reported that its concentration decreases rapidly during pregnancy in the mare [41]. The concentration dynamics in the follicular fluid also present a particular variation as the follicle develops. It has been reported that inhibin A concentration decreased from follicle size 25 to follicles size 40 mm, with a slight increase in the follicles that were about to ovulate [56]. Another study reported that inhibin A

concentration increase occurs in the follicle before follicular deviation, suggesting that inhibin A could have a role in the selection of the dominant follicle in the mare ovary [39]. Data presented in this study expand this current knowledge by monitoring the concentration in a wider range of follicle sizes, and it shows a linear increase in the concentration as the follicle increase size from small antral follicles to preovulatory size follicles. These results are consistent with those reported previously in the bovine follicular fluid that showed a positive correlation between the concentration of inhibin A and follicle size [48]. These data support the possibility that inhibin A participates through paracrine communication and regulation of follicle development and probably oocyte maturation, as it has also been reported that there is a positive effect on maturation and fertilization rates by supplementing inhibin A during the maturation culture of primates' oocytes [57]. It appears that there is abundant knowledge about these molecules and their endocrine function. However, their precise paracrine or autocrine roles still need further investigation. The observations of the current study are insufficient to determine the exact effect of AMH or inhibin A on the maturation of the oocyte in the horse, but this report at least describe them as a playing actor during this process.

Another important TGF-β molecule participating in follicle development is the Activin-binding protein Follistatin (FST) which opposes the effect of Activin thus it is considered to have a role in promoting atresia or luteinization [48]. In this study, it was found that *FST* expression is increased (log2FC: 4.25; adj-P: 0.000000000933) in CC-MII samples (log2CPM: 16.4) relative to CC-GV (log2CPM: 12.4). It appears that FST has an important role during the maturation of the oocyte and acquisition of developmental competence as a higher abundance of *FST* mRNA has been associated with a faster time

to the first cleavage in bovine zygotes [58]. Moreover, it has been shown *that in vivo* matured COCs present a higher expression of *FST* compared to *in vitro* matured bovine COCs [59]. There is no report of *FST* expression in the CC of horses. This information highlights the importance of TGF-β molecules on the maturation of oocytes in the horse.

An important event occurring during the maturation of the COC is the expansion of the cumulus. Cumulus expansion has been used as a marker for maturation success and blastocyst development [60]. This expansion is the result of a series of modifications to the extracellular matrix (ECM) of the CC surrounding the oocyte. During this process, hyaluronic acid is produced and secreted by CC and organized in a mesh-like network in the ECM which is stabilized by proteins and proteoglycans [61]. In this study, although the expression of the membrane-bound enzyme responsible for the synthesis of hyaluronan, hyaluronan synthase (*HAS2*) was detected, no significant gene expression difference was found between CC-GV and CC-MII. It is proposed that the synthesis and accumulation of hyaluronan seem to be stimulated more potently by FSH rather than LH [62]. Additionally, it has been described in horses that the level of *HAS2* transcripts in mural granulosa cells can be transiently increased with human chorionic gonadotropin (hCG) treatment [63]. Therefore, it can be suggested that the lack of increased expression of *HAS2* in CC-MII samples could be the cause for the reduced cumulus expansion observed in the *in vitro* matured COCs. Again, the results highlight the need for further investigation of the role of gonadotropins during equine oocyte maturation, including the effect on the expression of *HAS2* and cumulus expansion.

Pathways related to extracellular space and collagen trimer were significantly represented in the DEGs of CC samples. The results showed that multiple collagen genes

had a lower expression on CC-MII. Collagens are trimeric molecules of the extracellular matrix that provides structural integrity to cells and tissues [64]. Collagens participate in the formation of the ECM due to their relatively high affinity to hyaluronan and integrins [61]. Based on the literature reviewed, there is no reference to collagen synthesis by CC in the horse. However, the disruption of collagen during ovulation is a well-documented and necessary event [65]. It is important to consider that while the oocyte is maturing, the follicle prepares for ovulation which implies changes in the extracellular space that require proteolytic degradation of connective tissue that constitute the ECM [66]. Even though a significant difference was not detected in the gene expression of collagenases in this study, the expression reduction pattern of collagen genes observed in CC-MII samples partially agrees with this biological event.

CONCLUSIONS

This study is the first report on gene expression dynamics of equine oocytes and CC during IVM. These data demonstrated the changes in the expression of LH and FSH receptors in CCs, as *LHCGR* expression is reduced while *FSHR* expression was increased. Additionally, molecules of the TGF-β family seem to be important in the communication pathways of equine oocyte maturation, as AMH, inhibin A, and Follistatin genes are differentially expressed between GV and MII samples. It was also described that genes associated with the extracellular space, especially those related to collagen trimer showed a decreased expression in CC-GV samples. With the increasing growth in ART in the horse breeding industry, the advancement in knowledge regarding regulatory mechanisms of oocyte maturation is essential to optimize the outcome of IVP. The

information from this study will serve as the foundation to understand the dynamics of oocyte maturation and optimize the oocyte maturation system, ultimately increasing the efficiency of IVP in horses.

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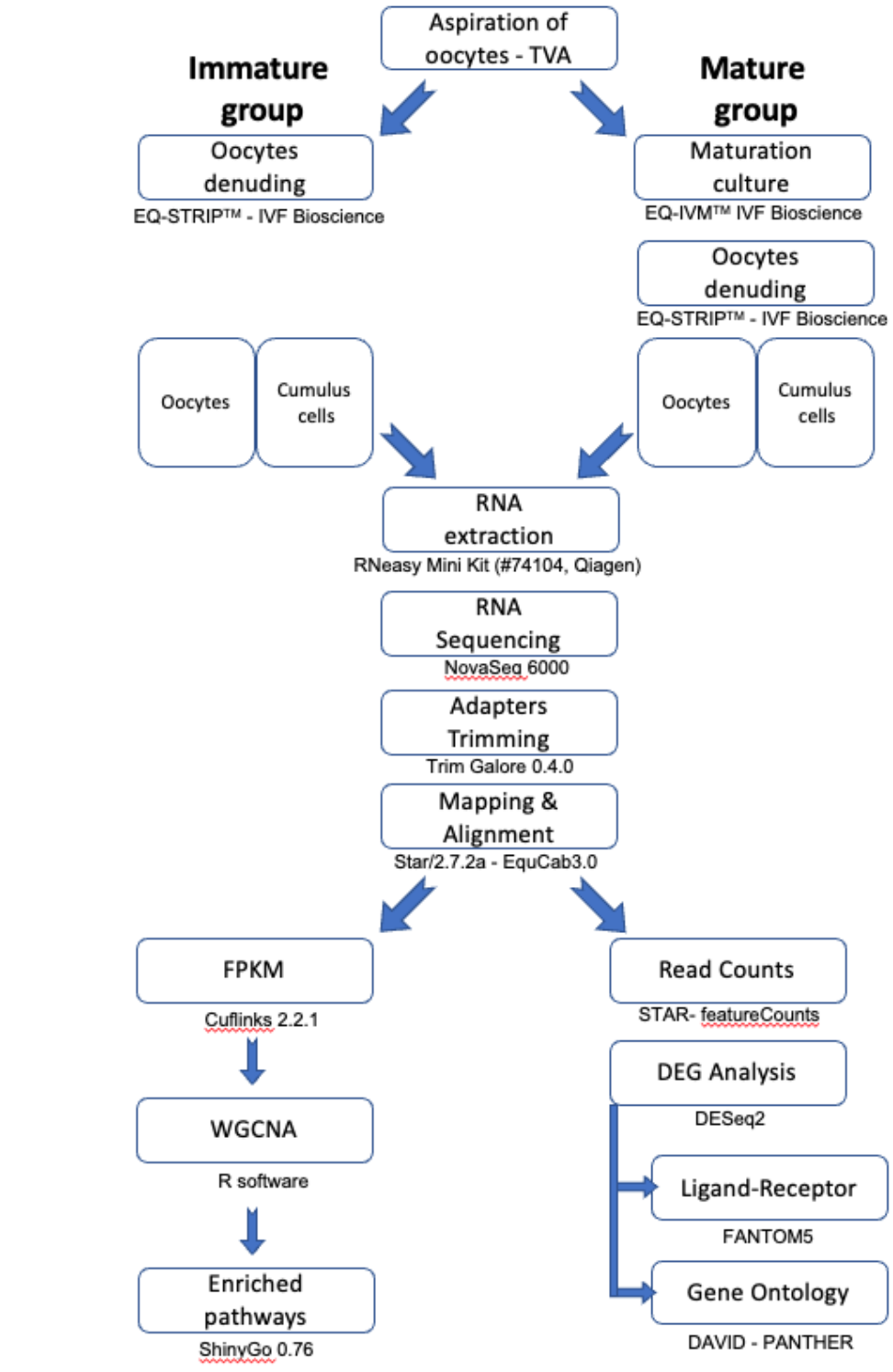
SUPPLEMENTAL MATERIAL

Supplementary Table 2.1. Description of the number of reads and mapping rates obtained for all samples. Reads were aligned to the horse reference genome (EquCab3.0) using STAR (Release 2.5.3a)

Sample type	Sample name	Raw reads	Clean reads	Raw bases	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)	Mapping rate (%)
OC	A	69621124	60824386	10.5G	9.2G	0.03	96.3	90.82	46.42	95.43
OC	B	91590548	81935972	13.8G	12.4G	0.03	96	90.16	46.47	94.79
OC	C	84620160	75650030	12.8G	11.4G	0.03	96.2	90.74	46.95	95.4
OC	D	92070144	81791056	13.9G	12.4G	0.03	96.33	90.95	46.73	95.02
OC	E	82758958	73201326	12.5G	11.1G	0.03	96.1	90.61	47.37	95.02
OC	F	83451480	75297696	12.6G	11.4G	0.03	96.26	90.74	46.7	95.33
OC	G	80441024	72704926	12.1G	11G	0.03	96.25	90.63	46.41	95.41
OC	H	81377828	73851862	12.3G	11.2G	0.03	95.91	89.51	46.42	95.92
CC	I	70610794	63037856	10.7G	9.5G	0.03	96.34	91.01	46.52	95.11
CC	J	78461870	69341788	11.8G	10.5G	0.03	96.12	90.64	45.36	93.43
CC	K	75977276	67901884	11.5G	10.3G	0.03	96.24	90.84	46.76	95.08
CC	M	87424856	78254408	13.2G	11.8G	0.03	96.26	90.87	47.92	95.64
CC	N	80570034	72383012	12.2G	10.9G	0.03	96.33	90.8	46.68	95.7
CC	O	70810840	63641466	10.7G	9.6G	0.03	96.29	90.8	48	95.49

Supplementary Figure 1.



Supplementary Figure 2.1. The pipeline of the methodology used for sample acquiring, processing, and RNA work.

CHAPTER 3

Gene expression profile of equine cumulus cells from oocytes matured *in vivo* and *in vitro*

ABSTRACT

With the continuing growth of assisted reproductive techniques (ART) like IVM of oocytes followed by ICSI in the equine breeding industry, it has become critical to determine the physiology of maturation occurring during IVM. *In vivo* matured oocytes (IVV) present a superior developmental competence when compared to the *in vitro* matured oocytes (IVM), which suggests that *in vitro* maturation culture conditions are suboptimal and fail to recreate the correct environment for the proper gene expression and control of natural physiologic pathways occurring during oocyte maturation. Thus, the aim of this study was to define the transcriptome profile of cumulus cells (CC) associated with cumulus-oocyte complexes (COC) of IVV and IVM and determine the differences in their gene expression and related pathways that might be affected by obtaining CC immediately after COCs are denuded for ICSI following two maturation protocols. It was found that 6661 genes were differentially expressed between IVM and IVV, of which 2,180 were highly expressed and lowly expressed 4,481 in the IVM samples compared to IVV samples. Enrichment analysis demonstrated that highly expressed genes in IVM were associated with the cell cycle, chromosome segregation, and nuclear division. The expression of gonadotropin receptors in the IVM group was determined to be different from the IVV group, showing a higher expression of the *FSHR* and a lower expression of *LHCGR* in IVM samples. Moreover, genes and pathways related to the extracellular matrix and cumulus expansion showed a lower expression in CC-IVM compared to CC-

IVV. These data contribute valuable information to begin comprehending the differences observed between the two types of oocyte maturation and the developmental success of each one.

INTRODUCTION

The use of *in vitro* production of embryos (IVP) has become an important part of the equine breeding industry. IVP requires the collection of oocytes from donor mares which commonly are performed by the transvaginal aspiration (TVA) technique. TVA can be done by aspiration of an oocyte from a dominant pre-ovulatory follicle in a mare that has been previously induced to ovulate with gonadotropins [1]. In the clinical ICSI practice, these are referred to as *in vivo* matured (IVV) oocytes and can be fertilized with only a short period of *in vitro* culture post aspiration [2]. Immature oocytes aspirated from antral follicles in the ovaries (> 5 mm diameter) can also serve as oocytes for IVP. This method requires IVM of collected immature oocytes before they can be fertilized by intracytoplasmic sperm injection (ICSI). The IVV oocytes have a greater maturation rate and developmental competence than the IVM oocytes, leading to a higher fertilization and blastocyst rate [3]. However, only one oocyte can be retrieved for each dominant follicle that undergoes the TVA procedure. Although they have lower developmental competence, the aspiration of immature oocytes presents advantages over the IVV method. The number of oocytes that can be obtained on each aspiration session is greater. Moreover, these aspiration sessions can be performed without time constraints [4] and year-round for many mares, not having significant adverse effects over maturation and blastocyst rates during the non-breeding season [5]. Regardless of these

advantages, IVM in horses is one of the important limitations in equine IVP, due to our limited understanding about oocyte maturation and the specific requirement for the culture conditions during this time.

Maturation success and acquisition of developmental competence in the oocyte are highly dependent on the cells of the cumulus [6]. Cumulus cells (CC) take part in an orchestrated control over the oocyte during its development, by maintaining meiotic arrest [7], inducing meiosis resumption (nuclear maturation) during luteinizing hormone (LH) signaling [8], and regulating the proper changes in the cytoplasm for the developmental potential of the embryo after fertilization (cytoplasmic maturation) [9]. This meticulous symbiosis between cumulus and oocyte is also important to allow the proper metabolic activities in both parts, as the CC helps the oocyte with specific amino acids, cholesterol biosynthesis, and glycolytic functions, due to a reduced ability of the oocyte to perform these processes [10]. Overall, the intimate relationship mediated by regulatory signaling molecules between the cumulus and oocyte supports the development of a fertilizable oocyte to undergo embryo development [11], [12].

Since CC play an important role during oocyte maturation, their gene expression patterns hold critical information regarding the biological processes responsible for the fate of the oocyte. Moreover, the information stored in these cells might be direct markers of the future developmental fate of the oocyte [13]. Cumulus cells are discarded after oocytes are denuded for fertilization in the clinical practice of ICSI, making CC a great sample for assessment of maturation and gene expression, and potentially a future marker for the developmental competence of oocytes. Thus, the objective of this study was to determine the transcriptomic profile of equine CC obtained from IVV cumulus

oocyte complexes (COCs) and compare them to those obtained from IVM COCs to identify differences in the gene expression that could explain or give hints regarding the differences in development observed in oocytes from IVV and IVM methods.

MATERIALS AND METHODS

Animals

Samples for this study were collected from mares from two different geographical locations. Cumulus cells from *in vivo* matured (**IVV**) COCs were obtained from a commercial *in vitro* fertilization laboratory located in Columbia, MO (https://equmed.com/reproductive-services/icsi/) with owner permission. These mares were client-owned Quarter horses (n=12) of age ranging in age from 4 to 20 years and were maintained under routine management of a private horse farm and did not require Institutional Animal Care and Use Committee approval. Cumulus cells from *in vitro* matured (**IVM**) COCs were obtained from university-owned mixed and light breed mares (age range: 6-15 years) housed in group paddocks at the Center for Equine Health (CEH), University of California, Davis (https://vetart.vetmed.ucdavis.edu) and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

In vivo matured (IVV)

First, it is important to clarify that these oocytes are not completely matured *in vivo* as the name indicates, since the protocol includes a period of *in vitro* culture. However,

they are labeled as *in vivo* matured oocytes and the protocol to obtain an IVV oocyte includes a gonadotropin stimulation of a dominant follicle. As described previously [2], donor mares were monitored by transrectal ultrasonography daily. When the diameter of a dominant follicle reached >35 mm and the uterus presented edema, 1.8 mg of deslorelin (Sucromate, Bioniche Animal Health) was administered i.m. followed by aspiration of the induced follicle 24-27 hours later. Aspiration of the dominant stimulated follicle (DSF) was performed using a Sonosite Micromax ultrasound machine (SonoSite) with 5-10 MHz micro convex probe and Minitube transvaginal needle guide. When the probe was in place and the DSF was visualized, a 12-gauge double lumen needle was introduced through the follicle wall and flushed with embryo flushing medium (Vigro Complete Flush) supplemented with 5 IU/mL sodium heparin kept at 37 °C using a vacuum pressure of 150 mmHg. The follicle was repeatedly filled and collapsed at least 10 times while the ovary and follicle were being massaged per rectum during aspiration. The collected COCs were then placed in *in vitro* culture for a period of 15-18 hours in 45% Medium 199 with Earle's salts, 45% DMEM [Dulbecco's modified Eagle's medium]/F-12 with 15 mmol/l HEPES, 10% newborn calf serum, 0.15 mmol/l sodium pyruvate and 25 µg/ml gentamicin) in 30 mm Petri dishes and incubated at 37.9°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (mixed gas). After this culture, oocytes were denuded, and CC mechanically removed by repeated pipetting in Medium 199 with Hanks' salts with 10% newborn calf serum, 25 µg/ml gentamicin, and 0.1% hyaluronidase. Oocytes in the metaphase II stage confirmed by the presence of a polar body were used for commercial ICSI and IVP for these privately-owned mares. Only CCs obtained from those MII oocytes were used in this study. Upon retrieval, CC were immediately placed in 100 uL of

RNALater®, frozen at -80 °C, and express-shipped to the laboratory at UC Davis until RNA extraction. It is important to emphasize that these oocytes are not entirely matured *in vivo*, since this method encompasses a period of *in vitro* culture. However, they have higher developmental success when compared to IVM oocytes, thus the aim of this study was to determine a gene expression signature that could be associated with that higher developmental competence.

In vitro matured (IVM)

Candidate mares were examined weekly using transrectal ultrasonography to determine the number of antral follicles (5-30 mm) present in the ovaries. Mares with the highest number of follicles were selected for TVA to increase the oocyte pool collected during each procedure. Retrieval of immature COCs was performed as previously described [14], [15]. Donor mares (n=5) were restrained in stocks, the rectum was emptied of feces, and the perineal area was thoroughly cleaned with water and soap and then carefully dried. The sedation protocol included 0.02 mg /Kg i.v., butorphanol (Torbugesic, Zoetis) and 0.01–0.02 mg/kg, i.v., detomidine (Dormosedan, Pfizer). To facilitate manipulation and decrease the risk of rectal tears, 0.16–0.24 mg/kg, i.v., N-butylscopolamine bromide (Buscopan, Boehringer Ingelheim, Ingelheim, Germany) was administered to relax the smooth muscles of the rectum. Each mare was treated with 1mg/kg, i.v., flunixin meglumine (Banamine; Schering-Plough) before the procedure. A urethral Foley catheter (28 Fr) was aseptically placed in the mare's urethra. A sterile needle guide was placed in a clean transvaginal ultrasound probe (6.5-mHz) (IVM Imaging©) and this was covered with a sterile sleeve filled with sterile lube (Therio-gel®,

AgTech Inc) that was then introduced into the vaginal vault. Once the operator manually secured the ovary transrectally and visualized the follicles using the ultrasound machine, a 60-cm long double-lumen 12-gauge needle (Minitube) was inserted in the needle guide, perforated the vaginal wall, and introduced into the ovary to aspirate all accessible antral follicles. The aspiration needle was connected through a sterile 180-cm tubing to a vacuum aspiration pump (Minitube) and follicular fluid was aspirated (150-200 mmHg pressure) until the follicle was emptied completely. The needle was rotated vigorously, and the follicle was flushed and emptied eight times with EquiPro OPU Recovery Medium (Minitube) to dislodge the hillock and detach the COC from the follicular wall. Bottles containing the aspirated follicular fluid were kept at room temperature and transported to a clean laboratory where filtration and COC searching was performed. Recovered immature COCs were rinsed and kept in the dark at room temperature (22 °C) for ~ 24 hrs in a holding medium (Equi-Hold, Minitube). Then, COCs were individually placed in Miri Culture Coin dishes with 25 µL of maturation medium composed of 54% Dulbecco's modified Eagle's medium (DMEM)/F-12 with 15 mM HEPES, 25 mg/mL gentamicin, 36.0% Global medium (LifeGlobal), 0.1 mM sodium pyruvate, 6.0% fetal bovine serum (FBS; F2442; Sigma), 10 µL/mL insulin–transferrin–selenium (ITS) solution, 10% DSF fluid, 8.8 mU ovine FSH (National Hormone And Peptide Program) and 1.1 mU/mL porcine somatotropin (Harbor UCLA Research and Education Institute) [14]. MiriTL® Culture Coins were cultured for 24-27 hours in an atmosphere of 5.8% CO_2, 5% O_2 at 37,8 °C in the MiriTL® Time-Lapse Imaging Incubator (Esco Technologies, Inc) for IVM. The MiriTL® captures each image in 5 focal planes using an IW single red LED (635 nm) with total light exposure of 0.064s per captured image which enables video recording

of the expansion of the cumulus. After IVM, CCs were detached from the oocyte by incubation in G-MOPS™ (Vitrolife) 0.2% hyaluronidase for a maximum of 2 minutes and repeated pipetting. Immediately after detachment from the oocyte, oocytes were assessed for their maturity status by observing under a stereomicroscope the presence of a polar body. CCs from each single COC were placed in individually labeled tubes with 100 µL of RNA*Later*® and frozen at -80 °C until posterior RNA extraction.

Time lapses recorded during the maturation culture of COCs in the MiriTL® were retrospectively analyzed to determine the change in cumulus dimensions (diameter and area). Due to the varying size of retrieved COCs, those with a cumulus surface bigger than the image field of MiriTL®, were not subjected to measurements considering that the borders could not be determined in the acquired image. The built-in measuring tools of the MiriTL® software was used to calculate the average diameter by measuring from end-to-end on two perpendicular sides of COCs and then imputed the radius (r) into the area (A) of a circle formula: ($A = \pi r^2$). Additionally, the surface area (µm^2) was determined using a second MiriTL® measuring tool capable to determine the area of a drawn circle. One of the conflicts for this measurement was the irregular non-perfectly circular shape of COCs, thus the drawn circles aimed to include most of the cumulus area, a representation of these measurements is shown in Supplementary Figure 3.3. To reduce variation due to measurement error, the average of both types of measurement was used for data analysis and graphical presentation. These measurements and calculations were performed every hour during the 24 h extent of IVM culture.

RNA extraction and assessment

Total RNA from each sample was obtained using the Single Cell RNA Purification Kit (#51800, Norgen) following the manufacturer's instructions. After extraction, RNA quality and concentration was determined using the High Sensitivity RNA ScreenTape assay for TapeStation System® (Agilent Technologies, CA, USA) following the manufacturer's instructions. Each pool of CC obtained from a single COC was considered one sample (n=24), resulting in a total of twelve *in vivo* (n=12) and twelve *in vitro* (n=12) matured samples.

Library preparation and Next-generation RNA sequencing

Next-generation RNA-seq was performed using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, USAA) to generate sequencing libraries [16], [17]. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). The first strand of complementary DNA (cDNA) was synthesized using random hexamer primer and M- MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends using exonuclease/polymerase activities. After the adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min, followed by 5 min at 95°C before PCR. PCR was subsequently performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and

Index Primer. PCR products were purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on NovaSeq 6000, and 6Gb of raw 150 bp paired-end reads were generated per sample.

Bioinformatics analysis

The reads were trimmed and mapped to EquCab3.0, using TrimGalore 0.4.0 for quality and adapters, and then mapped to EquCab3.0 using STAR 2.7.2a. [18]–[20]. All mapped reads were quantified using FeatureCount of the package Subreads v2.0.0. For clustering and principal component analysis (PCA), read counts were normalized with the EdgeR function to calculate the minimal count per million (CPM) using the log2(CPM+c) transformation [21]. A minimum of 0.5 (CPM) was set as a cutoff for sample analysis and only the 1500 most variable genes were used to construct the heatmaps and perform the PCA analysis. Differentially expressed genes (DEGs) analysis was performed using DESeq2 with a false discovery rate (FDR) adjusted p-value (adj-P) of 0.1 and a minimum fold change (FC) of 1.5. The results of the expression difference are reported in the results including the (log2FC) and (adj-P) for genes that presented significant expression differences between IVM and IVV and it was also reported the average normalized gene expression (log2CPM) value for each group (IVM and IVV) of the genes described. Gene ontology analysis for DEGs was performed using DAVID Bioinformatics Resources version 6.8 [22] along with the PANTHER classification system version 13.0 [23].

RESULTS

Gene expression profile of cumulus cells from IVV and IVM

On average, 24 million reads per sample with >92.3% presenting quality over Q30 Phred score were generated (Supplementary Table 3.2). Data were mapped to the current equine reference genome (EquCab3.0). with an average of 91.1% mapping rate (range: 77.9 - 96.11%; Supplementary Table 3.2).

A total of 14,479 genes were detected in CC with 69% being categorized as protein-coding genes. The heat map representing the 1500 most variable genes is presented in Figure 3.1 showing clear expression patterns among the two groups. When samples were graphed using the principal component analysis plot, a clear clustering can be observed for the IVV samples while IVM samples were more scattered (Figure 3.2).

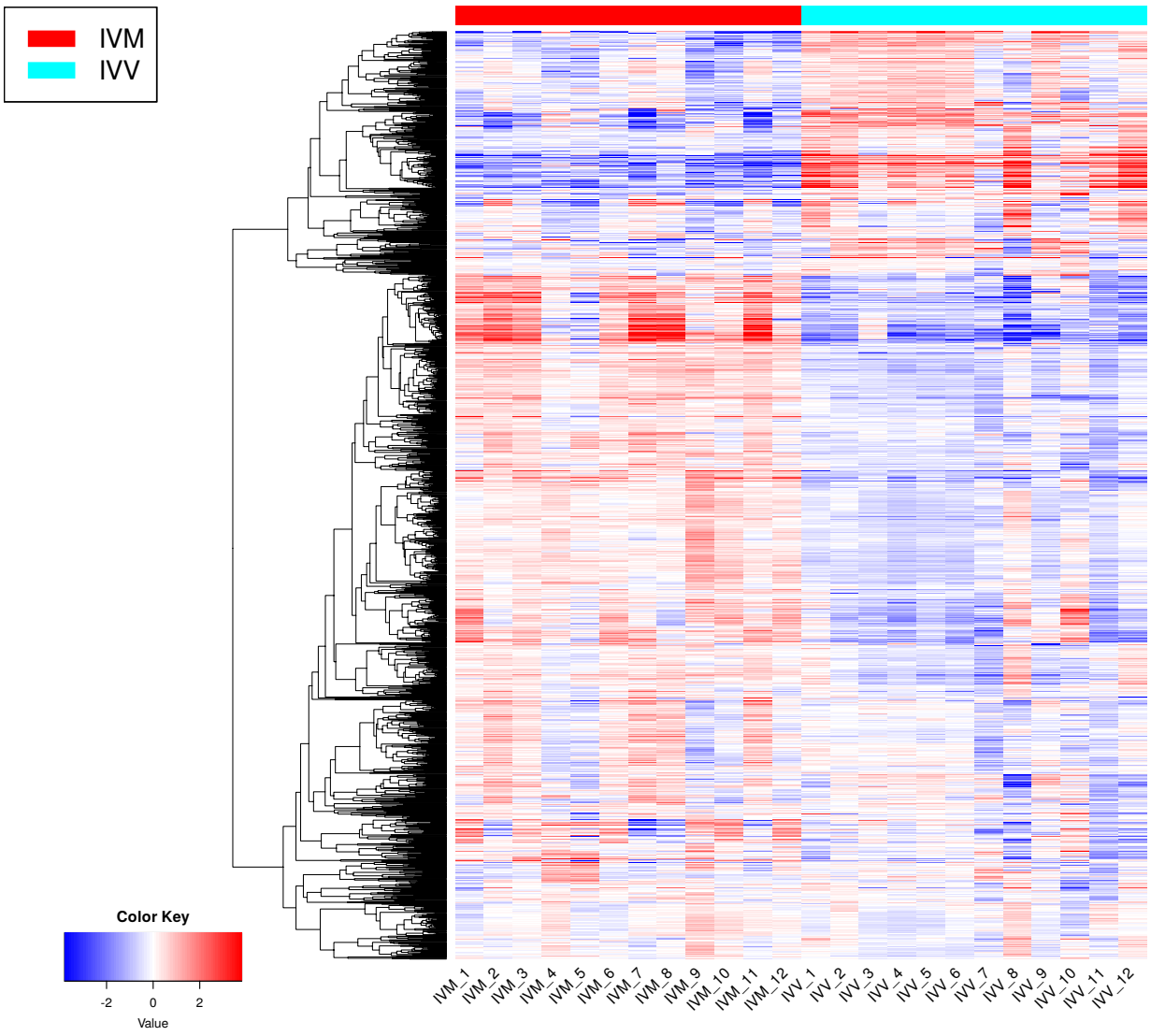


Figure 3.1. Heat map of the 1500 most variable genes comparing CC from IVM (A) to IVV (B) samples. The heat map was created using read counts values with a step of data normalization using the EdgeR: log2(CPM+c) transformation. Normalized gene expression is indicated in a color scale with red indicating high expression and blue low expression.

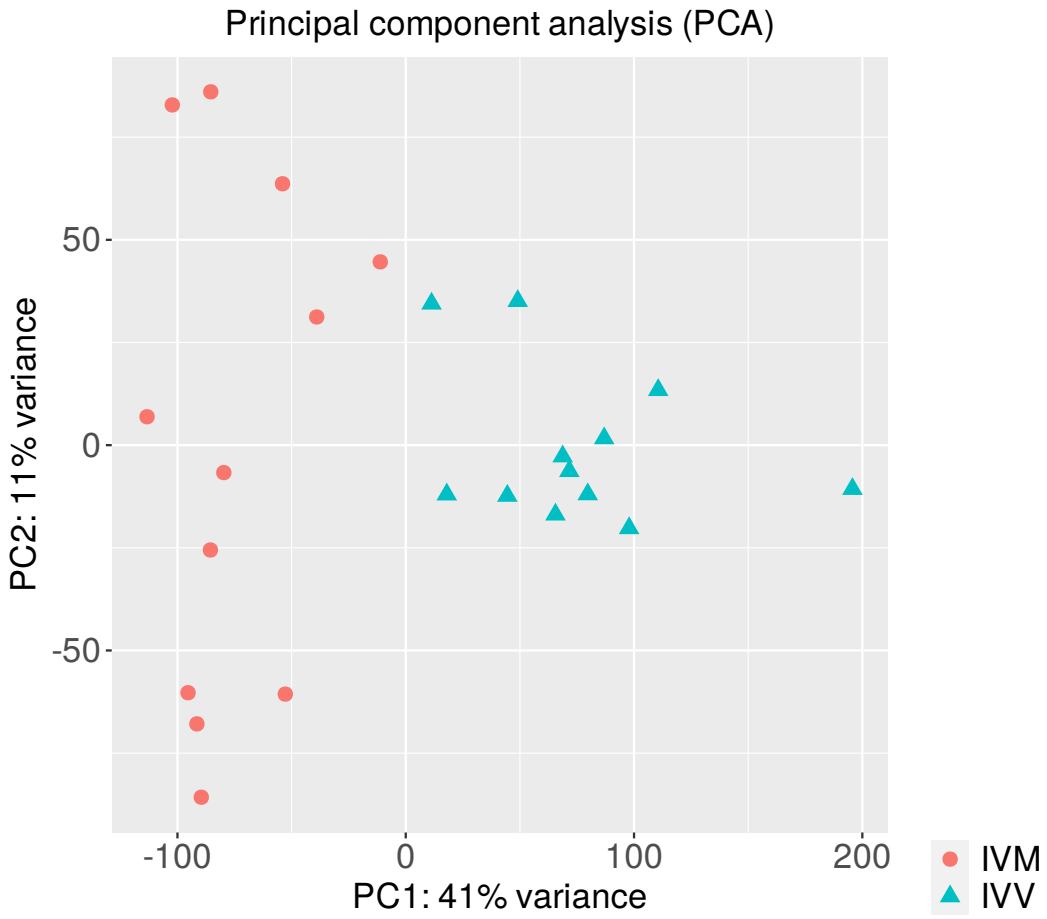


Figure 3.2. Principal Component Analysis (PCA) represents the divergence in gene expression between samples and between groups. Samples from the IVV groups clearly group closer compared to the samples from IVM. Read counts were normalized using the log2(CMP+c) transformation and defined a minimal count per million (CMP) of 0.5 to perform PCA and construct the plot.

Differentially expressed genes between IVV and IVM

A total of 6,661 differentially expressed genes (DEG) were observed when comparing IVM to IVV (fold change 1.5; FDR <0.1) with 2,180 highly expressed and 4,481 lowly expressed genes in the IVM samples relative to IVV samples. The relative higher or lower expression in IVM samples used the IVV group as the base for the comparison, under the assumption that IVV holds a transcriptomic associated with better

developmental competence. The number of genes showing lower expression in IVM is twice as much as those presenting higher expression and this difference can be appreciated in the volcano plot (Figure 3.3). The GO analysis of enriched pathways associated with DEGs that identifies biological, cellular, or molecular components enriched in the DEG list revealed that genes with lower expression in the IVM group were associated with components as cell periphery, plasma membrane, extracellular space, extracellular region, cell surface, and integral component of the plasma membrane. While genes with higher expression in the IVM samples were related to the cell cycle, chromosomes, mitotic cell cycle, nuclear division, DNA metabolic process, nuclear chromosome segregation, and organelle fission (Table 3.2).

Following the finding from Chapter 2 of this dissertation which discussed the potential most relevant genes associated with maturation, a more targeted analysis was conducted for this data set. One of the aspects of transcriptomic dynamics during maturation observed previously was the increase in the expression of transcripts of the Follicle-Stimulating Hormone receptor (*FSHR*) in the CC-MII group. In the current study, it was observed that IVM samples (log2CPM: 10.4) presented an increased expression (log2FC: 3; adj-P: 0.000000000268) of *FSHR* compared to IVV (log2CPM: 8.0). Also, the Luteinizing Hormone/Choriogonadotropin receptor (*LHCGR*) presented a lower expression (log2FC: -2.1; adj-P: 0.0000669) in IVM samples (log2CPM: 4.9) compared to IVV samples (log2CPM: 7.15). Just like in the previous study, it was not detected the transcription of *FSHB* nor *LHB*.

The Anti-Mullerian hormone (*AMH*) (log2FC: 2.7; adj-P: 0.0000000626) showed a higher expression in IVM (log2CPM: 10.7) compared to IVV samples (log2CPM: 8.19)

and Anti-Mullerian hormone receptor (*AMHR2*) (log2FC: 1.2; adj-P: 0.0000629) also was part of the DEG list with a higher expression in IVM (log2CPM:10.9) versus the IVV samples (log2CPM: 9.6).

It was also observed that the inhibin subunit α (*INHA*) (log2FC: 1.4; adj-P: 0.000873) displayed a higher expression in IVM (log2CPM: 17.4) compared to the expression in IVV (log2CPM: 16.1) same as and inhibin subunit β -A (*INHBA*) (log2FC: 1.34; adj-P: 0.0000657) that showed a higher expression in IVM (log2CPM: 15.5) samples relative to IVV (log2CPM: 14.1). Finally, the Follistatin gene (*FST*) showed a higher expression (log2FC: 2.1; adj-P: 0.00000429) in the IVM group (log2CPM: 12.3) compared to the IVV group (logCPM: 10.1).

This analysis also included the assessment of genes related to cumulus expansion that have been reported in the literature for other species. These include the Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) which shows a lower expression (log2FC: -2.9; adj-P: 0.00144) in IVM samples (log2CPM: 3.6) compared to IVV (log2CPM: 6.4). The TNF Alpha Induced Protein 6 (*TNFAIP6*) (log2FC: -2.5; adj-P: 0.00048) that displayed a lower expression in IVM (log2CPM: 6.1) compared to IVV (TPM:10), Finally, we reported differences in the expression of two important genes associated to cumulus expansion, there were the Hyaluronidase Synthase 1 (*HAS1*) that displayed a relative high fold change compared to other genes assessed (log2FC: -4.7; adj-P: 0.0000000225) with a lower expression in IVM samples (log2CPM: 3.1) compared to the IVV (log2CPM: 6.2), and the Hyaluronidase Synthase 2 (*HAS2*) (log2FC: -1.4; adj-P: 0.0246) that also presented a higher expression in IVV samples (log2CPM: 9.0) compared to the IVM group (log2CPM: 6.9).

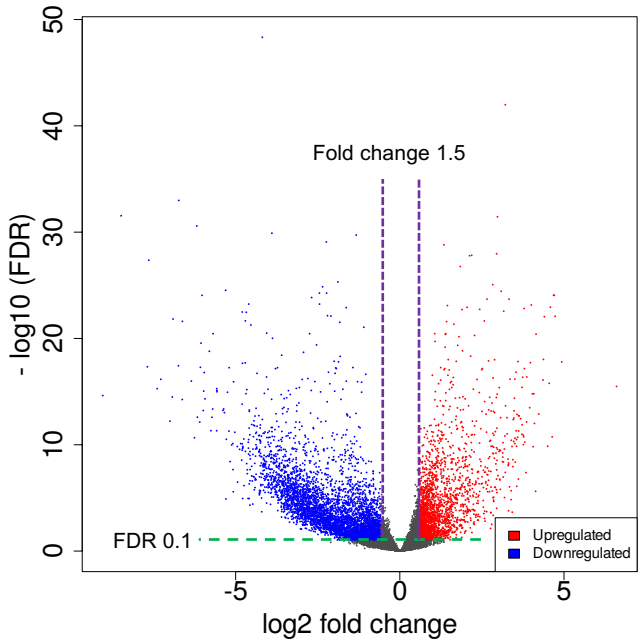


Figure 3.3. Differentially expressed genes (DEG) are represented in a volcano plot graphically denoting the greater number of downregulated genes (blue) on the left compared to upregulated genes (red). The dotted green line denotes the FDR cutoff of 0.1 and the purple dotted line the fold change setting of 1.5.

Table 3.1. List of enriched pathways in DEGs of downregulated (blue) and upregulated (red) genes.

Direction	adj. P value	n Genes	Pathways
Down regulated	1.7E-40	1211	Cell periphery
	5.9E-29	1076	Plasma membrane
	2.4E-27	343	Intrinsic component of plasma membrane
	1.4E-26	324	Integral component of plasma membrane
	2.4E-20	426	Cell projection
	1.2E-18	408	Plasma membrane bounded cell projection
	2.8E-15	129	External encapsulating structure
	5E-15	128	Extracellular matrix
	1.4E-13	232	Plasma membrane region
	4.2E-13	368	Cell junction
	8.7E-13	119	Receptor complex

	1.6E-11	80	Collagen-containing extracellular matrix
	0.000000001	190	Cell surface
	8.8E-09	215	Neuron projection
	0.000000059	142	Anchoring junction
Up regulated	1.1E-52	645	Non-membrane-bounded organelle
	1.1E-52	645	Intracellular non-membrane-bounded organelle
	4E-45	622	Membrane-enclosed lumen
	4E-45	622	Organelle lumen
	4E-45	622	Intracellular organelle lumen
	2.4E-44	242	Chromosome
	1.9E-36	558	Nuclear lumen
	8.6E-34	681	Protein-containing complex
	8.6E-34	95	Chromosomal region
	4.3E-30	72	Chromosome, centromeric region
	1.3E-28	490	Nucleoplasm
	1.2E-26	76	Condensed chromosome
	4.1E-26	57	Condensed chromosome, centromeric region
	1E-25	54	Kinetochore
	7.5E-21	445	Cytosol

Cumulus Expansion Measurements

Since it is well described in the literature that the cumulus expansion is notoriously less in IVM compared to IVV, it was reported the rate of expansion for COCs in IVM that reached the MII stage. As explained in M&M, due to the limited image field size of MiriTL®, the measurements only included COCs that fitted the image and the edge of the cumulus could be identified, thus the interpretation of cumulus expansion does not include that of COCs that contain a big number of cells and this need to be noted for any data interpretation. It was observed that the cumulus mass expands in a linear fashion

throughout the 24 h of IVM culture. Also, the growth tended to be greater in the first twelve hours while in the last twelve hours the expansions reached a plateau as can be observed in Supplementary Figure 3.2. The average values used to construct the plot are presented in Supplementary Table 3.1.

DISCUSSION

As mentioned before, the "*in vivo* mature oocyte" definition is technically incorrect, and it should probably be referred to as "dominant stimulated follicle oocyte" or "*in vivo* maturing oocyte" since at the moment of collection, they have physiologically initiated the process of maturation in the dominant follicle due to the gonadotropin stimulation. However, the protocol used in this study, similar to that used widely in equine ICSI laboratories, includes a period of *in vitro* culture that potentially can affect the gene expression on CC observed in a strictly "real" *in vivo* matured oocyte. Nonetheless, the main aim of this study was to determine the gene expression in CCs of those COCs following this protocol of gonadotropin induction and posterior in vitro culture.

In vitro matured oocytes are known to have a lower developmental competence compared to their *in vivo* matured counterparts [24], [25]. The developmental success achieved in our lab is around 30% for those IVM MII oocytes injected by ICSI compared to a substantially higher 70% success reported in IVV MII oocytes [2]. IVV oocytes have the advantage of natural selection since they were aspirated from the dominant follicle present in the ovary, moreover, this follicle provides the appropriate condition to stimulate the oocyte and CCs to correctly mature, while the IVM culture seems to lack most or at

least some of the key signaling mechanisms necessary to activate the critical pathways necessary for the correct maturation of a competent oocyte. Cumulus cells of the innermost corona radiata cells contain membrane extensions known as transzonal cytoplasmic processes (TZP) that penetrate the zona pellucida into the oocyte. This arrangement permits regulatory crosstalk between the oocyte and CC [26]. For this reason, CC studies can provide a useful array of potential markers that could be associated with the future developmental fate of their associated oocytes.

The results showed a similar expression signature among samples for each group as can be observed in the heat map. Also, IVV samples formed a close cluster, opposite to the IVM samples that were more scattered in the PCA plot. The gene expression similitude observed among IVV samples could be due to the homogeneous follicle population from where they are obtained from, which creates a population of COCs that were subject to very similar conditions before the CC were removed.

It was also determined that there is a great number of DEGs between IVV and IVM, even though both groups of oocytes are theoretically in the same MII stage. The analysis of enriched pathways, and biological, cellular, and molecular components among the DEGs, revealed pathways related to the cell cycle, nuclear division, DNA replication, and chromosomal and sister chromatid segregation were associated with highly expressed genes in the IVM group. To the best of the authors' knowledge and after an exhaustive literature search, there is no indication of increased mitotic activity of CC during oocyte maturation. Moreover, the observation of the COC expansion on the time-lapse recording obtained in the MiriTL® also supports that CC are not actively dividing during this time and that the physical expansion of the cumulus is not due to an increase

in the number of cells but rather to an extensive reorganization of cytoskeleton structures through the assembly of acting microfilaments and the synthesis of proteoglycans and glycosaminoglycans, especially Hyaluronic acid, that allow the cumulus to transform from a tightly compacted group of cells into an enlarged complex of mucified cells [27], [28]. However, this finding could indicate that the cell cycle regulation is relatively hyperactivated in IVM CC compared to those IVV CC group. This could potentially be explained by the fact that IVM maturation medium is basically formulated on a base of a cell culture media that include ingredients like growth factors that stimulate cell growth. Although, this explanation loses validity since the IVV COCs were also cultured in a medium formulated with cell culture media, even though for a relatively shorter period. Another hypothesis is that this upregulation of genes related to the nuclear division could be part of the controlling function of CC over the oocyte. It was presented in Chapter 2 that genes associated with cell cycle and nuclear division were highly expressed in IVM OC-MII (compared to OC-GV). Potentially suggesting that IVM might be actively regulating pathways associated with nuclear maturation in the oocyte and in CC. Unfortunately, the extent of the results in this study is insufficient to suggest the real cause of this high expression of genes related to nuclear division.

Another finding in the DEGs list corresponds to differences in the expression of inhibin genes. Inhibin is a heterodimer disulfide-linked glycoprotein of the transforming growth factor -β (TGF-β) superfamily [29] formed by a common α-subunit (*INHA*) linked to one of two β-subunits, β-A (*INHBA*) or β-B (*INHBB*), forming two biologically active forms, inhibin-A, and inhibin-B, respectively [30]. There was an increased expression of *INHA* and *INHBA* and a reduced expression of *INHBB* in IVM compared with IVV samples.

The upregulation of INHA and INHBA suggests that during IVM there might be an increase in the synthesis of inhibin A by CCs. As shown in Chapter 2, the concentration of inhibin A increases proportionally to the follicle size being the highest concentration in pre-ovulatory follicles. Another relevant TGF-β family molecule relevant to oocyte maturation is the anti-Mullerian hormone (AMH) which is a homodimer with a disulfide-linked glycoprotein [31], [32]. *AMH* and its receptor 2 (*AMHR2*) were found to have a higher expression in the IVM group compared to IVV. It has been shown that AMH expression in CC is regulated by the oocyte depending on the follicular stage, having an up-regulatory stimulus during pre-ovulatory follicle development [33]. Furthermore, this high expression of AMH in the CC of IVM COCs was determined in Chapter 2. This suggests that the IVM culture conditions as compared to the *in vivo* environment, are causing this higher expression of the AMH pathway. These gene expression differences observed in Chapters 2 and 3 of this dissertation, suggest that AMH and inhibins might be key candidates for further studies determining their precise function in oocyte maturation in horses.

It was observed that gonadotropin receptors displayed differences in their expression in the two groups. This suggests that they could be an important factor causing the developmental disparities observed between IVM and IVV oocytes. As determined in Chapter 2, the expression of *FSHR* is higher, while the expression of *LHCGR* is lower in CC-MII compared to CC-GV. In this study, it was also found that these two genes are differentially expressed between the IVV and IVM groups, with *FSHR* presenting a higher expression while *LHCGR* had a lower expression in the IVM than in the IVV samples. One factor to consider in these results is the content of gonadotropins in the IVM media

used during maturation culture. The used formulation includes ovine FSH (8.8 mU) along with 6 % equine follicular fluid (FF) obtained from a DSF [14]. FF is a non-defined medium; thus, the levels of gonadotropins present are unknown. Most of the maturation media formulations described in the literature for equine oocytes only include FSH, however, commercially available options claim to contain LH as well, although the hormone source and concentrations are not disclosed. The exact effect of FSH on the CC and the subsequent chain effect on the oocyte is not clearly described for the horse. However, it has been shown that FSH stimulates bovine CC to produce a meiosis-activating substance which is then secreted to the oocytes. Moreover, the author showed that such results cannot be caused by LH or hCG [34]. Cadenas et al 2021 [35], showed that the addition of at least 70 IU/L FSH caused an upregulation of *LHCGR* and a downregulation of *FSHR* in CC of matured human oocytes, however, when no FSH is added or a lower concentration of FSH (<70 IU/L) is used, the opposite effect is observed on the CC. This finding suggests that the concentration of gonadotropin used in the IVM media of this study could be the reason for the differences in gonadotropin receptor expression observed between IVV and IVM groups. Alternatively, the difference observed could also be caused by the gonadotropin concentration that IVV are subject to during the period of time before they are aspirated. Regardless, this suggests the next step in the research that would look into the effect of different concentrations of FSH and possible different FSH source on CC culture as a model for oocyte maturation.

Cumulus expansion could also be affected by FSH, as FSH stimulation of production of hyaluronic acid has been shown in murine COCs [27]. It is postulated that one possible pathway through which FSH directs the cumulus expansion of porcine COCs

is through a G protein-coupled receptor of the C family, the calcium-sensing receptor (CASR). The expression of *CASR* increases in CC during the GV breakdown period occurring when oocytes resume meiosis. Moreover, when a CASR- agonist is used, it results in the upregulation of the *HAS2* while a CASR-antagonist results in the downregulation of *HAS2*, *PTGS2* and *TNFAIP6* [36]. The results of the current study show that *HAS2*, *PTGS2*, *TNFAIP6*, and *HAS1* have a lower expression in the IVM compared to the IVV samples. In bovine COCs, its was shown that *TNFAIP6* and *FST*, both genes related to cumulus expansion, were upregulated in the CC of IVV COCs [13]. This also has been shown in COCs from humans that reported the downregulation of genes *TNFAIP6, FST and PTGS2* in *in vitro* matured COCs [37]. Our results show that *FST* has a higher expression in the IVM group relative to IVV. The gene profile of the other genes related to cumulus expansion seems to coincide to that reported in the literature. Moreover, we reported that genes of the DEG list with lower expression in IVM were associated with components of the extracellular matrix. This is important since it provides a logical explanation for the differences in cumulus expansion observed between IVM and IVV COCs that have been previously reported [38].

Finally, the morphometric analysis of the time lapses of COCs during IVM showing the volumetric expansion of the cumulus with hourly measurements of the area (μm^2) indicates that measured COCs expanded at a similar rate. As discussed above, it is suggested that FSH could have a direct effect on the expansion of the cumulus, however, the presence of substrate for hyaluronic acid synthesis is also a key factor. Chen et al., [39] demonstrated that when rat COCs are matured in the absence of fetal bovine serum (FBS), the expansion is significantly affected, regardless of the presence of FSH or not.

The maturation medium used for the IVM groups in this experiment includes 6% FBS. Other protocols described in the literature for IVM of equine oocytes include up to 10% FBS [2], [40]. However, a comprehensive study comparing the outcome of different maturation media in maturation rates or cumulus expansion for equine COCs has not been published.

CONCLUSIONS

The results obtained in these experiments confirm the results of Chapter 2 and show the expression of gonadotropin receptors in CC of IVM and IVV groups. Moreover, it was determined that there is a higher expression of *FSHR* and a lower expression of *LHCGR* in the IVM samples. It was also determined that genes and pathways related to the extracellular matrix and cumulus expansion show a lower expression in IVM samples relative to the IVV group, which is suggested as a logical explanation for the reduced cumulus expansion of IVM COCs compared to the greater expansion exhibited by COCs obtained from pre-ovulatory follicles. Additionally, IVM was associated with a higher expression of genes related to the cell cycle, chromosome segregation, and nuclear division.

These data provide a list of suggested important genes and pathways in the IVM culture conditions and serve as a solid starting point for potential targeted modifications of the IVM protocols.

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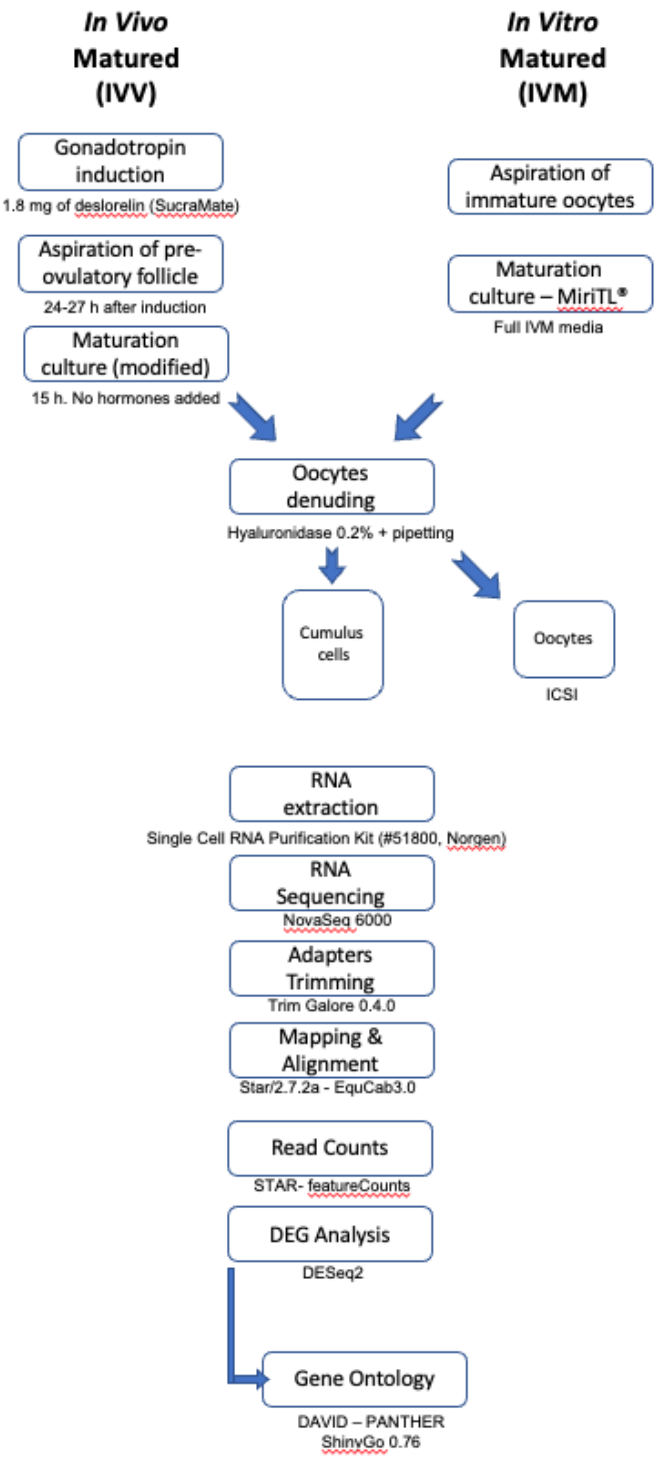
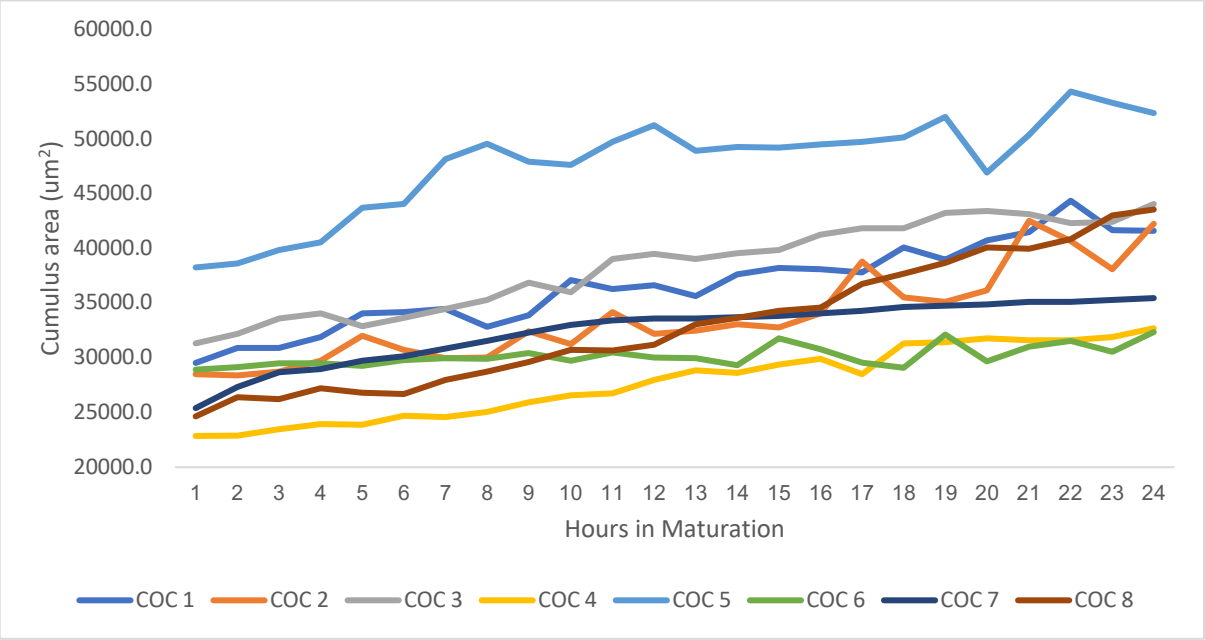
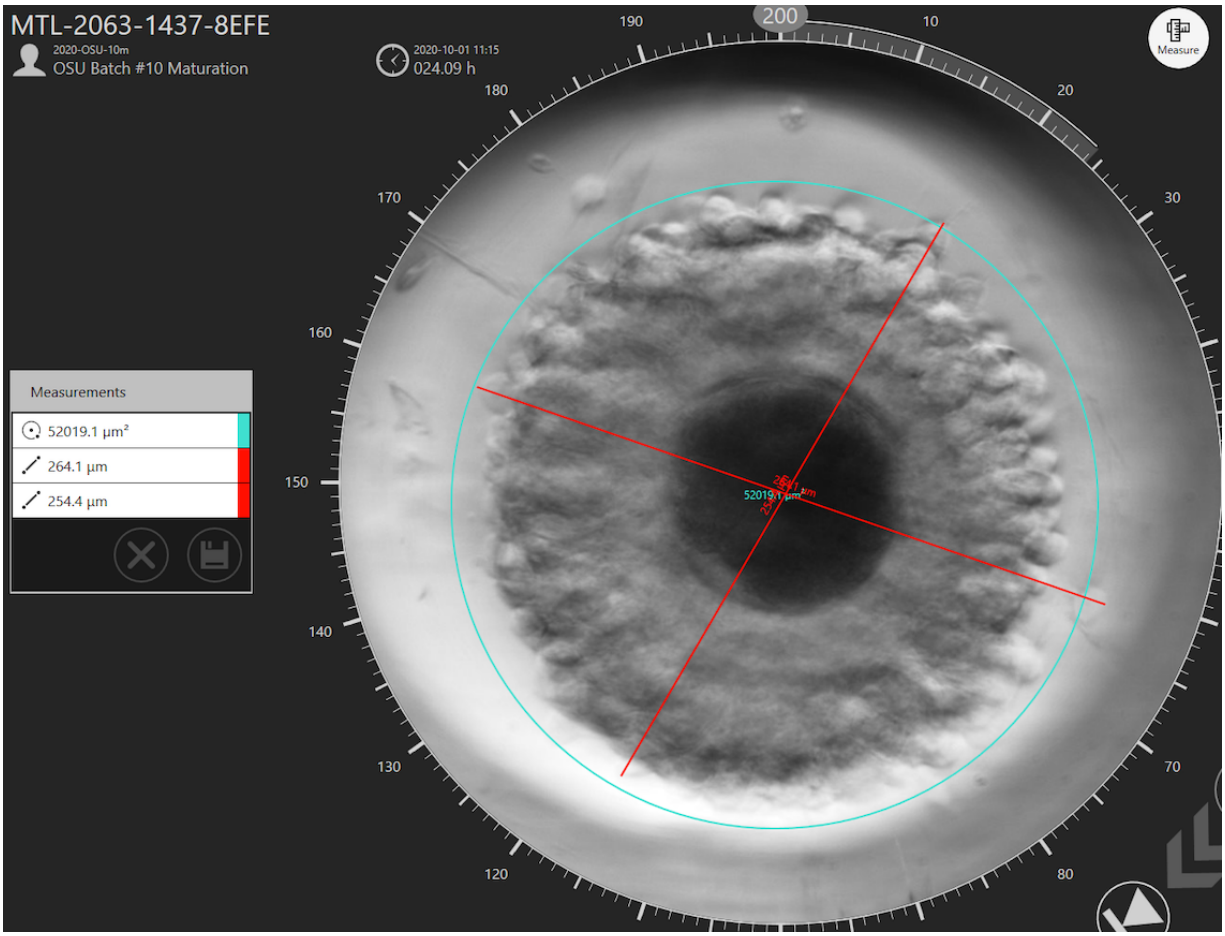
SUPPLEMENTARY DATA



Supplementary Figure 3.1. A pipeline representation of the methodology used for sample acquiring, processing, and RNA analysis.



Supplementary Figure 3.2. Linear plot of cumulus area (μm^2) measurements of eighth COCs matured *in vitro* for 24 hours in the MiriTL® Time-lapse imaging incubator. All these COCs were associated with an oocyte that reached the MII stage by the end of IVM. Measurements were obtained using the software built-in diameter- and area-measuring tool. I calculated the average diameter of the cumulus from two different points, this value was averaged with the measurement of the area-measuring tool to obtain the final value used to construct this plot.



Supplementary Figure 3.3. Example of one of the cumulus area measurements. The measurements were performed every hour during the 24 hours of IVM culture using the MiriTL® software built-in measuring tool for diameter (red lines) and area (green circle).

Supplementary Table 3.1. Cumulus area measurements were obtained every hour during the 24 hours of IVM culture using the MiriTL® software. The average diameter was calculated by measuring two perpendicular sides of COCs and then using the area (A) of a circle formula: ($A = \pi r^2$). The surface area (um^2) was also determined by using a second MiriTL® tool capable to determine the area of a drawn circle. The average value from both methods are presented in this table.

Hours	COC 1	COC 2	COC 3	COC 4	COC 5	COC 6	COC 7	COC 8
1	29541.8	28484.0	31327.9	22862.6	38268.0	28926.0	25405.2	24649.2
2	30921.6	28395.8	32215.4	22883.0	38651.9	29158.2	27360.2	26417.7
3	30927.9	28752.9	33594.5	23493.5	39865.6	29511.4	28704.8	26204.5
4	31882.9	29761.0	34049.7	23960.6	40577.7	29500.8	28962.6	27215.7
5	34043.9	32007.5	32900.7	23914.3	43726.2	29256.0	29746.0	26825.5
6	34207.6	30724.5	33654.9	24715.3	44064.0	29803.4	30164.3	26708.9
7	34503.2	29971.2	34481.5	24583.2	48166.4	29989.3	30849.3	27976.5
8	32828.9	30052.3	35304.6	25048.9	49554.8	29887.1	31563.1	28758.2
9	33864.3	32456.0	36887.4	25949.6	47950.5	30423.1	32342.3	29630.9
10	37084.2	31245.1	35973.3	26576.5	47656.7	29733.7	33007.4	30718.7
11	36269.7	34176.2	39071.0	26747.9	49726.8	30482.4	33409.6	30664.4
12	36641.9	32204.8	39526.1	27989.1	51253.9	30060.2	33589.7	31223.3
13	35631.4	32500.5	39067.7	28842.0	48927.9	29946.0	33617.7	33089.9
14	37615.0	33063.1	39558.2	28612.3	49272.2	29349.3	33724.5	33683.4
15	38218.8	32803.6	39875.9	29407.0	49217.6	31769.1	33863.3	34313.1
16	38115.0	34094.8	41275.5	29941.8	49530.9	30767.2	34049.8	34579.0
17	37838.7	38779.9	41864.9	28506.9	49725.1	29570.3	34317.9	36733.1
18	40076.9	35525.2	41831.4	31347.3	50161.7	29102.3	34649.9	37670.1
19	39002.3	35126.7	43244.2	31422.6	52046.6	32117.8	34759.4	38666.7
20	40753.8	36159.3	43432.0	31776.7	46944.6	29700.3	34908.0	40079.8
21	41508.4	42535.6	43114.3	31625.3	50379.0	31031.3	35098.5	39961.2
22	44372.2	40656.1	42309.6	31599.3	54349.0	31566.2	35142.2	40868.3
23	41653.0	38111.9	42416.8	31893.7	53294.8	30558.1	35293.6	43023.2
24	41634.9	42261.0	44069.6	32708.7	52389.7	32361.8	35465.5	43565.5

Supplementary Table 3.2. Quality control of sequenced data with a description of the number of reads, mapping rates, and quality control (%>=Q30) obtained for all CC samples. Reads were aligned to the horse reference genome (EquCab3.0) using STAR (Release 2.5.3a).

Sample type	Mapping rate (%)	Raw reads	%>=Q30
InVitro	96.11	25,389,028	92.35
InVitro	95.47	25,804,287	92.3
InVitro	94.22	26,172,728	92.22
InVitro	92.84	22,630,489	89.71
InVitro	92.73	24,020,971	92.65
InVitro	95.34	24,043,687	92.25
InVitro	94.98	23,621,634	91.43
InVitro	94.54	24,635,197	91.55
InVitro	91.4	23,788,558	92.79
InVitro	90.35	23,840,329	92.71
InVitro	94.77	24,912,327	91.47
InVitro	90.49	24,407,971	92.23
InVivo	90.75	22,106,316	93.24
InVivo	89.46	23,867,488	92.95
InVivo	89.1	22,097,680	93.11
InVivo	87.54	22,039,360	92.65
InVivo	92.11	22,440,536	92.69
InVivo	89.86	23,591,014	92.85
InVivo	82.92	22,106,316	92.81
InVivo	92.87	21,495,441	91.59
InVivo	93.02	27,113,384	92.42
InVivo	89.38	24,391,520	92.43
InVivo	77.9	25,599,018	92.76
InVivo	88.63	24,138,150	92.2

DISSERTATION CONCLUSIONS

The experiments presented in this dissertation expand our understanding of the IVM of the oocyte in the horse and provided new insight into the differences observed between *in vitro* and *in vivo* maturation methods, giving a starting point and guided suggestions for the next steps in our investigation of oocyte maturation in the horse.

In Chapter 2, it was hypothesized that the gene expression of oocytes and CC changes during IVM and that their transcriptome signature could reveal important cellular, molecular, and/or biological pathways relevant to the physiology of maturation. Based on the findings, It was determined that the gene expression of gonadotropins receptors is different between CC-MII and CC-GV, showing upregulation of *FSHR* and a downregulation of *LHCGR* in CC-MII samples relative to CC-GV. Additionally, it was reported the expression of *CGA* in OC samples. It was also determined the expression of TGF-ß family molecules like *AMH*, *INHA,* and *INHBA* in CC samples and that the concentration of AMH in follicular fluid peaks in follicles of size 15 mm and then decreases in the growing follicle. In contrast, the concentration of inhibin A increases as the follicle grows.

In Chapter 3, the aim was to determine the differences in the transcriptomic profile of CC between IVV and IVM, two types of maturation used to obtain an MII oocyte for IVP in the horse. It was found numerous DEGs between IVV and IVM samples. Moreover, significant differences in the expression of *FSHR* and *LHCGR* between IVM and IVV samples were reported. This helps sustain the idea that gonadotropins play an important role in the physiology of oocyte maturation in horses and further studies regarding these molecules and receptors is a logical continuation of this research. Finally, it was

determined that genes associated with cumulus expansion were exhibited a lower expression in the IVM than IVV samples. Additionally, it was reported the expansion rate of horse COCs, and that the cumulus presents a linear increment in the area during IVM culture with a constant increase during the first twelve hours reaching a plateau during the last twelve hours.

In conclusion, these results suggest that IVM of equine COCs involves changes in the transcriptome of the oocyte and the surrounding CC and the genes and associated pathways described here might be important aspects of maturation that need to be revised more in depth for the optimization of maturation media formulations and enhancement of maturation culture protocols.