Gonadotropin treatment increases the expression of genes involved in follicular growth and oocyte maturation in granulosa cells of prepubertal Holstein heifers

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“What you plant now, you will harvest later.”

- Og Mandino

ABSTRACT

There is evidence suggesting that acquisition of developmental competence in bovine oocytes may be linked to the expression profile of genes in the mural granulosa cells (GC). To gain insight into the potential molecular changes occurring within GCs during the acquisition of oocyte competence, we have performed bi-weekly laparoscopic ovum pick-ups (LOPU) and collected cumulus-oocyte-complexes (COC) and mural GCs from 2-10mm follicles in 12 Holstein heifers from 2-6 months of age. While 3 heifers remained untreated (control) for the duration of the experiment, 9 received varying follicle stimulating hormone treatments (FSH-treated) to mimic an active hypothalamus-pituitary-ovarian axis. Adult GCs were collected from 8 FSH-treated adult cows. The COCs from prepubertal animals were matured, fertilized and cultured *in vitro* to assess development to the blastocyst stage. The relative mRNA abundance of *FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP* in GCs were quantified by RT-qPCR. The mRNA abundances of *FSHR, CYP19A1, 3β-HSD,* and *XIAP* were significantly decreased, and *STAR* and *3β-HSD* were significantly increased in prepubertal control samples compared to adult and prepubertal FSH groups. When comparing the mRNA abundance at 3 prepubertal ages (<100, 100-130, and >130 days), we observed significantly higher expression of *STAR* (all ages)*, 3β-HSD* (≤130 days), and *FOXO1* (>130 days) in control calves, and significantly higher expression of *FSHR* (≤130 days)*, CYP19A1* (100-130 days), and *XIAP* (>100 days) in FSH-treated calves. Increasing the FSH treatment duration from 2 to 4 days lead to a significant decrease in the mRNA abundance of *STAR, 3β-HSD,* and *CALM* in prepubertal animals. *LHR* mRNA was detected in a significantly higher number of GC samples from animals treated with longer FSH protocols. Lastly, we observed significantly lower *3β-HSD* and *CX43* mRNA, and significantly higher *FOXO1* mRNA in samples from animals that resulted in above median cleavage (>70%) and blastocyst (>15%) rates, respectively. These findings revealed that GCs of prepubertal animals respond to FSH treatment by increasing mRNA levels of genes promoting estradiol synthesis and follicular growth, and by decreasing mRNA levels of genes promoting progesterone production and follicular atresia. We also observed that the relative mRNA abundance of *3β-HSD, CX43*, and *FOXO1* in GCs is correlated with embryo cleavage and development in prepubertal heifers.

RÉSUMÉ

Il existe des études suggérant que l'acquisition de la compétence au développement des ovocytes bovins peut être reliée au profil d'expression de gènes dans les cellules de la granulosa murale (CG). Pour mieux comprendre les potentiels changements moléculaires se produisant dans les CGs pendant l'acquisition de compétence des ovocytes, nous avons performé des prélèvements de complexes ovocytes-cumulus (COC) et de cellules de la granulosa murale. Ces cellules ont été prélevées de follicules mesurant entre 2-10mm par laparoscopie (LOPU) aux deux semaines sur 12 génisses Holstein âgées de 2-6 mois. Tandis que 3 taures sont resté non-traitées (contrôle), 9 taures ont reçu des montants variants d'hormone folliculo-stimulante (traitée-FSH) afin d'imiter un axe hypothalamo-pituito-ovarien actif. De plus, des CGs adultes ont été prélevées de 8 vaches Holstein traitées par injection de FSH. Les COCs des animaux prépubères ont été mis en maturation, fécondés, et culturés *in vitro* afin de déterminer leur potentiel de développement au stage de blastocyste. L'abondance relative de mARN de *FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1* et *XIAP* dans les CGs a été quantifiée par la méthode du RT-qPCR. L'abondance de mARN de *FSHR, CYP19A1, 3β-HSD* et *XIAP* était significativement réduite, et celle de *STAR* et *3β-HSD* était significativement plus élevée dans les échantillons de CGs prépubères contrôles à comparér aux échantillons adultes et prépubères provenant d'animaux traités par injection de FSH. Lors de la comparison de l'abondance de mARN aux 3 âges prépubères (<100, 100-130 et >130 jours), des expressions significativement plus élevées dans les gènes de *STAR* (touts les âges)*, 3β-HSD* (≤130 jours) et *FOXO1* (>130 jours) ont été observée dans les taures contrôles, et des expressions significativement plus élevées de *FSHR* (≤130 jours)*, CYP19A1* (100-130 jours) et *XIAP* (>100 jours) dans le groupe prépubère FSH. L'augmentation de la durée du traitement de FSH de 2 à 4 jours a mené à une réduction des mARN de *STAR, 3β-HSD* et *CALM* dans les animaux prépubères. Le mARN de *LHR* a été détecté dans un nombre significativement plus élevé de d'échantillons de CGs d'animaux qui ont reçu un long traitement de FSH. Les quantités de mARN des gènes *3β-HSD* et *CX43* étaient significativement réduites, et celles de *FOXO1* augmentent chez les échantillons qui ont mené à des taux de clivage (>70%) et de blastocystes (>15%) supérieures à la médiane, respectivement. Ces découvertes ont

révélé que les CGs d'animaux prépubères répondent aux traitements de FSH par l'augmentation des niveaux de mARN de gènes promouvant la production d'estrogène et la croissance folliculaire, et par la réduction des niveaux de mARN de gènes promouvant la production de progestérone et d'atrésie folliculaire. Nous avons aussi observé que l'abondance relative de mARN de *3β-HSD, CX43* et *FOXO1* dans les CGs est corrélée avec le clivage et le développement embryonnaire dans les génisses prépubères.

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Vilceu Bordignon and Hernan Baldassarre designed experiments, analyzed data, reviewed the manuscript and supervised the primary author. Laura Michalovic conducted all the experiments, analysed the data and wrote the manuscript. Luke Currin, Karina Gutierrez, Werner Glanzner, Yasmin Schuermann, Rodrigo C. Bohrer, Rafael G. Mondadori, Naomi Dicks, Paulo Antunes da Rosa, Matheus Pedrotti De Cesaro, Rosalba Lopez, Fernando Altamura, Paula Lunardelli, Mariana Priotto de Macedo, Milena Taibi, Ejimedo Madogwe, Audrey St-Yves, Denyse Laurin, and Jim Gourdon helped conducting the experiments and data analysis.

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LIST OF ABBREVIATIONS

3β-HSD:	3-Beta-hydroxysteroid dehydrogenase
17β-HSD:	17-Beta-hydroxysteroid dehydrogenase
A4:	Androstenedione
AC:	Adenyl cyclase
ACTB:	Beta-actin
AKT:	Protein kinase B
AMH:	Anti-Müllerian hormone
ARC:	Arcuate nucleus
ART:	Assisted reproductive technologies
ATP:	Adenosine triphosphate
AVPV:	Anteroventral periventricular nucleus
CA:	Corpus albicans
CAMII:	Calcium/calmodulin-dependent protein kinase II
cAMP:	Cyclic adenosine monophosphate
cDNA:	Complementary deoxyribonucleic acid
CG:	Cortical granule
CGA:	Chorionic gonadotropin α
CIDR:	Controlled internal drug release
Chol:	Cholesterol
CH:	Corpus hemorrhagicum
CL:	Corpus luteum
COC:	Cumulus-oocyte-complex
c-Src:	Proto-oncogene tyrosine-protein kinase Src
CYCLO:	Cyclophilin
CYP11A1:	Cytochrome P450 Family 11, Subfamily A, Member 1
CYP17A1:	Cytochrome P450, Family 17, Subfamily A, Polypeptide 1
CYP19A1:	Aromatase
CX43:	Connexin 43
DNA:	Deoxyribonucleic acid

DHEA: Dehydroepiandrosterone

DHT: Dihydrotestosterone

E1: Estrone

E2: Estradiol

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EPAC: Exchange proteins directly activated by cAMP

FOXO1: Forkhead box protein O1

FSH: Follicle stimulating hormone

FSHR: Follicle stimulating hormone receptor

GnRH: Gonadotropin-releasing hormone

GD: Gonadotropin-dependent

GI: Gonadotropin-independent

GPCR: G protein-coupled receptor

GPR54: G protein-coupled receptor 54

G_S: Stimulatory G-protein

HPG: Hypothalamus pituitary gonadal

IM: Intra-muscular

IVC: *In vitro* culture

IVEP: *In vitro* embryo production

IVF: *In vitro* fertilization

IVM: *In vitro* maturation

kDa: Kilodalton

Kiss1: Kisspeptin

LH: Luteinizing hormone

LHR: Luteinizing hormone receptor

LOPU: Laparoscopic ovum pick-up

MAPK: Mitogen-activated protein kinase

ME: Median eminence

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

mRNA:	Messenger ribonucleic acid
OPU:	Ovum pick-up
P4:	Progesterone
P5:	Pregnenolone
PBS:	Phosphate buffered saline
PDK1:	Pyruvate dehydrogenase kinase 1
PG:	Prostaglandin
PGC:	Primordial germ cell
PIP_2:	Phosphatidylinositol di-phosphate
PIP_3:	Phosphatidylinositol tri-phosphate
POA:	Preoptic area
PMSG:	Pregnant mare serum gonadotropin
RAS:	Rat sarcoma
RNA:	Ribonucleic acid
STAR:	Steroidogenic acute regulatory protein
T:	Testosterone
TC:	Theca cells

CHAPTER 1: INTRODUCTION

Canada has become an international leader in dairy genetics through the development of genetic markers for the detection of high milk production. As a result, it is no longer necessary for a producer to wait until a cow's first lactation to evaluate that animal's production capabilities. These genetic advances are not only profitable for the producer, but also for the dairy industry: one that contributes over $6 billion annually to the Canadian economy [1].

Genomic tools, like single-nucleotide polymorphism (SNP) analyses, are currently available to predict which offspring will have a higher probability of inheriting a specific genotype leading to a desired phenotype [2]. Assisted reproductive technologies (ARTs) are regularly applied in the field of animal production to decrease the intergeneration interval of animals of high genetic value [3]. These technologies consist of in vitro oocyte collection from prepubertal females by laparoscopic ovum pick-up (LOPU), followed by in vitro embryo production (IVEP). However, in cattle, it has been reported that oocytes collected from prepubertal donors yielded lower rates of cleavage and blastocyst development when compared to their adult counterparts [4-6]. The latter has been associated with lower oocyte competence at the time of follicular aspiration [7].

Prior to ovulation, oocytes require the materials exchanged during bi-directional communication with the follicle's somatic cells to grow and mature, to ultimately, acquire developmental competence. When this communication is interrupted or incomplete, the oocyte does not reach its full developmental potential leading to lower blastocyst development following fertilization [8, 9].

The focus of this research was to determine if the prepubertal follicular microenvironment lacks important factors involved in oocyte competence. This was achieved by evaluating how prepubertal calf oocytes respond to different gonadotropin-stimulation protocols. These protocols aimed at mimicking an active adult hypothalamus-pituitary-ovary (HPO) axis and at improving the acquisition of competence during the last stages of oogenesis. The mRNA abundance of genes expressed in mural granulosa cells involved in these processes were measured and compared between prepubertal and

mature cows to determine the molecular conditions associated with higher quality oocytes.

This thesis is organized in chapters that present background information about ovarian development and signalling within the ovary, followed by an article presenting experimental design, analyzed results, and discussion.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 The beginning: Primordial germ cell migration

Primordial germ cells (PGC) are the germline founders, also known as the sex cell precursors, and the only cells of the body to undergo meiosis following mitotic proliferation [10]. Mammalian PCGs are derived from the embryonic inner cell mass, more specifically, the epiblast [11]. In early embryonic life, PGCs migrate from an extraembryonic site into XX or XY gonadal/genital ridges, which will later develop into the two embryonic gonads on either side of the dorsal wall of the abdomen. Together, the germ and somatic cells assemble in order to form the backbone to functioning reproductive organs [12]. In the bovine model, PCGs are first observed within the caudal wall of the proximal yolk sac of a trilaminar (endoderm, mesoderm, ectoderm) embryo on embryonic d18. Through morphological folding of the trilaminar disc between d18-d23, creating a cylindrical embryonic body, the PGCs relocate into the embryonic hind- and mid-gut. From d23-d25, PGCs are predominantly housed within the embryonic mesonephros. Around d27, the mesonephros develops into the gonadal ridge and consequently houses the PGCs. Finally, from d27-d39, the germ cells become unevenly distributed within the sexually indifferent gonadal fold [13].

Successful migration of these stem-cell like cells is made possible by the expression of set genes, by the utilization of their filopodia [14] and integrins [15], and by guidance from the extracellular matrix (fibronectin) [16] and chemoattractants leading to the genital ridges. PGCs make their way into the bipotential gonadal ridge. Following a few rounds of cell proliferation, PCGs enter a proliferative arrest. Male PGCs, expressing the *SRY* gene on the Y chromosome, will remain arrested in the primitive testes in mitosis until puberty while female PGCs, not expressing *SRY*, will undergo meiosis and become oocytes in the primitive ovaries [10].

2.1.1 Gametogenesis

Gametogenesis is defined by the *Oxford Dictionaries* as the process in which diploid cells undergo meiosis to form gametes, also known as mature haploid germ cells. There are many contrasting differences in the gametogenic processes of males and

females to produce spermatozoa and oocytes, respectively, and even more so between species [17]. Most interestingly, in males, four gametes can be produced by meiotic cycle and meiosis is continuously initiated in a mitotically dividing stem cell population. On the other hand, in females, only a single gamete is produced per meiosis and meiosis is initiated only once after the establishment of a finite population of cells within the ovaries [18]. In addition, sperm itself only contains a haploid nucleus compared to the oocyte's cytoplasm, which carries all the necessary equipment (haploid nucleus, mRNAs, cytoplasmic enzymes, metabolic substrates, and organelles) for initiation and maintenance of embryo metabolism and development [17].

2.1.2 Oogenesis

In the mammalian female, the PGCs develop into oogonia, which are self-renewing stem cells. These cells remain with the animal for their lifetime. Oogonia further divide into a restricted number of egg precursor cells known as primary oocytes. These diploid cells undergo a first meiotic division, however, arresting in the diplotene stage of prophase 1. This arrest is maintained for different periods of time between oocytes, however, it is observed at least until puberty [17]. In addition, oocyte chromosome decondensation and transcription is observed during this time lapse, resulting in the enormous growth of the oocytes [19]. The combination of hormonal influences and the onset of estrus cycles after puberty lead to the random recruitment of oocytes to resume meiosis. As a result, secondary oocytes containing the majority of cytoplasmic content and first polar bodies are yielded. Both these haploid entities further divide. Although the first polar bodies divide into 2 new polar bodies, the secondary oocytes undergo another meiotic division, however, arresting in metaphase. Only once fertilization occurs will meiosis II resume and lead to the extrusion of the second polar body. In summary, the maturation of a single oogonium produces four progeny; 3 polar bodies and 1 mature fertilizable egg [17].

2.1.3 The ovary

The mammalian ovary ("ovary" derived from the Latin word "ovum", or egg) is the female reproductive gland and product of bipotential gonad differentiation during

embryonic life [12, 13]. It is also a paired intra-abdominal and dynamic organ essential in reproduction [20]. The ovary is involved in the production of steroid hormones (estradiol (E_2) and progesterone (P_4)), in follicular growth and development, in the coordination of cellular events leading to the development of fertilizable oocytes, and lastly, ovulation [12].

2.1.4 Ovarian anatomy

The ovary is composed of an inner and outer zone, the medulla and cortex, respectively, and of a surface epithelium found enveloping the cortex. Early embryonic migration of primordial germ cells (PGCs), or oogonia, occurs from extraembryonic sites into the genital ridges, followed by a meiotic division, allowing for the establishment of a finite population of oocytes within the ovary [13, 17]. Each oocyte is swallowed up by a primary follicle and rests within the peripheral and avascular cortical tissues awaiting recruitment. On the other hand, growing and atretic follicles can be observed in the cortical medullary border due to its rich vascularization. Stromal cells, blood vessels, branches of the autonomic nervous system, lymphatic drains, and embryological remnants can also be observed in the amygdaloid-shaped reproductive organ [20].

2.1.5 Folliculogenesis

At birth, females possess a select number of oocytes in each ovary making up their ovarian reserve or follicular pool [21]. In cattle, this fetal reserve is estimated around 130,000 healthy oocytes, varying between individuals and breeds [22, 23]. Throughout reproductive life, follicles are cyclically recruited to grow and to mature oocytes for fertilization, although the greater part of the follicular pool will never reach a preovulatory status and will undergo atresia [21, 24]. Endocrine and paracrine factors determine each follicle's fate [25]. Until ovulation, oocytes housed within primordial follicles are maintained dormant and in meiotic arrest in the diplotene stage of prophase 1 [26-28]. The primordial follicles then develop into primary, secondary, antral, and finally, Graafian/pre-ovulatory follicles. Mature oocyte(s) are expulsed from pre-ovulatory follicles at ovulation and are then capable of spontaneously resuming meiosis [17, 29, 30]. Organized and coordinated signalling events, from the hypothalamus to the anterior

pituitary, and finally to the ovary (Hypothalamus-Pituitary-Ovarian axis (HPO axis)), must occur to enable the release of a fertilizable oocyte [31].

During folliculogenesis, development, growth and maturation of ovarian follicles are achieved predominantly by somatic cell proliferation and differentiation [32]. Folliculogenesis is a three-step process: recruitment, selection, and dominance. In the first step, dormant primordial follicles undergo continuous recruitment and are enlisted into the growing follicle pool [24]. From the primordial follicle onwards, ovarian crosstalk via gap junctions and paracrine factors between somatic cells (granulosa and theca cells) and the oocyte is essential for the progression of folliculogenesis [33]. Next, follicles from the growing pool are further selected based on their ability to escape apoptosis and continue their development [24]. Lastly, subordinate follicles become suppressed by the rapid development of the dominant follicle(s) [32].

In addition, folliculogenesis can also be separated into two phases: the gonadotropin-independent (GI) and gonadotropin-dependent (GD) phases [34]. GI development is observed in germ cells, and primordial, primary and secondary follicle stages. As germ cell nests breakdown, individual germ cells become surrounded by a single layer of squamous pre-granulosa cells and together known as the primordial follicle. Next, the pre-granulosa cells observed in the primordial stage become cuboidal shaped granulosa cells, and together with the oocyte, form the primary follicle. Lastly, the secondary follicle is defined by acquiring two or more layers of cuboidal granulosa cells covered by a basement membrane that will in turn become enveloped by a layer of theca cells [12]. On the other hand, antral follicles and ovulation are dependent on gonadotropins [35]. Antral follicles are characterized by the development of fluid-filled spaces between the granulosa cells and the oocyte. Next, these spaces merge to become the larger antrum of the preovulatory or Graafian follicle. Two distinct types of granulosa cells are observed in the Graafian follicle: the mural granulosa cells which lie right beneath the basement membrane and the cumulus cells which directly surround the oocyte. Cumulus cells and the oocyte form the intricate cumulus-oocyte-complex (COC). In addition, two or more layers of theca cells are observed surrounding the basement membrane [20]. Gonadotropins, being follicle stimulating hormone (FSH) and luteinizing hormone (LH), are secreted into circulation from the anterior pituitary gland following

stimulation from hypothalamic hormone gonadotropin-releasing hormone (GnRH). Both FSH and LH target ovarian cells and heavily contribute to follicular growth and steroidogenesis, oocyte competence, and ovulation [34, 35]. The ovulating follicle(s) undergoes luteinization and a progesterone-producing corpus luteum (CL) is formed [36].

Regarding mammalian females, individual reproductive life spans are determined by the number of primordial follicles in their finite pools of oocytes. As many developing follicles undergo recruitment, the majority undergo atresia, contributing to the slow depletion of the oocyte ovarian reserve leading to reproductive senescence [12].

2.1.6 Preparing the oocyte: Bi-directional cell communication

Female fertility is dependent on the coordinated development of oocytes and their ovarian follicular environments [8]. During folliculogenesis, the latter is accomplished through bi-directional signalling, or ovarian cross-talk, between the oocyte and somatic cells (cumulus, mural granulosa, and theca cells), Figure 1 [9, 37]. Intrafollicular communication is most commonly achieved by the dynamic exchange of small molecules through specialized gap junctions and by receptor-mediated paracrine signalling [9]. These processes are observed from primordial follicular stages up to ovulation [38].

In addition, previous studies have linked the developmental competence of bovine oocytes with gene expression patterns of follicles' mural granulosa cells [39]. Similar findings have been reported in rats [40] and in humans [41]. A competent oocyte is set apart from an incompetent counterpart partly based on the differential expression of specific gene markers in granulosa cells associated with oocyte developmental competence. However, the underlying mechanisms allowing for the synchronous maturation of ovarian follicles and the oocytes housed within leading to oocyte competency are not completely understood [39].

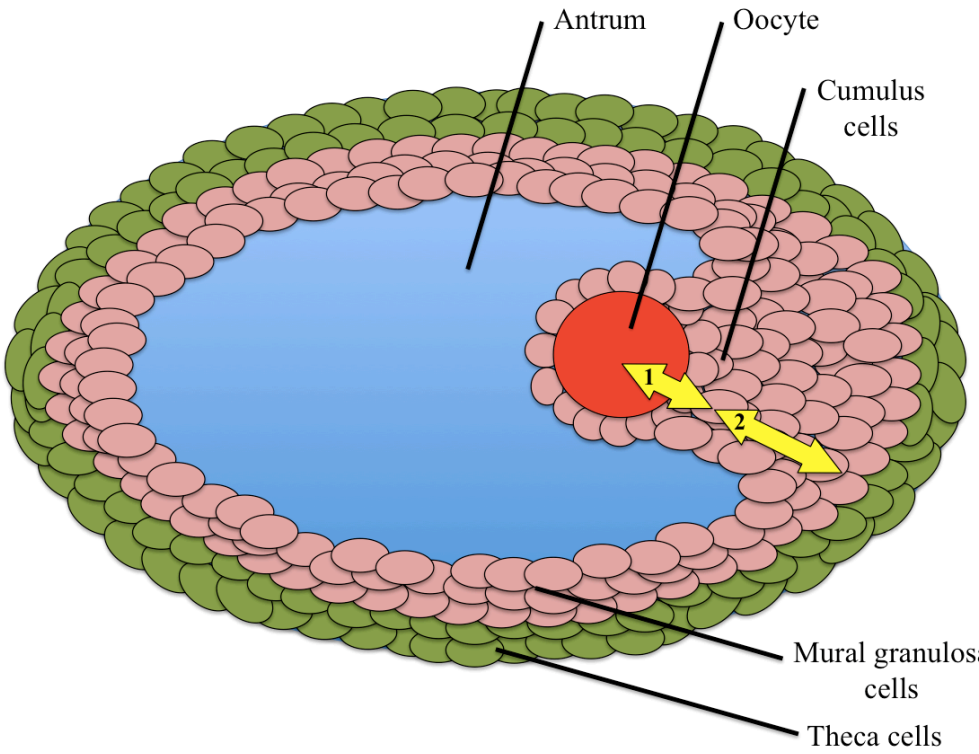

Figure 1: Antral follicle displaying bi-directional communication between the oocyte and cumulus cells (Arrow 1), and between the cumulus and mural granulosa cells (Arrow 2). Together, they allow for exchange of materials between oocyte and mural granulosa cells contributing to oocyte competence.

2.1.7 Oocyte Competence

An oocyte is deemed competent when possessing the ability to undergo meiosis, be fertilized, and produce a healthy embryo [9]. Individual oocyte quality varies tremendously and not all develop to an embryo stage [39, 42]. The latter may be associated with poor oocyte maturation throughout folliculogenesis [39]. However, it is unknown which mechanisms are at play in order for oocytes to gain developmental competence during follicular maturation [43]. Literature suggests that the developmental competence of mammalian oocytes is highly dependent on intercellular communications between oocytes and surrounding follicular cells. More specifically, due to their close association, granulosa cells (GC) are thought to reflect the degree of maturation of the oocyte they support [9, 38, 43]. Consequently, oocyte developmental failure is most commonly associated with the interruption of these communications [44]. In addition, it has been shown that individual oocytes' protein profiles do not influence developmental

competence implying that other factors are responsible for the latter [45]. For example, in bovines, ovarian follicular size [46] have been reported to affect oocyte competence. In addition, oocytes' fates depend on the development undergone within the ever-changing follicular microenvironments to which they are exposed [47].

2.1.8 Follicular Size

It has been well established that oocyte quality is affected by the oocyte's follicular environment [42]. In cattle, ovarian follicles measuring <4mm, 4-8, and >8mm in diameter are classified as small, medium and large, respectively [39]. Positive correlations between follicular size and oocyte competence have been made. More specifically, it has been shown that oocytes collected from larger follicles are more competent to reach the blastocyst stage compared to those collected from smaller follicles [48, 49]. In regards to follicular size, oocytes recovered from follicles <2mm in diameter have been found to be less meiotically competent and less able to achieve the blastocyst stage. On the other hand, the majority of oocytes collected from 3-8mm follicles were meiotically competent, which, following in vitro maturation, fertilization and development, lead to blastocyst rates up to 30% [46, 49, 50]. In addition, oocytes matured in vivo have yielded far more competent oocytes, leading to normal embryo development rates, compared to ones matured in vitro [51, 52]. It has also been shown that oocyte quality can be improved in a time-regulated manner through the gonadotropin-pre-treatment of ovaries [53-55].

2.1.9 Physiological differences: Prepubertal versus adult cattle

A first ovulation marks the beginning of puberty in heifers and many other female animals [56]. Prepuberty, on the other hand, is the physiological period spanning from birth to puberty where ovarian follicles grow in the same wavelike patterns observed in adult animals, however, regressing at a preovulatory stage [57, 58]. Holstein females are considered prepubertal from birth to 9 months of age and hit puberty between 9-10 months old, although inter-animal variability is often observed [56, 59]. These same animals will only be breed after reaching sexual maturity around 14-15 months old [56].

Poor developmental competence of oocytes retrieved from prepubertal animals has been identified in a variety of species, including cows [6], mice [60], goats [61], sheep [62], and pigs [63]. In cattle, prepubertal donor oocytes have been reported to yield 10-15% of viable blastocyst on average compared with adult donor oocytes yielding closer to 30% [7, 64]. Research groups have identified multiple potential causes for the decrease in embryo development observed with prepubertal oocytes: incomplete cytoplasmic maturation, decreased oocyte size, modified protein synthesis, and reduced metabolism [49, 65-67]. It has also been proposed that variations in the expression patterns of specific genes in prepubertal and mature GCs directly affects oocyte competence, which is highly dependent on the constant communication between these two cell types during follicular and oocyte maturation [39, 40].

2.1.10 Ovarian steroidogenesis

Steroidogenesis is the production of active steroid hormones from a cholesterol precursor. In the mammalian ovary, a variety of physiological processes require steroidogenesis and the ensuing steroid-mediated cell signalling for normal function [68]. More specifically, follicular growth, oocyte maturation, and ovulation depend heavily on gonadotropins, androgens, estrogens, and progestins [69].

The two main somatic cell types involved in steroidogenesis in the ovary are the theca cells and mural granulosa cells. First, high- and low-density lipoproteins, i.e. cholesterol (Chol), are sequestered from the circulation and bind to their respective receptors on the surface of theca cells and then undergo receptor-mediated endocytosis [70]. Once Chol has entered the theca cell, it is transported from the outer to the inner membrane of mitochondrion by steroidogenic acute regulatory protein (STAR). Since most of the steroidogenic enzymes are located within the mitochondrion, STAR protein is the rate-limiting regulator of steroidogenesis [68, 71]. Within the mitochondrion, cholesterol is converted to pregnenolone (P5) by enzyme Cytochrome P450 Family 11, Subfamily A, Member 1 (CYP11A1). Next, P5 exits the mitochondrion and enters the cytosol where it is converted either into dehydroepiandrosterone (DHEA) by enzyme Cytochrome P450, Family 17, Subfamily A, Polypeptide 1 (CYP17A1) or into progesterone (P4) by enzyme 3-Beta-hydroxysteroid dehydrogenase (3β-HSD). DHEA

and P4 are then converted into androstenedione (A4) by enzymes 3β-HSD and CYP17A1, respectively. Lastly in the theca cell, a portion of A4 is converted into testosterone (T) by enzyme 17-Beta Hydroxysteroid Dehydrogenase (17β-HSD).

Similarly to theca cells, mural granulosa cells also express Chol receptors on their surface and also endocytose the steroid hormone precursor found in circulation. Chol is converted to P5 by CYP11A1, and subsequently, P5 is converted to P4 by 3β-HSD. Since granulosa cells lack CYP17A1, steroid hormone production ceases at the latter stage. However, steroid products synthesized in the theca cells, being A4 and T, diffuse past the basement membrane and enter the mural granulosa cell layer [72]. Within individual cells, A4 and T are converted to estrone (E1) and estradiol (E2), respectively, by enzyme aromatase (CYP19A1). E2 then diffuses into the antral space (i.e. follicular fluid) and will prove itself crucial to follicle health and survival [73]. It has been shown that in cattle, E2 stimulates the proliferation of granulosa cells and elevates granulosa cell survival through resistance to apoptosis [74] Figure 2.

Important features present primarily on the surface of theca and granulosa cells, respectively, are luteinizing hormone receptors (LHR) and follicle stimulating hormone receptors (FSHR). Both these G protein-coupled receptors are triggered by the binding of their specific gonadotropin and induce second messengers. These messengers help regulate STAR expression and therefore, steroidogenesis. More specifically, the binding of luteinizing hormone (LH) and follicle stimulating hormone (FSH) to their receptors stimulates adenylyl cyclase, which induces an important elevation of cyclic adenosine monophosphate (cAMP) in both theca cells and mural granulosa cells. In turn, cAMP activates protein kinase A (PKA), which upregulates the expression of STAR by phosphorylating serine 195. In addition, cAMP plays an important role in androgen and estrogen production by regulating CYP17A1 and CYP19A1's enzymatic activities in theca cells and mural granulosa cells, respectively [68, 75].

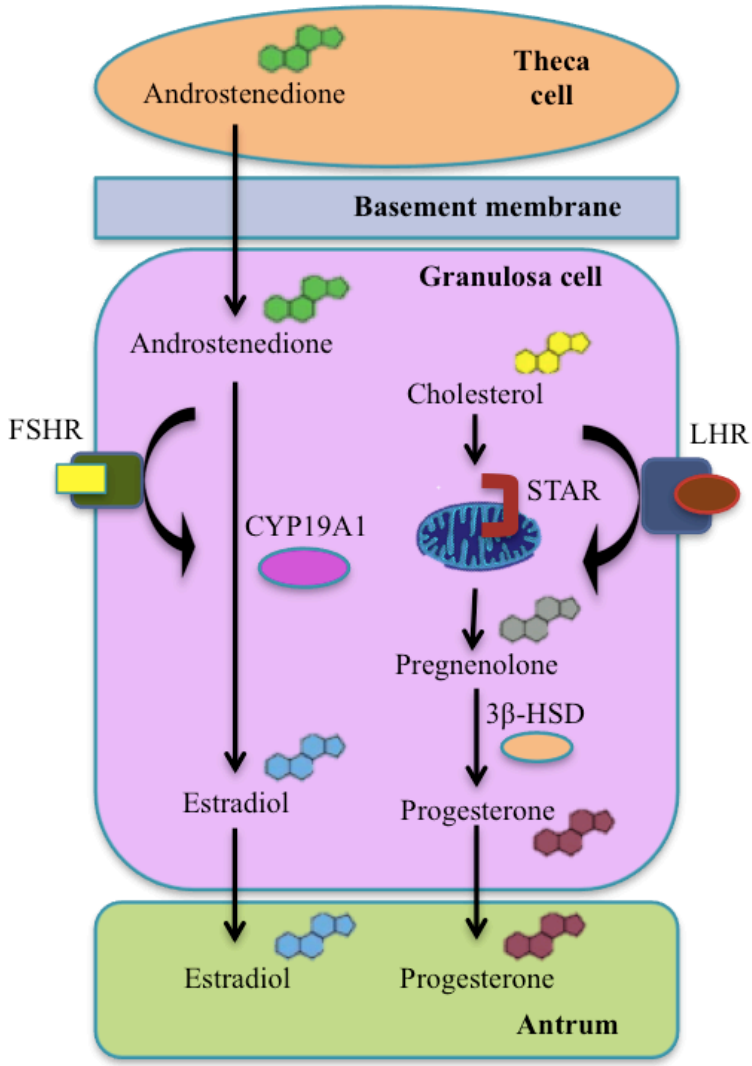

Figure 2: Steroidogenesis within the ovarian follicle**.** During follicular development, androstenedione is translocated from the theca cell layer into granulosa cells, and is ultimately converted into estradiol by enzyme aromatase (*CYP19A1*). During follicular atresia, cholesterol is sequestered into granulosa cells from the blood and binds *STAR* on the mitochondrial membrane to be later internalized in this same organelle. Cholesterol is converted to pregnenolone, and later into progesterone by enzyme *3β-HSD*. Both estradiol and progesterone have the ability to diffuse into the follicular antrum.

2.1.11 Hypothalamus-pituitary-ovarian axis in ruminants

The mammalian hypothalamus-pituitary-ovarian (HPO) axis interconnects the

hypothalamus, the anterior pituitary gland and the ovaries via endocrine communications fuelled by feedback mechanisms, both stimulatory and inhibitory, Figure 3. One of the most important players in this signalling cascade is a peptide known as kisspeptin, encoded by gene *kiss1*. In ruminants (sheep, goats, and cattle), kisspeptin neurons, which release kisspeptin, are located in the arcuate nucleus (ARC) of the brain [76]. These kisspeptin neurons are involved in both positive and negative feedback mechanisms, being the regulation of GnRH at the preovulatory LH surge and the forwarding of signals for the regulation of GnRH secretion, respectively. Kisspeptins mediate their effects by binding their G-protein coupled-receptors, encoded by genes *GPR54* or *kiss1r*, expressed on gonadotropin releasing hormone (GnRH) neurons. The latter are located in the preoptic area (POA) and in the median eminence (ME) of the brain. GnRH neurons do not express receptors for estrogen or progesterone as opposed to kisspeptin neurons, which do. As a result, fluctuating levels of circulating estrogen regulate key steroidogenic functions by inhibiting kisseptin expression (negative feedback on GnRH pulse generator) or stimulating it (positive feedback) in the ARC [77].

The binding of kisspeptins to their receptors on GnRH neurons in the ARC leads to a pulsatile release of GnRH into the blood where the hormone will then leave the hypothalamus and travel to the anterior pituitary via the pituitary hypophyseal portal veins. GnRH binds to receptors gonadotrope cells in the anterior pituitary gland allowing for the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into circulation. These two gonadotropins target the ovary, ultimately resulting in the activation of downstream steroidogenic pathways leading to estradiol and progesterone synthesis necessary for successful folliculogenesis and preparing the uterus for embryonic implantation, respectively [78, 79].

For the majority of the prepubertal stage, the dominant neuronal presence in the hypothalamus is the *Kiss1* neuron population that responds negatively to estrogen (negative feedback). Although it is still not fully understood in ruminants, hypotheses suggest that the onset of puberty coincides with body growth and development, and with the activation of estrogen-reactive kisspeptin neurons (positive feedback) in the ARC. This hypothalamic activation is linked with the secretion of endogenous estrogen in response to ovarian activation [80, 81]. The latter acts to increase *Kiss1*/kisspeptin

mRNA/peptide levels in the ARC, the number of projections spanning from the kisspeptin to GnRH neurons, *GPR54*'s sensitivity to kisspeptins, and to increase *GPR54* expression [82, 83]. The root cause for lack of ovulation in prepubertal animals is the negative feedbacks within the HPO axis. Low doses of circulating estrogen produced in the ovary from non-ovulation-yielding follicular waves bind to receptors in the hypothalamus and inhibit the release on LH, preventing ovulation, or the rupture of the follicular wall. It has also been shown that the number of estrogen receptors in the hypothalamus decreases closer to puberty, and the frequency of LH pulses increases ultimately leading to ovulations [84].

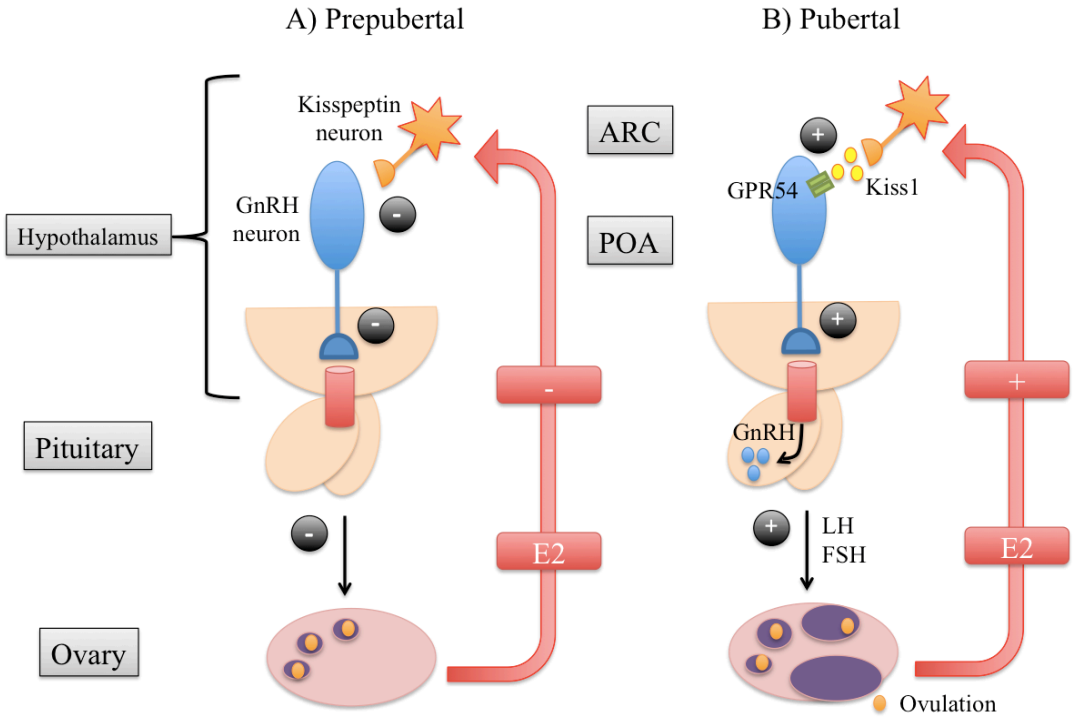

Figure 3: The HPO axis in ruminants. A) Prior to the onset of puberty, low levels of circulating estrogen inhibit GnRH secretion and downstream pathways, B) At puberty, endogenous estrogen binds kisspeptin neurons in the ARC leading to the release of kisspeptin. Kisspepting then binbs *GPR54* on GnRH neurons in the POA, which causes the release of GnRH. GnRH binds its receptors in the anterior pituitary gland and leads to the release of FSH and LH. FSH and LH target the ovary, promote steroidogenesis and folliculogenesis, and cause ovulations. Adapted from [82, 83].

2.1.12 Ovulation

Ovulation is a physiological event, which occurs every estrus cycle. Some mammalian species ovulate a single oocyte per estrus cycle (mono-ovulatory, e.g. cows, humans, and horses) compared to others, which ovulate many (poly-ovulatory, e.g. pigs, mice) [42, 85]. In response to an LH surge, downstream events are activated in the ovulatory cascade and the release of a fertilizable oocyte from a dominant, or Graafian follicle, is achieved [86, 87]. In cattle specifically, the LH receptor (LHr) gene is expressed on the plasma membrane of granulosa and theca cells from antral follicles >7mm in diameter [88-91]. In addition, antral follicles >8mm require gonadotropin support in order to reach a preovulatory stage [92]. As the dominant follicle(s) continues to grow, the levels of synthesized E_2 increase proportionally. E_2 in circulation positively feedbacks to the hypothalamus and stimulates the release of GnRH. The latter binds to its receptors in the pituitary leading to the release of an LH surge. Once LH binds its own receptors, the activation of the mitogen-activated protein kinase (MAPK) pathway occurs and the oocyte(s) secretes paracrine factors. In turn, the oocyte(s) resumes meiosis, cumulus cells expand, follicle(s) rupture, and lastly, theca and granulosa cells undergo luteinzation to form an active CL [12].

As previously mentioned, the lack of gonadotropin stimulation in prepubertal animals prevents the growth and maturation of follicles past the gonadotropin-independent (GI) development, and consequently prevents ovulation [84].

2.1.13 The bovine estrous cycle

Estrous cycles are hormone-dependent periods of dynamic ovarian and follicular development resulting in ovulation(s), or the release of a fertilizable oocyte [93]. The first cycle experienced by a mammalian female signifies the onset of puberty and the beginning of her reproductive life. In a normally cycling cow, a single estrous cycle last approximately 21 days. However, this length can vary anywhere between 17 to 24 days [94]. Cows are a polyestrus species meaning that, throughout the year, they have a uniform distribution of estrous cycles [95]. Ultimately, dairy herd profitability is highly dependent on estrus detection and reproductive efficiency [93].

One estrous cycle can be divided into three separate phases (estrus, luteal phase and follicular phase). The estrous cycle begins with estrus, or standing heat, which is the period of time prior to ovulation when a cow is sexually receptive and allows to be mounted by potential mates [95]. The latter can be a reliable sign indicating that the female is ready to be bred when the use of assisted reproductive technologies (ART) is involved [96]. The average duration of estrus in cattle is 15 hours, although ranges less than 6 hours to 24 hours have been reported. Ovulation can be observed between 24 and 32 hours after the initiation of estrus [95]. The oocyte, or egg, is expelled from its follicle and travels from the infundibulum into the uterine horn where it will be available for fertilization [97]. Cows are a monotocous, or mono-ovulatory, species implying that a single dominant follicle will ovulate each estrus cycle [98].

Following ovulation, the luteal phase is initated and typically last from days 1-17 of the cycle [94]. The ovulatory follicle undergoes luteinisation and a corpus hemorrhagicum (CH) structure is first formed. As its contained blood clot is absorbed, the CH becomes the corpus luteum (CL). Mural granulosa and theca lutein cells within the CL begin to produce progesterone (P4) in order to prepare the uterus and support a pregnancy if fertilization occurs [99].

The follicular phase, the last part of the estrus cycle lasting approximately 4 days (days 18-21), is defined by follicular development in a wave-like pattern. During each wave, a select number of primordial follicles are recruited from their finite pool under the influence of follicle stimulating hormone (FSH) [94]. These follicles are then selected and continue to grow and develop by luteinizing hormone (LH) stimulation. From the growing pool, a single follicle will become dominant, subsequently causing the death of other recruited follicles. However, when levels of progesterone produced by corpus luteum (CL) of the previous ovulation are high, ovulation of the new dominant follicle is inhibited and this same follicle undergoes atresia [99]. In the cow, it takes up to the duration of two or three follicular waves before levels of progesterone begin to drop in response to a lack of maternal recognition of pregnancy [100]. Uterine prostaglandins are responsible for the degradation of the CL, yielding a corpus albicans (CA), and ultimately, decreasing the levels of progesterone in circulation and allowing a new wave of primordial follicles to be initiated [101]. Although low levels of estradiol (E2),

produced by the granulosa cells, are inhibitory to gonadotropin-releasing hormone (GnRH) secretion, high E2 levels are stimulatory and cause the release of GnRH [102]. GnRH then stimulates the release of FSH, which in turn, recruits a new wave of follicles. GnRH then causes the pre-ovulatory LH surge, which leads to the release of a fertilizable oocyte from the dominant follicle and the beginning of another estrous cycle [103].

On the other hand, when the CL is intact and progesterone is produced due to maternal recognition of pregnancy, the dominant follicle of any subsequent follicular wave will not lead to ovulation [104].

2.1.14 Anestrus in bovine

Anestrus is a period of acyclicity, which is observed at two specific time points in a cow's reproductive life. Heifers and cows experience anestrus prior to the onset of puberty and post-parturition, respectively [105, 106]. Since estrus and ovulation are lacking during anestrus, neither heifers nor cows can be impregnated during that time. However, it has been found that since early follicular development is unaffected, oocytes can still be collected by follicular aspiration in prepubertal and pubertal heifers, and sexually mature cows [107].

2.1.15 Laparoscopic ovum pick-up (LOPU)

In 1974, Snyder and Dukelow first developed a technique for follicular aspiration and oocyte recovery *in vivo* by laparoscopic observation in a sheep model [108]. However, laparoscopic ovum pick-up (LOPU), a reliable technique for the collection of oocytes and their supportive cells from developing ovarian follicles, was established later on [109]. In cattle, the collection of ova by laparoscopy was first performed in 1980 [110]. It wasn't until 1994, that laparoscopy through the vaginal fornix was used as means of repeated aspiration of follicular oocytes in cows [111].

When knowledge of the health status of research animals is beneficial, LOPU is often chosen over classical oocyte collection by follicular aspiration from slaughterhouse ovaries [109]. Another technique commonly considered is ovum pick-up (OPU). As opposed to LOPU, OPU is performed transvaginally and guided by ultrasound. A Dutch team, in 1988, first established the use OPU in cattle [112]. Although both techniques

have been proven successful in oocyte recovery, the resulting choice of technique used can be strongly dependant on the site of least desired trauma; OPU is less traumatic on the vagina, fornix and abdominal organs, and LOPU is less traumatic on the ovaries [113]. In addition, the animal's anatomical size is an important factor to consider and that is why LOPU is performed in smaller animals. For larger animals, OPU is the practical and efficient, as it does not require general anaesthesia.

Nowadays, LOPU performed in animals, small ruminants for example, is primarily performed through the abdominal cavity [114]. Animals are restrained in dorsal recumbency and placed under general anaesthesia [109]. Three small incisions are made in the abdominal cavity and a laparoscope and two 'second puncture' trocars are inserted. In order to increase visualisation of reproductive structures, filtered air is inserted into the cavity. A telescope, an atraumatic grasping forceps, and a puncture pipette are inserted into the appropriate trocars. After successful visualization of the ovaries, follicles are punctured and the follicular content, i.e. follicular fluid, oocytes and supportive cells, is aspirated under vacuum [114]. In the majority of *in vivo* studies requiring the retrieval of zygotes and matured oocytes from small animals, LOPU is favoured over the surgically invasive alternative, a laparotomy [109].

2.1.16 *In vitro* embryo production (IVEP)

The oocytes, more specifically the cumulus-oocyte-complexes (COC), collected by LOPU are commonly used for *in vitro* embryo production (IVEP). This is achieved by placing the COCs in *in vitro* maturation (IVM), followed by *in vitro* fertilization (IVF), and lastly in *in vitro* culture (IVC) to a transferable/freezable stage of development. Cleavage rates, being the percentage of early embryos completing a first mitotic division and achieving the 2-cell stage, and blastocyst rates, being the percentage of embryos demonstrating an inner cell mass, a trophoblast layer, and a blastocoele, are typically determined on Day 2 and 7 of embryo culture, respectively [115, 116]. It has been shown that embryos cleaving early (Day 2) are more competent and of higher quality than those cleaving late (Day 3). In addition, it has also been demonstrated that early cleaving embryos correlate with higher rates of pregnancy/transfer, implantation, and birth [117].

IVEP is most commonly performed in valuable animals. These valuable embryos are then transferred into recipient females [3]. In all, LOPU allows for a valuable donor animal to undergo multiple oocyte recoveries, overcoming the primary limitation of IVEP in livestock [118]. The early propagation of animals has been proposed by performing LOPU followed by IVM/IVF in oocytes collected from prepubertal animals [109]. This assisted reproductive technology is of economic interest as it provides great promise for the shortening of intergeneration intervals of animals of high genetic gain in the animal production industry.

2.2 Genetic targets

The complete gene expression profile within bovine granulosa cells involved in follicular growth, oocyte maturation and competence is not yet fully understood. In addition, it has been suggested that the expression profile of some of these genes is age-dependent. As a result, determining which expression profile in GCs is the most favourable for increased oocyte competence is of great interest in the field of artificial reproductive technologies (ARTs) involving prepubertal cattle.

2.2.1 Follicle Stimulating Hormone Receptor

Follicular maturation, estradiol synthesis, and granulosa cell differentiation and proliferation are gonadotropin-dependent [38]. As follicles are recruited and begin to grow, they become increasingly vascularized. This exposure to circulating blood allows for the interaction of gonadotropins with their ovarian targets. More specifically, follicle stimulating hormone (FSH) becomes one of the key hormones regulating ovarian folliculogenesis [119]. FSH must bind its receptor, follicle stimulating hormone receptor *FSHR*, which is found exclusively on the surface of granulosa cells in order to mediate its effects [120]. However, *FSHR* expression is dependent on follicular stage in many species including bovine. In addition, FSH itself, members of the transforming growth factor β family (TFG-β) and epidermal growth factor (EGF) are factors that control *FSHR* expression [120]. However, follicle maturation brings on an increase in the expression of *LHR* on the granulosa cell layer, and ultimately, a decrease in the expression of *FSHR* in order to prepare the follicle for ovulation [89].

2.2.2 Luteinizing Hormone Receptor

Luteinizing hormone (LH), a gonadotropin produced in the anterior pituitary, enters in circulation and binds to its receptor, luteinizing hormone receptor (*LHR*), on the plasma membrane of the somatic cells within ovarian follicles [121]. LH signalling is required for folliculogenesis, oocyte maturation, ovulation and for the transition of theca and granulosa cells into their luteal counterparts in order to produce a functional progesterone-producing corpus luteum [122, 123]. In addition, *LHR* expression is temporal. Specifically, it has been reported that in mural granulosa cells, this expression is increased when approaching the preovulatory LH surge compared to theca cells which acquire these receptors in the early follicular growth phase [124], which allows these cells to commence testosterone synthesis [125]. *LHR*, however, is not expressed in cumulus cells reinforcing the concept that a physical distance does not prevent intercellular communications [124, 126]. In cattle, *LHR* mRNA was detected in granulosa cells of ovarian follicles between 3-7mm [43], and ≥8mm [90, 125, 127].

2.2.3 Protein Kinase B

Protein kinase B, or *AKT*, controls a variety of cellular functions and acts as an important regulator of physiology. *AKT* is involved in cell growth, proliferation, metabolism, and survival. More specifically, *AKT1*, an *AKT* isoform, is involved in cellular growth, and embryonic development and survival. *AKT1* is an active player in the PI3K/AKT pathway in GCs. *AKT1* phosphorylates *FOXO1* deeming it inactive and, as a result, promotes GC steroidogenesis and proliferation [128].

2.2.4 Forkhead Box Protein O1

Forkhead box protein O1, or *FOXO1*, is a FOXO family member and acts via the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway [129]. FSH and LH utilise the PI3K/AKT pathway to activate *FOXO1* and control its mRNA expression levels [130]. *FOXO1* mRNA levels regulate the downstream expression of genes involved in GC metabolism and development. Therefore, *FOXO1* expression tends to increase in granulosa cells of growing follicles. In addition, active nuclear *FOXO1* in GCs negatively impacts the cholesterol biosynthethic pathway by causing the absence of

cyclin D2, *CYP19A1*, *FSHR*, *LHR*, and steroidogenic enzymes [129]. As a result, *FOXO1*, in early stages of folliculogenesis, is thought to inhibit abundant steroidognesis in GCs [130]. *FOXO1* protein has been reported in high abundance in the cytoplasm of GCs and TCs of antral follicles. The nuclei of GCs from atretic follicles, on the other hand, have been found to contain the highest *FOXO1* protein levels. In all, *FOXO1* is known to negatively regulate steroidogenesis and proliferation [131].

2.2.5 FSH and PI3K/AKT signalling pathways

FSH signalling is indispensable for GC differentiation and follicular growth [129]. In addition, the PI3K/AKT signalling pathway is critical for oogenesis as it tightly regulates follicular growth, differentiation, and survival [132]. Together, these signalling pathways promote female fertility.

FSH begins signalling through follicle stimulating hormone receptor (*FSHR*), a G-protein coupled receptor (GPCR) expressed on granulosa cells' plasma membranes during the gonadotropin-dependent stage of folliculogenesis. This GPCR's stimulatory G protein (G_S) then activates adenyl cyclase (AC), which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [133]. cAMP then activates exchange proteins directly activated by cAMP (EPACs), which in turn, activate rat sarcoma (RAS). RAS targets, activates, and phosphorylates proto-oncogene tyrosine-protein kinase Src (c-Src) and subsequently epidermal growth factor receptor (*EGFR*) [134]. The phosphorylation of both c-Src and EGFR induces the binding of the catalytic and regulatory subunits of phosphoinositide 3-kinase (*PI3K*). *PI3K* phosphorylates phosphatidylinositol di-phosphates (PIP_2) into phosphatidylinositol tri-phosphate (PIP_3). PIP_3 then binds pyruvate dehydrogenase kinase 1 (*PDK1*), and *PDK1* phosphorylates *AKT1* [135]. *AKT1* targets and phosphorylates *FOXO1*, which promotes its translocation from the cell's cytoplasm to its nucleus for degradation [136]. As a result, the FSH and PI3K/AKT pathways lead to *FOXO1* degradation, increased steroidogenesis, and GC proliferation and differentiation [130] Figure 4. These events have proven indispensable in the reproduction of bovines.

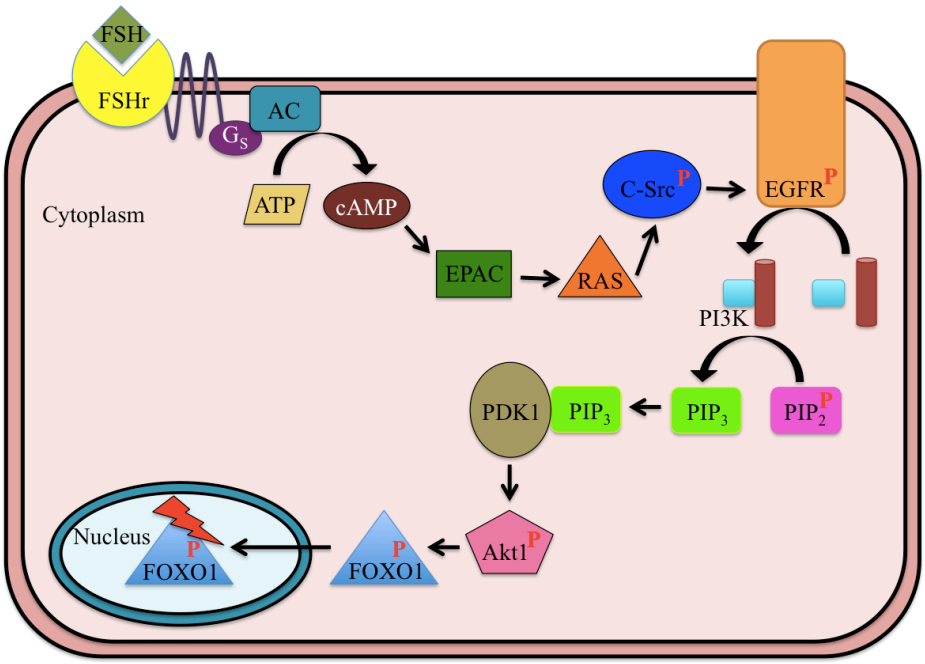

Figure 4: FSH and PI3K/Akt signalling pathways in granulosa cells of ovarian follicles. Adapted from [133, 135].

2.2.6 Steroidogenic Acute Regulatory Protein

Steroidogenic acute regulatory protein, *STAR*, is necessary for the synthesis of steroid hormones [137]. This mitochondrial protein is responsible for the transfer of extracellular cholesterol from the outer to the inner mitochondrial membrane [138]. The latter mobilization of cholesterol is the rate-limiting step of steroidogenesis [139]. In bovine granulosa cells, *STAR* expression is dependent on the stage, gonadotropin independent or dependent, of follicular development. Specifically, bovine granulosa cells cultured with FSH have been found to express significantly higher *STAR* mRNA levels compared to controls [125]. Similarly in mice, *STAR* is used as a marker of granulosa cells maturation since it has been reported that its expression is higher in mature GCs [140].

2.2.7 Cytochrome P450, Family 19, Subfamily A, Polypeptide 1

Cytochrome P450, Family 19, Subfamily A, Polypeptide 1, *CYP19A1*, is a gene encoding the enzyme aromatase. Aromatase is required for ovarian steroidogenesis, more specifically; it is the rate-limiting step in the biosynthesis of estradiol from a testosterone precursor [141]. Ovarian localization of *CYP19A1* occurs mainly in the granulosa cell

layer of developing follicles transitioning from preantral to preovulatory [142]. *CYP19A1*'s expression patterns have been found to change significantly throughout bovine follicular growth, plateau, and atresia. More specifically, this genes's mRNA abundance was significantly higher in healthy compared to atretic follicles. Consequently, this enzyme has proven to be a successful marker of follicular growth [143].

2.2.8 3β -Hydroxysteroid Dehydrogenase

3β-hydroxysteroid dehydrogenase (*3β-HSD*) is an important enzyme involved the steroidogenic casdades occurring within ovarian follicles; it is responsible for catalyzing the conversion of pregnenolone into progesterone within granulosa cells [144]. As a result, *3β-HSD* controls androgen production and its expression is commonly associated with *LHR* expression near ovulation [90]. In mono-ovulatory species, if the dominant follicle produces high concentrations of progesterone, it will enter atresia instead of following an ovulatory fate [145]. In bovine follicles, it has also been reported that basal atretic follicles <5mm express higher levels of *3β-HSD* and produce elevated levels of progesterone [146].

2.2.9 Connexin 43

Follicle cells have been found to directly influence oocyte growth and development through the supply of nutrients these cells provide it [147]. In vivo and in vitro communication between neighbouring ovarian cells is made possible through gap junctions [148]. More specifically, gap junctions are individually composed of two symmetrical connexons, one located on each of the neighboring cells, and which can be further divided into six connexin (CX) protein subunits [149]. These channels allow the direct exchange of ions, small molecules (<1kDa), and electrical impulses between adjoining cells [150-152]. In female reproductive tissues, these junctions play vital roles in folliculogenesis, steroidogenesis, oogenesis, and in corpus lutuem formation [153]. Connexin 43, *CX43*, is the predominant transmembrane protein in the granulosa/granulosa cell gap junction make-up [154].

Gonadotropins/cyclic adenosine monophosphate (cAMP) [155] and glucocorticoids [156] have been reported to regulate the expression of *CX43* in granulosa cells throughout folliculogenesis. cAMP is one of the main molecules exchanged between granulosa cells, and even with the oocyte, through gap junctions in order to maintain oocyte meiotic arrest prior to fertilization [157]. Furthermore, elevated concentrations of serum FSH or in vitro FSH treatments have been found to increase the amount of mRNA encoding *CX43* in large antral follicles [155, 158]. On the other hand, short in vitro exposures to LH (immediate response) have been associated with *CX43* phosphorylation and ubiquitination, and longer exposures to LH (late response) and preovulatory LH surges have led to the decrease in *CX43* mRNA abundance and to the elimination of *CX43* protein [158, 159] Figure 5. In addition, previous mammalian studies have correlated oocyte and embryo competence with granulosa cell *CX43* expression [160, 161]. Taken together, *CX43* has been found to play a critical role in the end stages of folliculogenesis and oogenesis [162].

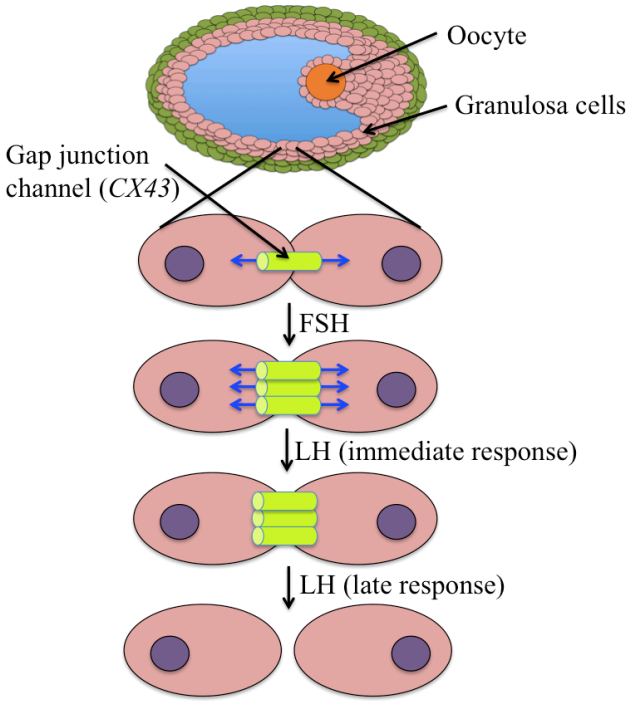

Figure 5: Gonadotropin-dependent gap junction protein *CX43* presence on antral follicles' granulosa cell plasma membranes. Adapted from [123].

2.2.10 X-linked Inhibitor of Apoptosis Protein

Apoptosis, or programmed cell death, in a normal physiological process, which readily occurs in the mammalian ovary due to its cyclical development. In addition, constant communication between pro-apoptotic and pro-survival molecules aid in the decisions of life or death of a cell. Autocrine and paracrine signalling between ovarian cells and involving growth factors are indispensable during follicular development. X-linked inhibitor of apoptosis protein, *XIAP*, is one of the important growth factors. [163]. *XIAP* frequently regulates early development and receptor-mediated intracellular signalling although its main function is to inhibit endogenous caspase activity terminating apoptotic signalling [164, 165]. Such activity is reported in many ovarian cell types: stromal cells, ovarian surface epithelium (OSE), granulosa cells, theca cells, oocytes, and luteal cells [166-170]. Although, oocytes undergo the highest rates of apoptosis during fetal development, adult life brings a marked increase in apoptosis of the granulosa cells of secondary and antral follicles. Granulosa cell apoptosis has been linked to meiotic anomalies, decreased survivial factors, and as a method of self-sacrifice [163]. In hopes of counteracting high rates of apoptosis, granulosa cells have been found to upregulate XIAP [164]. In addition, gonadotropin-induced XIAP expression has been linked to cell survival and control of follicular atresia in cow and rat granulosa cells [166, 171].

2.2.11 Calmodulin

Calmodulin, or the *CALM* gene, is known as the calcium-dependent regulatory protein. The binding of gonadotropins to their respective membrane receptors is said to promote the uptake of calcium into granulosa cells [172]. Cytoplasmic calcium ions (Ca^{2+}) become available to bind to calmodulin following their release from organelles, and together, the calcium/calmodulin complex activates numerous enzymes: one of which is calcium/calmodulin-dependent protein kinase II (CaMKII) [173]. CaMKII has been linked to cell cycle resumption and cortical granule (CG) exocytosis following parthenogenic activation or fertilization [174, 175]. Due to the latter, it has been hypothesized that calcium/calmodulin and CaMKII may play a key role in establishing the membrane block to polyspermy [176]. However, calf oocytes have found to have a decreased ability for migration and dispersal of CGs following ooplasmic calcium

oscillations. These organelles reportedly failed to disperse evenly below the plasma membrane and instead remained associated in aggregates [177]. Lastly, previous studies have also shown that the calcium/calmodulin complex plays a critical role in steroidogenesis in granulosa cells, independently of their stage of differentiation, by regulating the concentration of cellular cyclic adenosine monophosphate (cAMP) [178, 179].

CHAPTER 3: RATIONALE, HYPOTHESIS AND OBJECTIVES

Literature suggests that lower embryo development rates are observed from oocytes collected from prepubertal compared to mature animals [6, 64, 180]. Similarly, successful embryo development has been positively correlated with oocyte developmental competence. An oocyte's acquisition of competence occurs partially through the bi-directional communication between the oocyte and granulosa cells with ovarian follicles prior to ovulation [37]. Therefore, genes involved in steroidogenesis [38], apoptosis [164], cell-to-cell communication [160], and calcium metabolism [176] within ovarian follicles are being studied for their importance in follicular growth and oocyte maturation leading to improved embryo development in adults. Taken together, we hypothesized that genes involved in the acquisition of oocyte competence are defectively regulated in the mural granulosa cells of prepubertal compared to mature Holstein cows. The objectives of this study were to:

1. ***Evaluate the effect of different ovarian stimulation protocols on granulosa cell gene expression in prepubertal Holstein heifers.***
2. ***Determine the effect of age on granulosa cell gene expression in prepubertal Holstein heifers.***
3. ***Assess if the developmental competence of the oocytes is correlated with the expression profile of genes in granulosa cells.***

Using a prepubertal bovine model, we determined the gene expression pattern of mural granulosa cells involved in follicle maturation and oocyte competence at a very young age. The understanding gained from these studies may lead to the development of protocols for improved embryo development rates in prepubertal heifers, allowing the shortening of the generation intervals in production animals with desired characteristics, and contribute to the successful use of assisted reproductive technologies (ARTs) in prepubertal animals.

CHAPTER 4

Manuscript in preparation for submission to the Journal of Ovarian Research

Gonadotropin treatment increases the expression of genes involved in follicular growth and oocyte maturation in granulosa cells of prepubertal Holstein heifers

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Short title: Genetic markers in prepubertal bovine granulosa cells

Keywords: Cattle, prepubertal, granulosa cells, gene expression, mRNA, follicular growth, oocyte competence, embryo development.

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4.1 ABSTRACT

There is evidence suggesting that acquisition of developmental competence in bovine oocytes may be linked to the expression profile of genes in the mural granulosa cells (GC). To gain insight into the potential molecular changes occurring within GCs during the acquisition of oocyte competence, we have performed bi-weekly laparoscopic ovum pick-ups (LOPU) and collected cumulus-oocyte-complexes (COC) and mural GCs from 2-10mm follicles in 12 Holstein heifers from 2-6 months of age. While 3 heifers remained untreated (control) for the duration of the experiment, 9 received varying follicle stimulating hormone treatments (FSH-treated) to mimic an active hypothalamus-pituitary-ovarian axis. Adult GCs were collected from 8 FSH-treated adult cows. The COCs from prepubertal animals were matured, fertilized and cultured *in vitro* to assess development to the blastocyst stage. The relative mRNA abundance of *FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP* in GCs were quantified by RT-qPCR. mRNA abundance of *FSHR, CYP19A1, 3β-HSD,* and *XIAP* were significantly decreased, and *STAR* and *3β-HSD* were significantly increased in prepubertal control samples compared to adult and prepubertal FSH groups. When comparing the mRNA abundance at 3 prepubertal ages (<100, 100-130, and >130 days), we observed significantly higher expression of *STAR* (all ages)*, 3β-HSD* (≤130 days), and *FOXO1* (>130 days) in control calves, and significantly higher expression of *FSHR* (≤130 days)*, CYP19A1* (100-130 days), and *XIAP* (>100 days) in FSH-treated calves. Increasing the FSH treatment duration from 2 to 4 days lead to a significant decrease in the mRNA abundance of *STAR, 3β-HSD,* and *CALM* in prepubertal animals. *LHR* mRNA was detected in a significantly higher number of GC samples from animals treated with longer FSH protocols. Lastly, we observed significantly lower *3β-HSD* and *CX43* mRNA, and significantly higher *FOXO1* mRNA in samples from animals that resulted in above median cleavage (>70%) and blastocyst (>15%) rates, respectively. These findings revealed that GCs of prepubertal animals respond to FSH treatment by increasing mRNA levels of genes promoting estradiol synthesis and follicular growth, and by decreasing mRNA levels of genes promoting progesterone production and follicular atresia. We also observed that the relative mRNA abundance of *3β-HSD, CX43*, and *FOXO1* in GCs is correlated with embryo cleavage and development in prepubertal heifers.

4.2 INTRODUCTION

Modern genomic tools are being readily paired with assisted reproductive technologies (ART) in the animal production industry [181]. These technologies are essential as there is a growing interest in producing genetically superior offspring at prepubertal ages. However, it has been reported that in cattle oocytes collected from prepubertal animals yield lower rates of development when compared to their adult counterparts [4-6]. This has been associated with lower oocyte competence at the time of follicular aspiration [7].

Biology normally dictates that ovarian development occurs in prepubertal animals, although, follicular growth and maturation is limited to the gonadotropin-independent stages since animals at this age still have an inactive hypothalamus-pituitary-ovarian (HPO) axis [82]. In addition, granulosa cell (GC) mRNA patterns have been linked to the developmental competence of bovine oocytes [39], due to intra-follicular bi-directional communications occurring between these cell types [9].

Gonadotropin treatments have been tested to induce follicular growth in prepubertal heifers, but their effects on follicular cells were not systematically investigated. In order to evaluate the response at the molecular level to exogenous FSH treatments, the abundance of transcripts encoding steroidogenic (*FSHR, STAR, CYP19A1, 3β-HSD, FOXO1,* and *AKT1*) [120, 125, 128, 131, 143, 146], cell-to-cell communication (*CX43*) [162], apoptosis (*XIAP*) [166], and calcium metabolism (*CALM*) [176] regulators were investigated in mural GCs recovered from prepubertal heifers. The objectives of this study were to: i) evaluate the effect of different ovarian stimulation protocols on gene expression in GCs of prepubertal heifers; ii) determine if the effect of gonadotropin treatment on gene expression in GCs is affected by the age of the prepubertal heifers; and iii) assess if the expression profile of genes in GCs is correlated with the developmental competence of the oocytes in prepubertal heifers.

4.3 MATERIALS AND METHODS

4.3.1 Animals

All experimental procedures using cattle were approved by the Animal Care and Use Committee of McGill University. Twelve Holstein calves aged between 2 and 4

weeks and weighing approximately 45kg were purchased from various dairy farms in Québec. The calves were housed at the Large Animal Research Unit of McGill University. They were provided water and second-cut hay *ad libitum*, and were fed milk replacer (Optivia, Shur-Gain, Brossard, QC, Canada) until two months of age and slowly weaned onto grain (Optivia), which was later fed twice daily. Eight mature Holstein cows between 17 and 22 months of age were used as positive control animals and were housed and cared for at L'Alliance Boviteq (St-Hyacinthe, QC, Canada).

4.3.2 Ovarian stimulation

Two groups of 6 calves were used in this study. The first group consisted strictly of gonadotropin-stimulated animals (n=6). The 6 calves from the second group were selected at random and placed into 2 different treatment groups: control (n=3) and gonadotropin-stimulated (n=3). A total of 8 (group 1) and 7 (group 2) LOPUs were performed every two weeks in the same animals. Prior to the LOPUs, the animals received either a short or long stimulation protocol. Short stimulation protocols consisted of FSH (Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada) at 12-hour intervals starting 2 days prior to LOPU (FSH dose range: 60-128mg) combined or not with a single shot dose (200-400IU) of equine chorionic gonadotropin (eCG – Folligon; Intervet Canada Corporation, QC, Canada) given 36h prior to LOPU. Long stimulation protocols consisted of FSH at 12-hour intervals over 3 to 4 days prior to LOPU (FSH dose: 140mg). All injections were administered intra-muscularly (IM). A controlled internal drug release (0.3g progesterone/Eazi-Breed CIDR, Zoetis, MI, USA) was vaginally inserted in all animals 5 days prior to LOPU to prevent ovulations during the stimulation period. The mature cows (positive controls, n=8) were administered FSH (180mg total dose) every 12h for 3 days prior to ovum pick-up (OPU). Stimulation protocols were adjusted from one LOPU to another based on follicular response, oocyte recovery rates, and embryo development.

4.3.3 Laparoscopic ovum pick-up (LOPU)

Laparoscopic observation was used to recover cumulus-oocyte-complexes (COCs) and mural granulosa cells by aspiration of follicular contents under vacuum using

a 20-gauge hypodermic needle. The COCs and GCs were received in a collection tube containing 0.5mL of aspiration media. This aspiration medium (Vetoquinol, Lavaltrie, QC, Canada), was supplemented with 10U/mL of heparin (Fresenius Kabi Canada Ltd., Richmond Hill, ON, Canada), and 25µg/mL of gentamicin (Sigma-Aldrich, Saint Louis, MO, USA). LOPU was repeated up to 8 times in the same prepubertal animals throughout this study. Protocol adapted from [182].

4.3.4 In vitro embryo production (IVEP) procedures

The COCs from each animal were placed in HEPES-buffered TLH solution (supplemented with 0.3% bovine serum albumin, 0.2mM pyruvate, and 50mg/mL gentamicin) and washed three times to separate them from follicular fluid. COCs having homogenous cytoplasm and surrounded by more than 1 layer of non-expanded cumulus cells were placed in 50µL droplets of maturation medium under mineral oil. Maturation medium was composed of TCM199 (Gibco 1150059, Invitrogen Life Technologies), 10% fetal bovine serum (Wisent Bioproducts), 0.2mM pyruvate, 50mg/mL gentamycin, 0.5mg/ml FSH (Folltropin-V, Bioniche), 5mg/mL luteinizing hormone (Lutropin, Bioniche), and 1mg/mL prostaglandin E_2. Maturation droplets were incubated for 24 hours at 38.5°C with 5% CO_2 in 100% humidity.

Following *in vitro* maturation (IVM), the COCs were collected and washed twice in TLH medium before being transferred to 48µL droplets under mineral oil. The droplets consisted of modified Tyrode's lactate medium supplemented with fatty acid-free BSA (0.6% w:v), pyruvic acid (0.2mM), heparin (2µg/mL), and gentamicin (50µg/mL). Fifteen minutes prior to adding the semen, the oocytes were transferred under mineral oil. To enhance sperm motility, penicillamin (2µL), hypotaurine (1mM), and epinephrine (250mM) were added to each droplet. Selected spermatozoa (Semex, Canada) stored in liquid nitrogen were thawed for 1 minute in 35.8°C water, laid on a discontinuous gradient (45% over 90%) of BoviPure (Nidacon Laboratories AB, Göthenborg, Sweden) and centrifuged at 600 x *g* for 5 minutes. The supernantant containing the cryoprotector and dead spermatozoa was discarded, and the pellet was then resuspended in 1mL of modified Tyrode's lactate and centrifuged at 300 x *g* for 2 minutes. The resuspended spermatozoa were counted on a hemocytometer and diluted with *in vitro* fertilization

(IVF) medium to obtain final concentrations ranging between 0.5-1 x 10^6 sperm cells/mL. Lastly, 2µL of sperm suspension were added to the droplets containing the matured COCs. The fertilization medium was incubated at 38.5°C in a humid atmosphere of 94.5% air and 5.5% CO_2 for 18 to 22 hours.

Embryos from individual calves from Group 1 were cultured together *in* vitro (IVC) while embryos from calves from Group 2 that received the same ovarian stimulation protocol prior to LOPU were pooled and cultured together in 10µL droplets of modified synthetic oviduct fluid (mSOF) with nonessential amino acids, 3mM EDTA, and 0.4% fatty acid-free BSA (ICP bio, Auckland, New Zealand) under embryo-tested mineral oil. The embryo culture dishes were incubated at 38.5°C with 6.5% CO_2, 5% O_2, and 88.5% N_2 in 100% humidity. Embryos were transferred to new 10µL droplets of mSOF containing nonessential and essential amino acids 72 hours after fertilization. At 120 hours post-fertilization, embryos were transferred to 20µL droplets of mSOF containing nonessential and essential amino acids to prevent toxicity due to ammonium concentration and nutrient depletion caused, respectively, by amino acid degradation and embryo metabolism. Embryos were analyzed for cleavage on day 2, and blastocyst development on day 7 after fertilization. Rates of development were recorded. Adapted from [183].

4.3.5 Granulosa Cell Isolation

Granulosa cells from prepubertal animals were collected from follicles aspirated during LOPU. Under a stereomicroscope, granulosa cells from each individual calf per LOPU were separated from the follicular content and aspiration media using a 200µL pipette. The cells were placed in an eppendorf tube containing 600µL of phosphate buffered saline (PBS) (Life Technologies, Grand Island, NY, USA) and centrifuged for 1.5 minutes at 1300 x *g*. The supernatant was removed and the cells were rinsed again with PBS and centrifuged for 1.5 minutes at 1300 x *g* to yield the final granulosa cell pellet. Samples were immediately placed on dry ice and stored at -80°C until RNA extraction. In vivo granulosa cell collections from the mature cows were performed by transvaginal ultrasound-guided ovum pick-up (OPU). Granulosa cells were isolated and stored as mentioned above.

4.3.6 RNA extraction and Real Time PCR (qPCR)

Trizol was used to extract RNA from granulosa cell samples as per manufacturer's Mini-Trizol protocol. The quality and quantity of RNA from each sample was measured using the Nanodrop 2000 (Thermo Scientific). The 260/280 ratio for all samples was within a range close to 2.0. cDNA was synthesized from 250ng of total RNA using the iScript cDNA Synthesis kit (1708891, Bio-Rad, Mississauga, ON, Canada). All primers were purchased from Integrated DNA Technologies (Table 1) (Skokie, U.S.A.). The qPCR assays were performed according to the MIQE guidelines [184] (Bio-Rad). The following conditions were used for mRNA analysis: an initial denaturation at 95°C for 5 minutes followed by 39 cycles of 95°C for 15 seconds, 58°C for 30 seconds for annealing and 95°C for 10 seconds. Each primer sets were optimized so that the efficiency was between 85 and 115% and the correlation coefficient was between 0.99 and 1.00. Transcript abundance for a gene of interest in each sample was determined by taking starting quantity (SQ) values, as displayed in CFX manager TM software (Bio-Rad). Relative transcript abundance for each gene of interest was calculated by dividing their respective SQ values by the mean SQ values of two reference genes (*Beta-actin* and *Cyclophilin*).

4.3.7 Statistical Analyses

Data analyses were performed using JMP software (Cary, North Carolina, USA). The significance level employed for all experiments was $P<0.05$. The relative mRNA abundance between animal groups was checked for normality using the Shapiro-Wilk test. Sample groups not passing this normality test were further checked for normality using a log or ranks averaged transformation. Following normalization, experiments were analysed with Student's *t*-test or the Least Squares Means Student's *t*-test. The following model was used for analysis: $Y_{ij} = \mu + Treatment_i + e_{ij}$, where Y_{ij} represents the value for normalized mRNA abundance from the ith treatment of the jth animal, μ is the overall mean, $Treatment_i$ is the effect of the ith treatment on mRNA abundance, and lastly, e_{ij} is the random residual effect of the jth animal in the ith parameter (age, development rate or average aspirated follicular size). The proportion of *LHR*-positive samples was compared between groups using the Chi-Square test.

4.4 RESULTS

4.4.1 Effect of gonadotropin treatment on mRNA levels.

The relative mRNA abundance of *FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP* was determined in granulosa cells of control and FSH-treated prepubertal heifers and compared with FSH-treated adult cows. Significantly higher levels of *FSHR* mRNA were detected in adult cows compared to prepubertal heifers. Samples from the prepubertal control group had higher *STAR* and lower *CYP19A1* mRNA than adult and prepubertal FSH-treated groups ($P<0.05$; Fig. 1). In addition, samples from the prepubertal control group had higher *3β-HSD* and lower *XIAP* mRNA than FSH-treated calves ($P<0.05$; Fig. 1). There were no statistical differences between groups in mRNA abudance of *CX43*, *FOXO1*, *CALM*, and *AKT1* ($P>0.05$; Fig. 1). The average follicle size was significantly different ($P<0.05$) between adult FSH (9.38mm), prepubertal FSH (4.01mm), and prepubertal control (1.94mm) animals.

4.4.2 Effect of calf age and treatment on mRNA levels.

The relative mRNA abundance of *FSHR*, *CYP19A1*, *CX43*, and *XIAP* was higher in FSH-treated compared to control calves at <130 days, 100-130 days, 100-130 days, and at >100 days of age, respectively ($P<0.05$; Fig. 2). On the other hand, *STAR*, *3β-HSD*, and *FOXO1* mRNA was higher in prepubertal control animals at the three different ages, <130 days, and at >130 days, respectively ($P<0.05$; Fig. 2). There were no significant differences in the mRNA abundance of *CALM* and *AKT1* in granulosa cells across treatment groups at all ages ($P>0.05$; Fig. 2). The average follicle size was significantly higher in FSH-treated and control calves at ages of 100-130 days (4.93mm vs. 1.63mm, $P<0.05$) and of >130 days (3.39mm vs. 1.94mm, $P<0.05$).

4.4.3 Effect of gonadotropin stimulation duration on mRNA levels.

The relative mRNA abundance of *3β-HSD* and *CALM* was higher in animals treated for 2 days with FSH ($P<0.05$; Fig. 3). On the other hand, *STAR* mRNA was lower in animals treated with FSH for 4 days prior to LOPU ($P<0.05$; Fig. 3). There were no significant differences in the mRNA abundance of *FSHR, CYP19A1, CX43, FOXO1*, *AKT1,* and *XIAP* in granulosa cells across treatment groups tested ($P>0.05$; Fig. 3). The

average follicle size between the animals receiving 2 (3.31mm), 3 (3.9mm) or 4 days (4.3mm) of FSH treatment was not significantly different (P>0.05).

4.4.4 mRNA levels in samples with superior cleavage and blastocyst rates.

The mRNA abundance of *3β-HSD* and *CX43* was higher in samples that resulted in below median cleavage (≤70%) compared to above median cleavage (P<0.05; Fig. 4). On the other hand, the mRNA abundance of *FOXO1* was higher in samples that produced above median blastocysts (>15%) compared to below median development (P<0.05; Fig. 4). No significant differences were observed in the mRNA abundance of *FSHR, STAR, CYP19A1, CALM*, *AKT1*, and *XIAP* (P>0.05; Fig. 4) between samples of above and below median cleavage and blastocyst rates. There were no significant differences in the average follicle size when comparing the lower and higher groups of cleavage (3.54mm vs. 3.75mm, P>0.05) and blastocyst development (3.52mm vs. 3.83mm, P>0.05).

4.4.5 Effect of treatment on expression of *LHR* mRNA.

Presence of *LHR* mRNA was determined in GC samples of prepubertal control and FSH-treated animals. Significantly higher numbers of *LHR* positive samples were found in calves treated with FSH for 3 or 4 days compared to those treated for only 2 days and controls (Table 2; P<0.05).

4.5 DISCUSSION

The bi-directional mechanisms occurring between the mural granulosa cells and the oocyte in follicles leading to successful follicular growth and oocyte developmental competence are not fully understood. There is evidence from studies in adult cows that these events are influenced by genes expressed in mural granulosa cells [39]. However, it has not been demonstrated if granulosa cells of prepubertal heifers are able to regulate the same molecular mechanisms. Shedding light on critical genes controlling follicular growth and oocyte developmental competence could help improve the success of assisted reproductive technologies, including *in vitro* embryo production, in young genetically valuable animals. In the present study, relative levels of *FSHR, LHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP* mRNA in mural granulosa cells were

measured in control and FSH-treated prepubertal heifers, and FSH-treated adult cows. These genes were chosen based on their critical roles in the regulation of steroidogenesis [120, 125, 128, 131, 143, 146], cell-to-cell communication [162], apoptosis [166], and calcium metabolism [176].

Findings from this study first revealed that mRNA levels of *FSHR, STAR, CYP19A1, 3β-HSD,* and *XIAP* were altered in prepubertal control compared FSH-treated prepubertal and/or adult samples. This indicates that expression of genes involved in steroidogenesis and cell survival is altered in prepubertal heifers, but prepubertal granulosa cells are capable to regulate these genes in response to gonadotropin treatment. As expected, given their inactive hypothalamus-pituitary-ovarian (HPO) axis, prepubertal control animals have altered mRNA levels of genes encoding steroidogenic enzymes, which were evidenced by the lower abundance of *CYP19A1* and higher abundance of *STAR* and *3β-HSD*. It is possible that, by lacking the gonadotropins necessary to support follicular growth, a proportion of aspirated follicular pools in the control group had already begun to undergo atresia and switched from estradiol to progesterone production [185-187]. The fact that the mRNA expression of the apoptosis suppressor factor *XIAP* was lower in the control compared to FSH-treated samples, further suggests that more cells in the control group were committed to apoptosis [166].

Findings in this study revealed that exogenous gonadotropin treatment of prepubertal animals might mimic an active HPO axis and stimulate follicular development and granulosa cell functions as in adult cows. Indeed, mRNA levels of *FSHR*, *CYP19A1*, and *XIAP* were increased, and mRNA levels *STAR* and *3β-HSD* decreased in granulosa cells of FSH-treated compared to control prepubertal heifers, indicating less apoptosis and higher estradiol production in the follicles of the treated animals. It has been shown by previous studies that healthy ovarian follicles contain higher concentrations of estradiol compared to higher levels of progesterone and androgens in follicles undergoing atresia [185-187]. In this study, lower *FSHR* and *3β-HSD* mRNA levels were the only differences observed in samples from FSH-treated prepubertal compared to adult animals, which is likely due to the significant difference in the follicular size between these groups.

This study also showed that the age of the prepubertal heifers was not a determining factor affecting the response of granulosa cells to gonadotropin stimulation. Indeed, differences in mRNA abundance between control and FSH-treated animals were observed at different prepubertal ages: i.e., <100, 100-130, and >130 days. However, granulosa cells of treated and control calves older than 130 days had similar mRNA levels of *FSHR*, *CYP19A1*, and *3β-HSD*, which suggests that the HPO axis is more developed compared to young ages [89, 90, 144].

This study also revealed that extending the gonadotropin treatment from 2 to 4 days decreased the mRNA levels of *STAR* and *3β-HSD*, which suggests that more than 2 days of FSH treatment is necessary to select follicles with higher estradiol than progesterone production capacity. It is also possible that the number of follicles committed to atresia was reduced in the longer treatment. The numerically higher size of follicles in the longer compared to the short treatment further supports this hypothesis. *CALM* mRNA was also progressively decreased by extending the gonadotropin treatment. Calmodulin becomes activated by binding cytosolic calcium ions, and together, the calcium/calmodulin complex activates important enzymatic pathways [176]. Our findings suggest that longer gonadotropin treatment is required to modulate the calcium/calmodulin pathways in granulosa cells of prepubertal heifers. Extended FSH treatment from 2 to 3-4 days resulted in *LHR* expression in GCs of prepubertal heifers, which is likely due to the larger size of aspirated follicles in the longer treatments. *LHR* is an indicator of follicular maturation and ovulatory potential [89], and is normally detected in bovine granulosa cells from follicles >8mm in diameter [125].

Next, this study showed lower mRNA levels of *3β-HSD* and *CX43* in the higher cleavage group and higher *FOXO1* mRNA levels in the higher blastocyst group. Together, these results suggest that steroidogenic and cell-to-cell communication targets may play a critical role in yielding the lower rates of cleavage observed from oocytes retrieved from prepubertal compared to postpubertal animals [4-6, 188, 189].

Lastly, in this study, strictly the mRNA abundance of genes was evaluated. However, since mRNA abundance is not always reflective of protein abundance [190], performing follow-up studies to evaluate protein abundance of these same genes by Western blot would be of value [191]. The immunoblotting results may contribute to our

overall understanding of the roles of granulosa cells in the promotion of follicular development in prepubertal Holstein heifers in response to gonadotropin treatment.

4.6 ACKNOWLEDGEMENTS

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4.7 FIGURES AND FIGURE LEGENDS

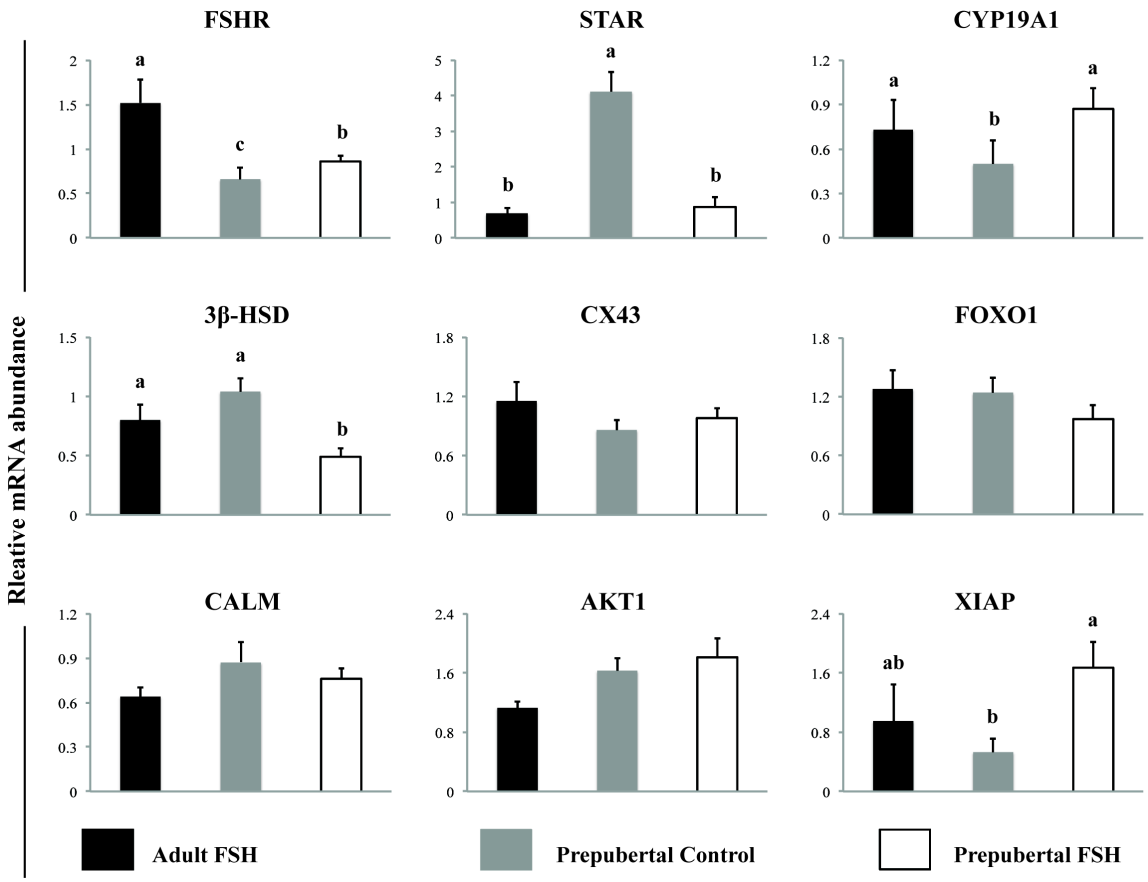

Figure 1: Effect of gonadotropin treatment on mRNA levels. Quantitative-PCR was performed to quantify the relative mRNA levels of genes (*FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP*) in granulosa cells from non-treated and FSH-treated prebubertal heifers, and adult FSH-treated cows (N=8-33/treatment). Data were normalized to control genes Beta-actin and Cyclophilin and are expressed as a mean SEM (+/-). Lower case letters denote significant differences (P<0.05).

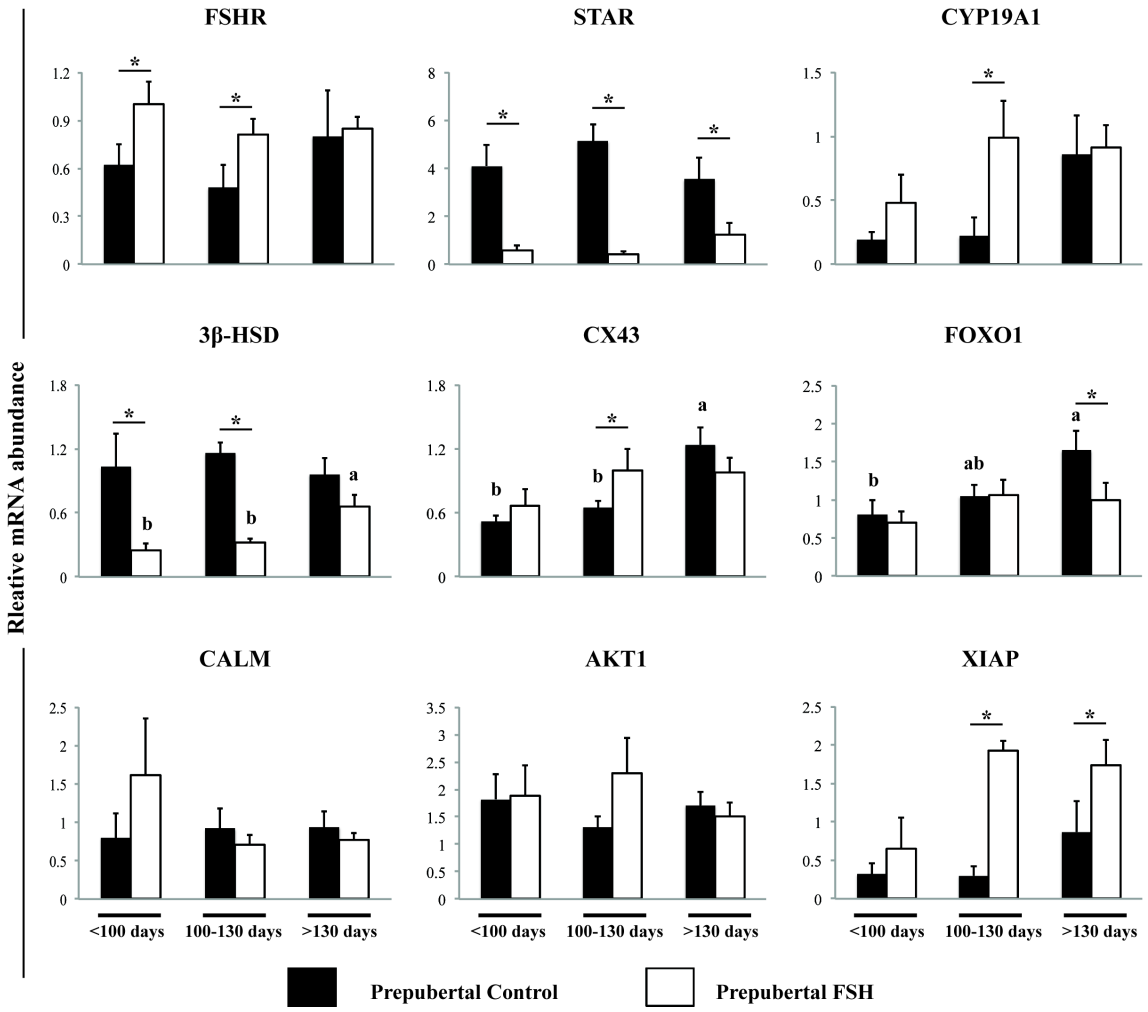

Figure 2: Effect of calf age and treatment on mRNA levels. Quantitative-PCR was performed to quantify the relative mRNA levels of genes (*FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP*) in granulosa cells of prepubertal animals receiving no treatment or a FSH treatment at the following ages: <100, 100-130, and >130 days old (N=5-17/treatment). Data were normalized to control genes Beta-actin and Cyclophilin and are expressed as a mean SEM (+/-). * denotes significant differences (P<0.05) between ages and lower case letters denote P<0.05 between stimulation protocols.

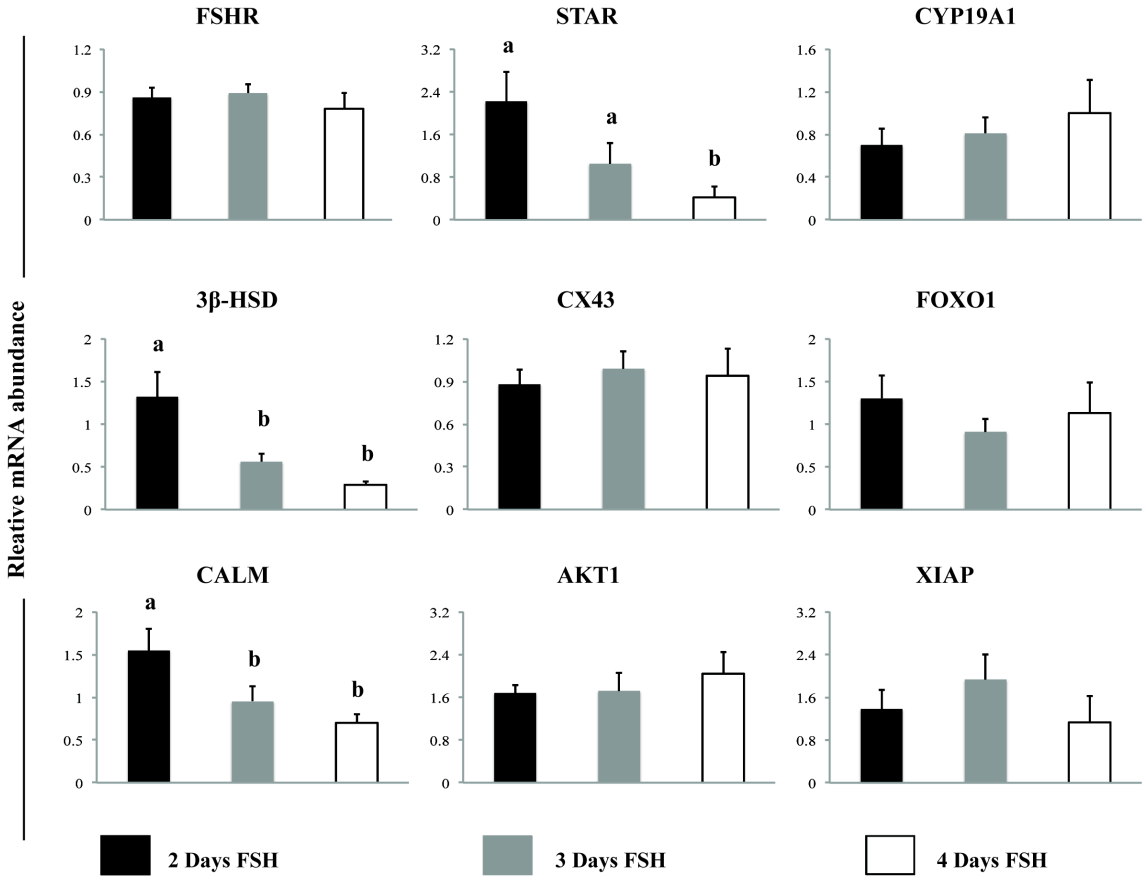

Figure 3: Effect of gonadotropin stimulation length on mRNA levels. Quantitative-PCR was performed to quantify the relative mRNA of genes (*FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM*, *AKT1*, and *XIAP*) in granulosa cells of prepubertal animals treated with FSH for 2, 3 or 4 days prior to LOPU at the following ages: <100, 100-130, and >130 days old (N=9-33/treatment). Data were normalized to control genes Beta-actin and Cyclophilin and are expressed as a mean SEM (+/-). Lower case letters denote significant differences ($P<0.05$).

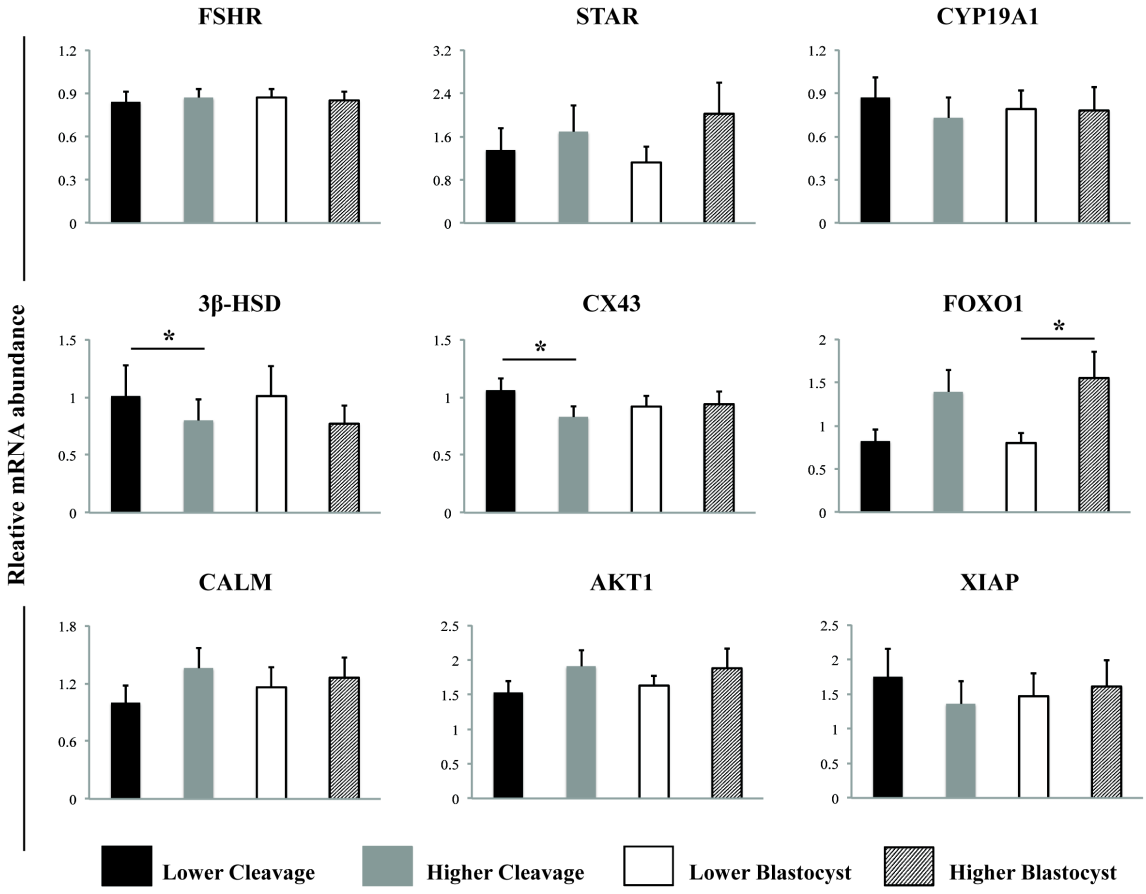

Figure 4: mRNA levels in samples with superior cleavage and blastocyst rates. Quantitative-PCR was performed in prepubertal FSH-treated animal granulosa cell samples to quantify the relative mRNA abundance of *FSHR, STAR, CYP19A1, 3β-HSD, CX43, FOXO1, CALM, AKT1,* and *XIAP* correlating with embryos of lower (≤70%) and higher (>70%) cleavage rates (N=29-37), and with embryos of lower (≤15%) and higher (>15%) blastocyst development (N=30-36). Data were normalized to control genes Beta-actin and Cyclophilin and are expressed as a mean SEM (+/-). * denotes significant differences (P<0.05).

4.8 TABLES

Table 1: Bovine primer pairs used in Real-Time qPCR experiments.

Gene name	Forward Primer	Reverse Primer
ACTB	TGTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA
CYCLO	ACAGTCAAGGCAGAGAACGG	CCAGCATCACCCCACTTGAT
FSHR	AGCCCCTTGTCACAACTCTATGTC	GTTCCTCACCGTGAGGTAGATGT
STAR	GAGATGGCTGGAAGAAGGTG	GCCAGATAACCCCATCTCAA
CYP19A1	GTGTCCGAAGTTGTGCCTATT	GGAACCTGCAGTGGGAAATGA
3β-HSD	GCTAATGGGTGGGCTCTGAA	TGATTGGTCAGGATGCCGTT
CX43	GGGTGACTGGAGTGCCTTAG	GTCCCCAGTAGCAGGATTCG
FOXO1	CGCAGATTTACGAGTGGATGG	CACTCTTGCCTCCCTCTGG
CALM	GAGGTCTCTTGGGCAGAATCC	GCGGAGCTCTGCTGCACTAA
AKT1	GATTCTTCGCCAGCATCGTG	GGCCGTGAACTCCTCATCAA
XIAP	GGACGTGGATGTACTCCGTT	AGCATGTTGTTCCCAAGGGT
LHR	GCACAGCAAGGAGACCAAATAA	TTGGGTAAGCAGAAACCATAGTCA

Table 2: Effect of treatment on follicular size and expression of *LHR* mRNA in prepubertal animals. Quantitative-PCR was performed with prepubertal control and FSH-treated animal granulosa cell samples to quantify the relative mRNA abundance of *LHR*. Data were normalized to control genes Beta-actin and Cyclophilin. Data are expressed as ratios between groups or sums. Lower case letters (a,b) denote significant differences ($P<0.05$).

Treatments	Number of *LHR*-positive GC samples	Number and size (mm) of aspirated follicles where *LHR* was detected in GCs		
		Small (<3)	Medium (3-5)	Large (>5)
Prepubertal Control	1/21 (4.8%)[b]	13	0	1
2 Days FSH	1/33 (3.0%)[b]	4	0	17
3-4 Days FSH	7/33 (21.2%)[a]	8	59	77

CHAPTER 5: CONCLUSION

In conclusion, there is a growing interest in producing offspring from genetically superior animals at prepubertal ages. However, oocytes recovered from prepubertal heifers are known to be less developmentally competent than those recovered from adult cows [6, 7]. This study investigated the expression patterns of genes involved in follicular growth and acquisition of oocyte developmental competence in granulosa cells of prepubertal Holstein heifers. Findings from this study revealed that: i) prepubertal ovaries are capable of responding to exogenous FSH with follicular development; ii) prepubertal control animals have increased mRNA levels of genes promoting progesterone production and follicular atresia; iii) prepubertal FSH treatments lead to the increased mRNA levels of genes promoting estradiol synthesis and follicular growth; and, iv) FSH treatments lead to an increased ability to inhibit granulosa cell apoptosis and follicular atresia.

CHAPTER 6: REFERENCES

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