DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES:
FOLLICULAR STATUS AND ULTRASOUND IMAGE
CHARACTERISTICS

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ABSTRACT

This thesis investigates the relationship between the physiological phase of follicles in cattle, their echotexture attributes and the developmental competence of the oocytes that they contain. This study also investigates the local influence of ovarian structures (CL and dominant follicle) on developmental competence of oocytes and follicular echotexture. The drive behind this work was to improve the understanding of ovarian function in this species for the purpose of refining the techniques of follicle manipulation, and to determine ultrasound image characteristics useful to the development of diagnostic and prognostic tools for use in cattle and other species, including humans.

After stimulating the emergence of a new ovarian follicular wave in cows, images of dominant and subordinate follicles were digitalized at Days 2, 3, 5 and 7 of the follicular wave. Cumulus oocyte complexes from the same ovaries were collected from subordinate follicles ≥3 mm and underwent in vitro embryo production to the blastocyst stage.

Image analysis revealed differences in echotexture between dominant and subordinate follicles among Days 2 to 7 of the follicular wave. Follicles at Day 7 of the wave displayed consistently lower (P < 0.05) values of image attributes in peripheral antrum, follicular wall and perifollicular stroma. Oocytes collected on this day displayed low developmental competence. The proportion of oocytes that developed to the blastocyst stage was higher (P<0.05) in COC collected on Day 5 after wave emergence than on any other day analysed. All follicles displayed a consistent pattern of variation in echotexture among follicular phases. Data did not support the hypothesis of
a local effect of the CL or dominant follicle on oocyte competence, and no variation of echotexture attributes related to the presence of ovarian structures were detected by image analysis.

Our results demonstrate a positive relationship between early follicular regression and oocyte competence. Moreover, changes in follicular image attributes were consistent with changes in follicular status. We conclude that echotexture analysis of ultrasound images could provide, if developed further, a very useful non-invasive, safe and easy to use diagnostic tool in assisted reproduction.
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Chapter 1

GENERAL INTRODUCTION

In the last decade, there has been an unprecedented evolution of technologies devoted to assisted reproduction. Until recent years, oocyte maturation and quality have not even been considered as important factors in influencing the embryonic yield of in vitro procedures. For a long time, the technical difficulties of achieving fertilization and culture of embryos in vitro have somewhat overshadowed the importance of oocyte quality. Moreover, since it is relatively simple to obtain oocyte nuclear maturation in vitro, many researchers have regarded oocyte quality as a minor problem. The establishment of successful and repeatable in vitro fertilization and culture systems has in turn allowed the research to focus on gamete development and biology. The more the knowledge of oocyte physiology advanced, the more obvious became the paramount importance of gamete quality for successful embryo production. As a direct consequence, the need for oocyte quality indicators to be used in everyday clinical and commercial settings became apparent. The challenge was to identify parameters that are correlated with oocyte quality, non-invasive, relatively fast and easy to assess, reliable and, most important, that would not damage the female gamete or the reproductive
ability of the female. Follicular fluid composition, cumulus cell morphology, follicle
dimension, stage of the estrous cycle at collection, body condition score of the donor
animal, circulating hormones concentration and oocyte metabolic byproducts are
examples of the numerous parameters investigated.

The recent development of an electronic algorithm to analyze the echotexture of
captured ultrasound images has brought a new perspective to this fascinating field of
research. The present study is, to our knowledge, the first attempt to link oocyte quality,
measured as \textit{in vitro} developmental competence to blastocyst stage, to the echotexture
characteristic of the follicles from which the oocytes were obtained.

1.1 Correlation between oocyte and follicle developmental anatomy

1.1.1 \textit{Prenatal development}

When an oocyte interacts successfully with the sperm and generates a zygote, what
is seen is the final step of a long and complex process that starts when the female gamete
is a primordial germ cell in the undifferentiated fetal ovary. During embryonic
development, the primordial germ cells arise from the yolk sac and migrate to the
undifferentiated gonad. Upon arrival of the germ cells, the epithelium of the gonadal
ridge begins to thicken and proliferate. Germ cells also proliferate by mitotic division.
After proliferation, the number of germ cells in the gonadal ridge may reach as many as
2 million per animal, more than 90\% of which will degenerate by the time the calf is
born (Erickson, 1966). After a process of reorganization of cytoplasm organelles and
cell growth, the primordial germ cells in the fetal gonad multiply by mitosis and become oogonia. When the mitotic phase is completed, the oogonia start the meiotic prophase and differentiate into primary oocytes. Meiosis comprises two successive divisions in order to reduce the genetic content of the cell from the diploid (2n) to haploid (n) state. The oogonium becomes a primary oocyte when it enters the first meiotic division, and it becomes a secondary oocyte when it starts the second. During the first meiotic division, the oogonium enters the proleptotene and leptotene phases of prophase I. During preleptotene, the final DNA replication takes place in the oogonium. This synthetic activity signals the passage from oogonium to primary oocyte. The primary oocyte then proceeds through the leptotene and zygotene phases. During the first meiotic division, part of the growth of the cell is due to nuclear enlargement, but by far the greatest contribution to oocyte enlargement is due to the increased number of cytoplasmic organelles (Baker, 1971). The following step is pachytene, in which crossing over and recombination of chromatids occurs. At the end of prenatal life, the oocytes have passed through diplotene and are arrested in the dictyate phase, where they remain until stimulated to resume meiosis.

During growth and differentiation of the female gamete, the follicle changes as well. There are four basic types of follicle: (i) primordial, (ii) primary, (iii) secondary, and (iv) vesicular (antral or Graafian). Primordial follicles are very small (30-60 μm in diameter); the oocyte is surrounded by one layer of 4 to 8 flattened granulosa cells, and does not have a zona pellucida (ZP; Hulshof et al, 1992). In cattle, primordial follicles occupy the peripheral areas of the ovarian cortex. The primordial follicle is separated from the ovarian stroma by a basal lamina. There are reports suggesting the presence in
primordial follicles of thecal cells as well (Hirshfield, 1991). Primordial follicles constantly move towards the primary and secondary follicle stage, thus entering the growing pool.

Primary follicles have the same dimension as primordial ones, but the granulosa cells are present in higher number and are cuboidal in shape, even though they are still organized a single layer (Hulshof et al, 1992). At this stage, the ZP appears. At the beginning, it is constituted by islands of fibrillar material lying between adjacent granulosa cells and the oocyte surface. As the oocyte continues to grow, the ZP becomes denser and more complete. The contact between the granulosa cells and the oocyte is maintained by cytoplasmic processes arising from the granulosa cells and projecting through the ZP to reach the oolemma, where they eventually connect with the oocyte by means of gap-junctions. These gated channels are maintained open throughout the oocyte growth and maturation, and are interrupted several hours after the germinal-vesicle breakdown phase of meiosis resumption (Larsen et al., 1987; Sutovsky et al., 1993).

The secondary follicles can be distinguished from primary follicles by their several layers of cuboidal granulosa cells, and greater oocyte size (Hulshof, 1992). Follicular and oocyte growth are coordinated and they are somehow regulated within the ovary. The factors initiating and regulating their growth are not clearly elucidated yet, but it is generally accepted that, in the earliest stages, follicular growth is independent from the amount of circulating gonadotrophins (Braw-Tal and Yossefi, 1997).

Vesicular follicles first appear in the fetal bovine ovary around the eighth month of gestation, and by the ninth month the ovary usually contains many vesicular follicles (Russe, 1983). The antrum first appears when the follicle reaches a diameter of 115 μm,
and 90% of the follicles of 280 μm are antral (Monniaux et al, 1983). The vesicular follicle is formed by a primary oocyte, containing a large nucleolus and surrounded by many layers of granulosa cells; a cavity (the antrum) filled with follicular fluid; a somatic component formed by many peripheral layers of columnar granulosa cells; an outer thecal layer in communication with the ovarian blood vessels.

1.1.2 Postnatal life

During postnatal life, antral follicles continue to grow. In follicles measuring 1 to 2 mm, the oocyte is surrounded by several layers of granulosa cells (Marion et al., 1968). While the follicle is growing, the granulosa cells differentiate into two populations: the mural granulosa in contact with the basement membrane and the cumulus cells enclosing the oocyte. By the time of ovulation, the follicle is at least 10 mm in diameter, while the cumulus oophorus is 8-10 cell layers thick and attached to the mural granulosa by a thin neck. The mural granulosa layer increases its size as well, and at ovulation is around 100 μm thick. While the follicle is growing, the oocyte undergoes several changes and increases its dimension as well. Oocyte dimension stays relatively constant until the follicle reaches a diameter of 5-6 mm. By the time the follicle is 8 mm large, the oocyte has reached a mean diameter of 135 μm (Motlik and Fulka, 1986). Apart from increasing its diameter, the oocyte undergoes many other morphological changes during postnatal growth. The nucleus size increases, and nucleoli form around the time that the bovine oocyte attains its full size (Motlik and Fulka, 1986). Many cytoplasmic organelles change their appearance as well. Mitochondria increase in
number (Kruip et al., 1983) and change their position within the cell. During the growing phase they are associated with the smooth endoplasmic reticulum and are elongated in shape, but at the end of the growing phase and during oocyte maturation they cluster at the periphery of the cell and assume an oval to round shape. The Golgi apparatus increases its activity during oocyte growth to process and concentrate its secretory products in the cortical granules that are being formed. The cortical granules appear when the oocyte is in its growing phase (Cran and Esper, 1990), and are formed by the Golgi apparatus and the rough endoplasmic reticulum.

1.2 Correlation between oocyte competence and morphology

As mentioned before, mammalian primary oocytes usually become arrested in prophase I of meiosis during the fetal period. Completion of the first meiotic division occurs only after the oocyte and the follicle that encompasses it have undergone extensive growth. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division, subsequent progression to metaphase II, and the accompanying cytoplasmic events essential for fertilization and early embryonic development. In vivo, this process is triggered by the preovulatory surge of LH. In cattle, ovulation occurs about 24 hours after LH surge (Bernard et al., 1983). At his time, cumulus cells are expanded and loosely attached to the ZP, the oocyte cytoplasm is finely granulated and, when the oocyte is denuded, the first polar body is clearly visible in the perivitelline space. The oocyte ovulated during a normal estrous cycle by a healthy sexually mature female is able to successfully sustain embryonic development. A cumulus oocyte
complex (COC), after 22 hours of in vitro maturation (IVM), achieves roughly the same morphological characteristics of a naturally ovulated one. Cumulus cells are usually expanded and a polar body is present. These are the morphological criteria used to determine that in vitro maturation is completed. These morphological similarities between the in vivo and in vitro matured COC have led scientists to conclude that COC with the same appearance possess the same developmental competence. Relatively recent reports, and the results of this work, prove this to be untrue. More than 90% of in vitro matured COC attain the mature-like morphology, but about two thirds of them will never reach the blastocyst stage (Brackett and Zuelke, 1993). There are different reasons for this apparent paradox. First of all, the ovulated COC comes from one large follicle, usually >13 mm in diameter, while in vitro produced embryo come from a pool of follicles of 2-8 mm. Secondly, the in vitro maturation system alters the metabolism of the oocytes and the expression of some of their genes (Watson et al., 2000). Finally, the communications between the somatic compartment and the oocytes are interrupted early in in vitro systems (Vassena et al., 1999; Sutovsky et al., 1993). Although in vitro morphology is a fast, easy to assess and non-invasive parameter, there is the need for a more reliable and accurate indicator of oocyte competence.

1.3 In vitro embryo production in cattle

The first demonstration of successful in vitro fertilization (IVF) was the birth of young rabbits after fertilizing matured eggs in vitro, using sperms recovered from the uterus of the female 12 hours after mating (Chang, 1968). In cattle, the first success in
the IVF of an artificially matured oocyte was reported in Japan (Iritani and Niwa, 1977). The first calf resulting from IVF of an ovulated oocyte was born the 9th June 1981 in the USA (Brackett et al., 1982). A few years later, Lu and coworkers (1987) reported the birth of twins from cattle oocytes undergoing a complete *in vitro* embryo production (IVEP) process, that is maturation (IVM), fertilization, and culture (IVC). The development of an IVEP protocol has made a large number of embryos available to researchers and investigators. This in turn has led to the development and refinement of many new assisted reproduction techniques such as embryo sexing by blastomere biopsy and subsequent polymerase chain reaction (PCR), assisted hatching, embryo splitting, intracytoplasmic sperm injection (ICSI), sperm sexing and sorting, genetic cloning and nuclear reprogramming. The results of the development of new biotechnologies were such that, in 1997, Wilmut and colleagues at the Roslin Institute announced the successful cloning of a sheep by fusion of an adult somatic cell from the mammary gland and the cytoplasm of an oocyte (Wilmut et al., 1997).

Oocyte maturation is the first of the three steps involved in an IVEP protocol. Two main maturation systems have been developed in time; one involves the culture of the whole follicle *in vitro* while the second involves the removal of the COC from the follicle. The latter is the most widely used, while the first system is now reserved for particular research application such as the study of steroidogenesis in calves (Peluso and Hirshel, 1987, 1988) and in adult cows (Kruip and Dieleman, 1989) or of the effect of different hormones on the follicle-oocyte functional unit (Yoshimura et al., 1992). Maturation media can be broadly divided into two categories: simple and complex. Simple media are often bicarbonate buffered systems containing saline with pyruvate, lactate and glucose. They are usually supplemented with serum, mostly from fetal calf
(FCS), newborn calf (NCS) or estrus cow (ECS), and albumin to provide nutritional substrates and to ensure the correct osmolarity. In addition, they contain trace amount of antibiotics and antimycotic to prevent contamination of the culture by bacteria and fungi. Complex media contain the same components of simple ones, but amino acids, vitamins, purines and other substances are added. The aim of complex media is to mimic as closely as possible the environment that is lost when the COC is removed from the follicle. One of the most widely employed IVM media in cattle since the mid 1980s, and the one used in this work as well, is the tissue culture medium 199 (TCM-199), which is a complex media consisting of Earle's salts, buffered with HEPES and sodium bicarbonate and supplemented with pyruvate, lactate, vitamins, amino acids, traces of antibiotic/antimycotic and other substances found in FCS. For many years, TCM-199 has been the standard medium employed in cattle and sheep oocyte IVM in Europe; most cattle IVF laboratories all over the world now routinely use TCM-199 for IVM, and there have been very few reports suggesting that other complex media could be more appropriate (Hawk and Wall, 1993).

Together with an increased request for in vitro produced embryos to be shipped across national borders, and the consequent concerns of spreading infectious diseases in specific disease-free countries, there has been an intense search for a completely defined medium, that is, a medium containing only known substances. Serum, which often represents at least 10% of the medium final volume, is the main source of sanitary concern. Some studies have reported good maturation rates with defined media, but it is still difficult to support in vitro embryo development to the blastocyst stage with these media.
The second step for a successful IVEP protocol is the fertilization *in vitro* of the mature oocytes. Before fertilization, the semen has to be prepared and the quality of the sperm, especially if coming from frozen/thawed straws, has to be artificially increased. Motility, together with concentration, is probably the most important characteristic for IVF semen. There are at least three techniques for selecting and separating the motile fraction of the semen: swim-up, glass wool filtration and use of a bovine serum albumin (BSA)/Percoll density gradient. Swim-up involves covering the sperms with a layer of suitable medium (usually TALP) and incubating the swim-up tubes, leaning at 30°, for 30-60 minutes in an incubator at 39°C. Motile sperms will swim up into the TALP medium and can be recovered from the supernatant. Swim-up consistently yields samples with highly motile sperms, and it has been proven to increase IVF in cattle by 46% to 59% (Parrish et al., 1984; Keefer et al., 1985). The swim-up technique has been used in this work.

Another system for sperm selection is the passage of the semen sample through a column of glass beads or glass wool. In human IVF, this is a reliable and consistent method to separate poor quality sperms from motile ones (Daya et al., 1987; Lechtzin et al., 1991). In cattle IVF, a study comparing glass wool filtration and swim-up concluded that the two separation methods were both adequate, but glass wool filtration was significantly faster (it takes only 3 to 5 minutes) and therefore more suitable for busy commercially oriented laboratories (Stubbing and Wosik, 1991).

The BSA/Percoll density gradient method is based on the assumption that viable, motile, morphologically normal sperms can be isolated using a discontinuous BSA gradient because they are denser than abnormal sperm. With Percoll gradients, sperms
are separated because they migrate in the fraction of the gradient whose density is in equilibrium with their own density. In pigs, this procedure yields a fraction of more than 90% motile sperms (Estienne et al., 1988). The gradients used in cattle IVF can be 30% and 40% (Utsumi et al., 1988), 30% and 45% (Utsumi et al., 1991) or 90% and 45% (Rosenkrans et al., 1993). Many studies found that Percoll gradient separation is more effective than swim-up when used to separate poor quality semen (Lanu and Blanchard, 1988; Sapienza et al., 1993; Moohan and Lindsay, 1993). Unfortunately, because of suspected embryo toxicity and teratogeny of Percoll, its use has been prohibited in human assisted reproduction and, more recently, it has been completely withdrawn from the market.

Once selected and separated, sperm are washed and used for IVF. Substances such as caffeine (Ohogoda et al., 1987), glutathione (Slaweta and Laskwoska, 1987), heparin, or a mixture of penicillamine-hypotaurine-epinefrine (PHE; Leibfried and Bavister, 1982) are added to the final medium to increase sperm motility. Before fertilization, oocytes are washed and, in some species (humans), the cumulus cells are removed from the ZP to facilitate fertilization. In cattle, the effect of oocyte denuding before IVF not only does not improve the fertilization rate (Cox, 1991), but it also decreases the incidence of acrosomal reaction in sperms at fertilization (Fukui, 1990; Younis and Brackett, 1991) and therefore is not performed. Fertilization can be achieved in a variety of media and systems, including coculture with granulosa or oviductal cells, but the easiest way to perform IVF is with microdroplets of fertilization media covered with tissue culture oil and left in the incubator for a variable time (from 3 to 24 hours, according to the system and the concentration of sperms employed).
After incubation of mature oocytes with sperms, IVC takes place. This is the last and longest step of embryo production, and it starts with transferring the presumptive zygotes to the culture medium. Zygotes are washed in culture media and then placed either in a cell-free media, on a monolayer of different cell populations or surgically transferred into the ligated oviducts of surrogate mothers, usually rabbits or sheep (Lu et al., 1987). The use of an oviductal cells monolayer as the culture system in sheep was an important step towards successful IVEP (Gandolfi and Moor, 1987), and soon it became apparent that many different populations of somatic cells from different species (sheep, cattle, pigs, horses, rabbits, mice and humans) could be used as a viable substrate for IVC. Among in vitro culture systems that do not involve the use of cell monolayer, we can differentiate three main groups of media: the first one is the synthetic oviductal fluid (SOF) and its variants, developed for use in low oxygen concentration by Tervit and coworkers (1972) and successively improved and modified by various authors. The second group of media is formed by common culture media such as non-enriched TCM-199. These media do not yield a high percentage of blastocyst stage embryos (Wright and Bondioli, 1981). The last group of media comprises chemically defined media, of novel formulation and designed on the metabolic requirements of the embryo rather than modifications of more common tissue culture media. The Charles Rosenkrans 1 (CR1) media with both essential and non-essential amino acids (Rosenkrans et al., 1990; Rosenkrans and First, 1991), also used in this work, and the semidefined variation of the TCM-199 + BSA system including insulin, transferring and selenium (Shamsuddin et al., 1993) are examples of this category. The advantage of a complex chemically defined medium is the independence of the outcome (embryonic development) from the many variables of a coculture with somatic cells.
1.4 Follicular dynamics in ruminant

Rajakoski (1960) postulated that 2 waves of follicular activity occur during the bovine estrous cycle on the basis of gross and histological examination of ovaries recovered on known days of the cycle. However, it was not until the development and use of ultrasound techniques that the pattern of follicle development in cattle could be fully elucidated. Emergence of a follicular wave is defined as the synchronous development of a group or cohort of follicles during the final phase of growth (Ginther et al., 1989a, 1989b; Knopf et al., 1989). Follicular growth during the estrous cycle is characterized by two or three follicular waves (Pierson and Ginther, 1986, 1987, 1988; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a; Knopf et al., 1989). During each follicular wave, a cohort of follicles is initiated to grow beyond 4 mm in diameter. From this cohort, a single follicle is selected and grows larger than the rest of the follicles in the cohort to become the dominant follicle. The other follicles in the cohort regress, while the dominant follicle continues to grow until it reaches the maximum size. If the dominant follicle develops during the follicular phase, it ovulates. However, dominant follicles developing during the luteal phase of the estrous cycle regress, rather than ovulate, because of the lack of the preovulatory LH surge (Kastelic et al., 1990). Emergence of successive follicular waves during the estrous cycle has been associated with increases in circulating concentrations of FSH (Adams et al., 1999), which preceded wave emergence by 1 day (Adams et al., 1992). Furthermore, declining concentration of FSH after wave emergence has been implicated in the
mechanism of selection of the dominant follicle (Adams et al., 1999). A surge of FSH begins before a wave emerges, reaches a peak 1-2 days before wave emergence, and declines 1-2 days after; this decline is in temporal association with the appearance of a selection mechanism that allows one follicle of the wave to become dominant (Adams et al., 1992). The hypothesized mechanism of selection is that follicles in the cohort need FSH to continue their growth. There would be a competition for utilization of the FSH available, and the most successful follicle becomes the dominant. The competition among follicles becomes acute when FSH levels decline. Subordinate follicles can be “rescued” by higher concentrations of FSH (Adams et al., 1993), or by the removal of the dominant follicle (Ko et al., 1991), and can assume a dominant position. The competitive phase ends by Day 5 of the wave, and after this point subordinate follicles are committed to regression by atresia (Adams et al., 1992; Ko et al., 1991). However, the functional stage of the follicle in vivo is not always associated with the developmental competence of the oocyte that it contains. A slight follicular degeneration appears to be positively related to oocyte developmental competence in vitro (Blondin et al., 1997). Removal of the dominant follicle either by cautery (Ko et al., 1991) or by puncturing (Bergfelt et al., 1994; Gibbons et al., 1997) allowed a new follicular wave to emerge within the next two days (mean value: 1.5 days after ablation). Ultrasound guided transvaginal follicular aspiration has been used in the present study to elicit a new wave emergence.

Wave emergence has been detected, on average, on the day of ovulation (Day 0) and day 10 in cows with two follicular waves, and on days 0, 9 and 16 in cows with three follicular waves (Ginther et al., 1989a). Although 2-wave cycles were shorter than 3-waves cycle (20.4 versus 22.8 days), the growth dynamics of Wave 1 follicles were
similar in 2 wave and 3 wave cycles (Ginther et al., 1989a, 1989b). In the present study, only follicles from Wave 1 were used. During an anovulatory wave, the development of each follicle has been subdivided into growing (increasing diameter), static (no changes in diameter) and regressing (decreasing diameter) phases (Ginther et al., 1989a, 1989b; Knopf et al., 1989). These ultrasonographically classified phases have shown to be closely correlated with the follicle's ability to produce steroid and protein hormones, which are indicative of follicular health (Badinga et al., 1992; Guibault et al., 1993), and its ability to ovulate in response to prostaglandin induced luteolysis (Kastelic et al., 1990; Savio et al., 1990).

1.5 Ultrasound quantitative image analysis of ovarian functions in ruminants

An ultrasonographic image is composed by a two-dimensional matrix of an array of picture elements (pixels) that differ in their gray-scale value, displayed on a video screen (Kremkau, 1998; Ghinter 1995; Pierson and Adams, 1995). Each pixel represents a discreet tissue reflector as it relates to the reflection of the ultrasound beams and is represented by one of 256 shades of gray, 0 corresponding to black and 255 to white (Pierson and Adams, 1995). The characteristic appearance of the ultrasound image of a particular tissue is referred to as the echotexture of the tissue (Ghinter, 1995) and is ultimately determined by the morphology of the tissue (Kremkau, 1998; Ghinter 1995). In the past, echotexture of a tissue has been scored subjectively by visual examination (Pierson and Ghinter, 1985; Townson and Ghinter, 1989). Unfortunately, the human eye does not readily quantify tissue characteristics. In addition, each individual perceives
gray-scale data differently, leading to extremes of variability in image interpretation. Furthermore, visual examination depends on operator consistency and expertise, and differences may not be apparent on an individual-observation basis for diagnostic purposes. Computer-assisted analysis of gray-scale values provides a quantitative approach that minimizes inconsistencies between operators. Such computer algorithms specifically designed for ultrasound images have been custom-developed at the University of Saskatchewan (SYNERGYNE, Version 2.8, WHIRL, Saskatoon, SK, Canada; ©Pierson) and have been used in many studies (Adams and Pierson, 1995; Pierson and Adams, 1995; Singh et al., 1997, 1998; Tom et al., 1998a, 1998b) to characterize the echotexture of ovarian structures or of the male reproductive tract (Chandolia et al., 1997). The image analysis algorithms allow quantification of echotextural tissue characteristics of anatomical structures. Two of the analysis methods that can be used, spot analysis and line analysis, have been applied in this work.
1.6 Objectives

The overall objective of the thesis work was to study the developmental competence of the oocyte contained in bovine ovarian follicles. I attempted to find a relationship between oocyte competence, stage of the follicular wave in which they were collected and ultrasonographic attributes. The drive behind the work presented here was to establish a correlation between bovine follicular structure and oocyte competence in order to improve the understanding of ovarian function in this species for the purpose of refining the techniques of follicle manipulation, and to determine ultrasound image characteristics useful to the development of diagnostic and prognostic tools for use in cattle and other species, including humans.

The specific objectives of the study presented in this thesis were to:

1. Investigate whether morphology and developmental competence of cumulus oocyte complexes are related to the phase of development of the follicle and to the presence of the corpus luteum and/or the dominant follicle in the ovary from which the cumulus oocyte complexes were collected (Chapter 2).

2. Investigate whether ultrasonographic attributes of follicles are related to the phase of follicular development, to the presence of the corpus luteum and/or the dominant follicle in the ovary, and to the developmental competence of cumulus oocyte complexes contained in the follicles from the same ovary (Chapter 3).
Chapter 2

MORPHOLOGY AND DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES RELATIVE TO FOLLICULAR STATUS

2.1 Summary

In cattle, follicle dimension has been used as the main criterion for selection of oocytes for in vitro embryo production. However, follicles with similar diameters may be in very different physiologic phases. The aim of this study was to investigate whether the morphology and developmental competence of cumulus oocyte complexes (COC) are related to: 1) the phase of development of the follicle, and 2) the presence of the CL and/or the dominant follicle in the ovary from which the COC were collected. Cross-bred beef cows (n = 143), ranging in age from 4 to 14 years, were given a luteolytic dose of PGF$_{2a}$ and 8 days later underwent transvaginal ultrasound-guided ablation of all follicles $\geq$ 4 mm to induce emergence of a new follicular wave. Cows (n = 10 to 20 per replicate) were sent to the abattoir on Days 2, 3, 5 or 7 (Day 0 = follicular wave emergence), equivalent to the growing, early static, late static, and regressing
phases of subordinate follicle development, respectively. Ovaries were recovered and COC were collected from all subordinate follicles ≥ 3 mm. COC were morphologically classified as denuded, degenerated, or healthy, and underwent IVM-IVF-IVC irrespective of morphologic classification. No differences were detected among days in the proportion of COC that were classified as having a compact cumulus (59%, 65%, 60% and 68%, respectively), but fewer (P<0.05) denuded COC were collected on Days 5 (5%) and 7 (3%) than on Days 2 (11%) and 3 (16%). Data did not support the hypothesis of a local effect of the CL or dominant follicle; oocyte morphology and developmental competence were similar between COC collected from the ovary ipsilateral and contralateral to the CL or the dominant follicle.

The proportion of oocytes that developed to the blastocyst stage was higher (P<0.05) in COC collected on Day 5 after wave emergence (23%) than on Days 2 (12%), 3 (13%) or 7 (16%). We conclude that a positive relationship exists between early follicular regression and oocyte competence; COC from subordinate follicles in the late static phase had greater developmental competence. Moreover, morphologic characteristics used in this study were not predictive in identifying competent oocytes in this study.

2.2 Introduction

Calves have been produced for numerous years from oocytes that underwent in vitro maturation (IVM), fertilization (IVF) and culture (IVC) (Goto et al., 1988; Lu et al., 1987; Hanada et al., 1986). However, the proportion of oocytes that develop to the
blastocyst stage in vitro has not improved over time (Farin et al., 2001). Much attention has focused on the in vitro environment; however, the inability to improve in vitro embryo production may be a result of the inherent developmental competence of oocytes collected from small follicles (2 to 6 mm diameter; reviewed in Blondin and Sirard, 1998). The majority of COC collected from 2 to 6 mm follicles appear to mature in vitro, but many are not capable of producing blastocysts (Brackett and Zuelke, 1993). Oocyte competence, defined as the ability of an oocyte to sustain embryonic development to the blastocyst stage in vitro, has been examined in relation to follicular diameter in cattle (Pavlok et al., 1992; Tan and Lu., 1990), and current production methods use follicle diameter as the main criterion for selecting COC for in vitro embryo production (IVEP). However, follicles of the same diameter can be in very diverse physiological phases, according to their stage of development in the follicular wave at the time of collection.

Emergence of a follicular wave is defined as the synchronous growth of a cohort of follicles (Ginther et al., 1989a, 1989b; Knopf et al., 1989). Follicular growth during the estrous cycle in the cow is characterized by two or three follicular waves (reviewed in Adams, 1999). During each wave, a cohort of follicles begins to grow beyond 4 mm in diameter. From this cohort, a single follicle is selected to become the dominant follicle and continues to grow while the remaining follicles in the cohort begin to regress. The hypothesized mechanism of selection involves follicular competition for FSH, and the liberation of the incipient dominant follicle from FSH dependence through the acquisition of LH responsiveness. After the wave-inducing surge of FSH, competition among follicles becomes acute; the first follicle to acquire LH receptors
becomes dominant while those depending on FSH regress. The competitive phase ends around Day 5 of the wave; after this point, subordinate follicles are committed to regression (Adams et al., 1992; Ko et al., 1991). Ablation of the dominant follicle by either cautery (Ko et al., 1991; Adams et al., 1992) or puncture (Bergfelt et al., 1994; Gibbons et al., 1997) results in the emergence of a new follicular wave within the next two days (mean, 1.5 days after ablation). Ultrasound-guided transvaginal follicular ablation was used in the present study to elicit new wave emergence.

The purpose of this study was to test the hypotheses that 1) oocyte competence is related to the phase of the follicular wave in which it is collected rather than to the dimension of the follicle from which it is derived, 2) oocyte competence is influenced by the local presence of the CL or the dominant follicle in the ovary from which it was collected, and 3) the morphological characteristics of cumulus oocyte complex is related to the stage of the follicular wave and its developmental competence.

2.3 Materials and Methods

2.3.1 Animals and grouping

Cross-bred beef cows (n=152), 4 to 14 years of age, were used during July, August, and September. The cows were kept in 3 outdoor paddocks on a single farm, and maintained on a rising plane of nutrition (barley and hay) in preparation for slaughter. After an initial ultrasound examination of the reproductive system (Aloka
SSD-500 with a 5 MHz linear-array transducer; ISM Inc., St. Laurent, PQ, Canada), 9 cows were excluded because of advanced pregnancy. Thirteen of the remaining 143 cows were pregnant \( \leq 70 \) days and were given PGF to induce abortion (25 mg dinoprost; Lutalyse, Pharmacia & Upjohn Animal Health, Orangeville, ON, Canada). They were included in the last replicate of the study, at least 40 days after induced abortion. Cows were assigned randomly to groups in which ovaries were to be collected at slaughter on Days 2, 3, 5, or 7 of the follicular wave (Day 0 = wave emergence). Cows (10 to 20/replicate) representing 2 day-groups, were synchronized to permit slaughter on a given day. Cows were thus divided into 8 replicates. Synchronization involved a luteolytic dose of PGF (Lutalyse) followed 8 days later by transvaginal ultrasound-guided ablation of all follicles \( \geq 4 \) mm. The day after follicle ablation was considered to be the day of wave emergence (Bergfelt et al., 1994). The goal of the synchronization scheme was to electively induce a wave of follicular development during the luteal phase in all animals.

2.3.2 COC collection and classification

The ovaries were examined by transrectal ultrasonography \( \leq 2 \) hours before the cows were sent to the abattoir to record the dimensions and positions of the dominant and subordinate follicles and the corpus luteum (CL). The cows were grouped so that the ovaries could be collected in a time range not exceeding 30 minutes after slaughter. Each ovary was placed in an individual plastic bag filled with phosphate-buffered saline (PBS) + 1% (w/v) antibiotic/antimycotic (10,000 IU penicillin, 10 mg streptomycin and 25 \( \mu \)g amphotericin B/ml; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Each bag
was marked with an identification number different from the ear tag number so that the investigator was blinded from day groupings. The ovaries were maintained at 30°C and transferred to the laboratory within 3 hours of collection. The COC were aspirated into a 15 ml glass vial containing 3 ml of warm collection medium (TCM 199 + 0.75% kanamycin). Aspiration was accomplished with an 18 g 1.5 inch needle and a vacuum pump using a continuous controlled flow-rate of 35 ml/min. The needle was rinsed with warm collection medium before and after aspiration of each follicle to minimize sticking of the COC to the internal surfaces of the needle and tubing. The dominant follicle and, when in doubt, the largest subordinate follicle were aspirated separately. COC from all subordinate follicles ≥3 mm in the same ovary were collected and pooled in the same tube. COC were evaluated morphologically at a magnification 400x and categorized as compact, not compact, or denuded. Denuded oocytes were defined as those with less than one complete surrounding layer of granulosa cells. Compact COC were those with ≥3 surrounding layers of granulosa cells, a compact cumulus, smoothly granular oocyte cytoplasm, and no irregularities of the oocyte nucleus. Not compact COC were those with one or more of the following characteristics: expanded cumulus, ≥2 surrounding layers of granulosa cells; pyknotic granulosa cell nuclei; fragmented oocyte nucleus; misshapen, partially absent, or vacuolated oocyte cytoplasm; empty zona pellucida.

2.3.3 In vitro maturation, fertilization and culture (IVM, IVF, IVC)

All COC were matured, fertilized and cultured, regardless of initial morphology.
After collection, the COC were washed twice in the collection medium and once in the maturation medium. The maturation medium consisted of TCM 199 + 0.1% PVA + 1 μl/ml FSH/LH (Folltropin V, Vetrephe A Canada Inc., Belleville, ON, Canada) + 0.75% kanamycin + 10% (v/v) fetal calf serum (FCS, Gibco BRL, Burlington, ON, Canada). COC were matured for 22 hours in a drop of 20 μl of maturation medium in a petri dish previously conditioned under tissue culture oil at 38.5°C, 100% humidity and 5% CO2.

A few hours before the end of maturation, semen for IVF was prepared. Semen from a Charolais bull known from previous use to be effective for IVF was used in the present study. Two 0.5 ml straws were thawed in a water bath at 37°C for 1 minute and assessed for post-thaw motility. The semen was pooled in a 15 ml polypropylene test tube (VWR Canlab, Mississauga, ON, Canada) previously warmed to 37°C and then dispensed into four 1.7 ml Eppendorf tubes (250 μl of semen each) containing 1 ml of SP-TALP medium. The tubes were placed in the incubator at 39°C for 1 hour to allow selection by swim-up of the sperm. The supernatant (800 μl) containing the motile fraction of sperm was collected from each Eppendorf tube and pooled in a warmed 15 ml Falcon tube. The semen was washed twice by adding 5 ml of FERT-TALP medium followed by centrifugation (500 G for 8 minutes); the supernatant was discarded and replaced each time. A sample of 5 μl of semen solution was diluted 1:20 with H2O and sperm concentration was estimated using a modified Neubauer chamber (VWR Canlab) under a phase-contrast microscope (Diaphot; Nikon Canada Inc., Mississauga, ON, Canada) at a magnification of 400x. The concentration of spermatozoa in the fertilization solution was adjusted to 3.5 million cells/ml. Heparin was added to the
sperm suspension at a final concentration of 10 \( \mu g/ml \). Fertilization microdroplets of 15 \( \mu l \) were prepared in a petri dish and covered with conditioned tissue culture oil. Matured COC were washed twice in FERT-TALP medium, and then placed in the sperm suspension in an incubator at 38.5°C for 20 hours.

After exposure to the sperm suspension, the presumptive zygotes were washed once in FERT-TALP medium, vortexed for 3 minutes to remove the remaining cumulus cells, and washed again in IVC medium before being transferred to conditioned IVC microdroplets under tissue culture oil. The IVC medium was CR1 + 10% FCS (Rosenkrantz et al., 1990). The developing zygotes were maintained at 38.5°C, in an atmosphere of 5% CO\(_2\) and 100% humidity, and evaluated for development at the 2- to 4-cell, 8- to 16-cell, morula, and blastocyst stages (i.e., at 36, 84, 120 and 192 hours post-insemination). Regardless of the developmental stage attained, culture was terminated at Day 9 (216 hours post-insemination).

2.3.4 Statistical analyses

In vitro development of oocytes (i.e., proportion that developed to the cleavage, 8 to 16 cell, morula, or blastocyst stages) was compared among the 4 follicular phases (i.e., Days 2, 3, 5, or 7) and among the 3 morphological categories (i.e., denuded, degenerated, or healthy) by Chi-squared analyses. For analyses involving the local effects of the CL and dominant follicle, the ovary was considered the experimental unit (n). Within each day-group, animals were ranked according to the number of blastocysts.
they produced. The highest 4 producers were randomly assigned to 1 of 4 subgroups; the next highest producers were similarly assigned, and so forth until all cows were placed in one of the 4 subgroups. This was done in an attempt to overcome the intrinsic limitation of in vitro embryo production when done with few oocytes per culture droplet, and the number of ovaries that did not produce any embryos because of a small number of COC collected (1 to 4). Once formed, the subgroups were retained for all successive statistical analyses. Using a mean and variance derived from each of the subgroups, two-way analyses of variance were used to examine the local effects (ipsilateral vs. contralateral) of the CL or the dominant follicle on blastocyst production in the respective day-groups. To determine the local effects of ovarian structures (CL or dominant follicle) on oocyte morphology, and its interaction with follicular stage (Days 2, 3, 5 or 7), data were transformed (arcsin of the square root of the proportion of oocytes in a given morphologic category) so that 2-way analyses of variance could be done. The theoretical binomial error variance used for the analysis of variance was $821/n$ ($n =$ number of subgroups; Fisher and van Belle, 1993). A probability value of $< 0.05$ was considered significant (i.e., less than a 5% probability that the difference detected was due to chance alone).

2.4 Results

2.4.1 COC morphology

The effect of the day of collection on the morphology of COC is presented in
Table 2.1. The percentage of COC classified as compact ranged from 59.8% to 67.73%, and did not vary among Day-groups. The percentage of denuded oocytes decreased (P<0.05) from Day 3 (16%) to Days 5 (5%) and 7 (3%). Conversely, the percentage of not compact COC increased (P<0.05) from Day 3 (19%) to Days 5 (35%) and 7 (29%).

No differences were detected in the proportion of either compact or not compact oocytes collected from the ovary containing the dominant follicle or from the contralateral ovary on different days of the follicular wave. However, fewer (P < 0.0001) denuded oocytes were collected from the ovary containing the CL than from the contralateral ovary. Moreover, the proportion of denuded oocytes collected from ovaries containing the dominant follicle was higher than from the contralateral ovary (P = 0.03). An interaction (P < 0.0001) between CL-side and Day-group resulted from fewer denuded oocytes collected on Day 7 from the ovary containing the CL compared to the ovary contralateral to the CL. Similarly, an interaction (P = 0.03) between side of the dominant follicle and day resulted from a greater proportion of denuded oocytes collected on Day 7 from the ovary containing the dominant follicle compared to the contralateral ovary.

2.4.2 In vitro development

Technical problems with the in vitro culture procedures were experienced during the first 3 replicates. Therefore, the following results were obtained from the last 5 replicates (74 cows). The effect of day of the follicular wave on oocyte in vitro development is presented in Tables 2.2 and 2.3. A greater percentage (P<0.05) of
oocytes collected on Day 5 of the follicular wave developed to the morula and blastocyst stages than in any of the other Day-groups (Table 2.2). The percentage of oocytes that developed to the 2- to 4-cell stage was lower (P<0.05) in the Day 7 group than in the Day 2 and 3 groups.

Table 2.1. Morphology of COC collected from subordinate follicles (>3 mm) on successive days after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>Oocytes</th>
<th>Denuded (%)</th>
<th>Not Compact (%)</th>
<th>Compact (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>365</td>
<td>40 (11.0)a,b,c</td>
<td>109 (29.9)b,b</td>
<td>216 (59.2)a</td>
</tr>
<tr>
<td>3</td>
<td>319</td>
<td>52 (16.3)c</td>
<td>61 (19.1)b</td>
<td>206 (64.6)a</td>
</tr>
<tr>
<td>5</td>
<td>436</td>
<td>23 (5.3)a,b</td>
<td>151 (34.6)a</td>
<td>262 (60.1)a</td>
</tr>
<tr>
<td>7</td>
<td>189</td>
<td>6 (3.2)b</td>
<td>55 (29.1)a,b</td>
<td>128 (67.7)a</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1309</td>
<td>121 (9.2)</td>
<td>376 (28.7)</td>
</tr>
</tbody>
</table>

a,b,c Within columns, values with no common superscript are different (P<0.05).

Table 2.2. In vitro development of oocytes collected from subordinate follicles (>3 mm) at successive stages after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>IVC*</th>
<th>No Dev. (%)</th>
<th>2-4 Cell (%)</th>
<th>8-16 Cell (%)</th>
<th>Morula (%)</th>
<th>Blast. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>138</td>
<td>47 (34.0)b,c</td>
<td>91 (66.0)a,b</td>
<td>72 (52.2)a</td>
<td>37 (26.8)b</td>
<td>17 (12.3)b</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td>34 (29.3)c</td>
<td>82 (70.7)a</td>
<td>43 (37.0)b</td>
<td>30 (25.8)b</td>
<td>15 (12.9)b</td>
</tr>
<tr>
<td>5</td>
<td>216</td>
<td>94 (43.5)a,b</td>
<td>122 (56.5)b,c</td>
<td>96 (44.4)a,b</td>
<td>83 (38.4)a</td>
<td>49 (22.7)a</td>
</tr>
<tr>
<td>7</td>
<td>163</td>
<td>80 (49.0)a</td>
<td>83 (51.0)c</td>
<td>57 (34.9)b</td>
<td>44 (27.0)b</td>
<td>26 (15.9)b</td>
</tr>
</tbody>
</table>

a,b,c,d Within columns, values with no common superscripts are different (P<0.05).

* All COC, regardless of morphology, were matured, fertilized and cultured. Values at each stage of development are calculated as a percentage of the total number of oocytes undergoing IVC.

28
Table 2.3. In vitro development of oocytes collected from subordinate follicles (≥3 mm) at successive stages after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>IVC</th>
<th>No Dev. (%)</th>
<th>2-4 Cell* (%)</th>
<th>8-16 Cell* (%)</th>
<th>Morula* (%)</th>
<th>Blast.* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>138</td>
<td>47 (34)(^{b,c})</td>
<td>91 (66)(^{a,b})</td>
<td>72 (79)(^{a,b})</td>
<td>37 (51.4)(^{b})</td>
<td>17 (46)(^{a})</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td>34 (29.3)(^{c})</td>
<td>82 (70.7)(^{a})</td>
<td>43 (52.4)(^{b})</td>
<td>30 (69.7)(^{a})</td>
<td>15 (50)(^{a})</td>
</tr>
<tr>
<td>5</td>
<td>216</td>
<td>94 (43.5)(^{a,b})</td>
<td>122 (56.5)(^{b,c})</td>
<td>96 (78.6)(^{a})</td>
<td>83 (86.5)(^{a})</td>
<td>49 (59)(^{a})</td>
</tr>
<tr>
<td>7</td>
<td>163</td>
<td>80 (49)(^{a})</td>
<td>83 (51)(^{c})</td>
<td>57 (68.6)(^{a})</td>
<td>44 (77.2)(^{a})</td>
<td>26 (59)(^{a})</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) Within columns, values with no common superscript are different (P<0.05).

* Percentages for each stage of development are calculated as proportions of the previous stage of development.

Table 2.4. Local effect of the CL on the developmental competence of oocytes (measured as number of blastocysts produced per ovary; mean ± SEM) collected from subordinate follicles on successive days after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>Ipsilateral to CL</th>
<th>Contralateral to CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.5(^{b}) ±0.1</td>
<td>0.4(^{b}) ±0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.3(^{b}) ±0.2</td>
<td>0.8(^{a,b}) ±0.3</td>
</tr>
<tr>
<td>5</td>
<td>1.8(^{a}) ±0.3</td>
<td>1.3(^{a}) ±0.3</td>
</tr>
<tr>
<td>7</td>
<td>0.7(^{b}) ±0.1</td>
<td>0.8(^{a,b}) ±0.1</td>
</tr>
</tbody>
</table>

Day effect, P = 0.0002; local effect of CL, P = 0.9; interaction, P=0.19

\(^{a,b}\) Within columns, values with no common superscript are different (P<0.05).
Table 2.5. Local effect of the dominant follicle on the developmental competence of oocytes (measured as number of blastocysts produced per ovary; mean ± SEM) collected from subordinate follicles on successive days after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>Ipsilateral to dominant follicle</th>
<th>Contralateral to dominant follicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.6b ±0.1</td>
<td>0.3b ±0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.6b ±0.2</td>
<td>0.5a,b ±0.4</td>
</tr>
<tr>
<td>5</td>
<td>1.7a ±0.4</td>
<td>1.5a ±0.2</td>
</tr>
<tr>
<td>7</td>
<td>0.9b ±0.1</td>
<td>0.4a,b ±0.2</td>
</tr>
</tbody>
</table>

Day of collection, P = 0.0003; side of the dominant follicle, P = 0.14; interaction, P=0.83

a,b Within columns, values with no common superscript are different (P<0.05).

The mean number of oocytes that developed to the blastocyst stage per ovary was not affected by the local presence of the CL (Table 2.4) or the dominant follicle (Table 2.5). Consistent with the data in Table 2.1, however, more (P<0.05) blastocysts were produced from oocytes that were collected on Day 5 than from any other Day-group, regardless of the location of the CL or dominant follicle.
Table 2.6. Diameter (mm) of subordinate follicles observed on successive days after follicular wave emergence (Day 0).

<table>
<thead>
<tr>
<th>Day</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.3</td>
<td>4.4</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>5.8</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>5.8</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
<td>4.9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

2.5 Discussion

Developmental competence of oocytes is of paramount importance in determining the success of an in vitro embryo production protocol (Sirard and Blondin, 1996). Increasing evidence suggests that competence is acquired by the oocytes while they are still in the follicle (Blondin et al., 1997). In an attempt to develop criteria that are predictive of oocyte quality (competence), follicle dimension has been studied. Oocytes contained in follicles < 2 mm in diameter had very low developmental ability (Pavlok et al., 1992), while follicles between 3 and 8 mm in diameter contained oocytes with similar developmental ability (Tan and Lu, 1990; Pavlok et al., 1992, Lonergan et al., 1994). These findings suggested that the use of follicle dimension was a good predictor of oocyte competence. However, the range of dimensions of follicles observed in ovaries during the first 8 days of the estrous cycle (Table 2.6) did not change dramatically. If we had considered follicle dimension as the criterion for selecting
oocytes, we would have been unable to distinguish between different functional stages of follicles (Table 2.6). Elucidation of the wave-like pattern of follicle development in cattle (reviewed in Adams, 1999) has indicated that follicles with the same diameter can be in very different physiological stages of development, from growing to static to atretic. Results of this study show that the developmental competence of oocytes is related to the physiologic phase of the follicle from which it was obtained, regardless of its dimensions.

Day 5 after follicular wave emergence may be the most appropriate time for oocyte collection from subordinate follicles for optimal in vitro embryo production in cattle. This time corresponds to the stage of development in which most of the subordinate follicles are in late static or early atretic phases (Adams, 1999). These data confirm those of a previous study (Salamone et al., 1999) in which a higher percentage of expanded and hatched blastocysts were produced from oocytes collected during the static or regressing phases of the follicular wave compared to oocytes collected from follicles in the growing phase. Results of these studies are also consistent with an independent study (Blondin et al., 1997) in which higher percentages of blastocysts were obtained when ovaries were left in saline at 30°C for 4 hours after slaughter, prior to COC collection. It appears that the effects of early follicular atresia are beneficial to the developmental competence of oocytes contained in those follicles, but the reasons for this phenomenon are still speculative.

Maternal mRNA drives early stages of embryonic development until the
embryonic genome takes over (reviewed by Latham, 1999). Perhaps oocytes that are maintained in the follicular environment for a longer period of time accumulate a greater amount of, or more efficient, maternal mRNA into the cytoplasm. When the oocyte is removed from the follicle, it loses the ability to produce RNA in less than 2 hours (Sirard and Coenen, 1994). Insufficient cytoplasmic maturation of the oocyte may lead to an incorrect switch from maternal to embryonic control of development. This is consistent with the observation that the effects of improper maturation are mainly seen later in development rather than during IVM or IVF (Leibfried-Rutledge et al., 1987). Prolonged metabolic coupling between the oocyte and the granulosa cells of the cumulus oophorus may play an important role in this regard. In vivo, gap junctions between the two cellular compartments remain patent until after the preovulatory LH surge (Larsen et al., 1986). However, the process of gap junction break-down, as assessed by Connexin 43 rearrangement in intracellular clusters, began immediately upon removal of the COC from the follicular environment and was complete after 6 hours of IVM (Sutowsky et al, 1993). Perhaps a longer sojourn within the follicle permits greater storage of maternal mRNA and proteins within the oocyte, making the oocyte more capable of developing to the blastocyst stage. Further, a study found that germinal-vesicle breakdown was initiated in subordinate follicles in the late static and early regressing phases, without the surge of preovulatory LH (Salamone et al., 1999).

Asymmetry in follicular dynamics between the left and right ovaries of the same individual has been the subject of many studies, and has been used to elucidate local versus systemic mechanisms that control ovarian function (reviewed in Adams, 1999). Some have reported greater follicular activity in the right ovary in cattle and a higher
incidence of right-side ovulations (ca. 60%; reviewed in Pierson and Ginther, 1987b), whereas others report no such differences (Ginther et al., 1989c; Sirois and Fortune, 1988). Ginther and coworkers (1989c) concluded that the dominant follicle effects subordinate follicle suppression by systemic rather than local channels. Results of the present study are consistent with these findings. A positive intra-ovarian effect of the CL on the development of small antral follicles (3 mm) has been documented in cattle (Pierson and Ginther, 1987). However, no differences in the developmental competence of oocytes collected from follicles in the presence or absence of the CL were found in the present study. These findings have important implications regarding mechanisms controlling ovarian function and the roles of locally produced ovarian peptides. It appears that the 2 ovaries act primarily as a single unit, and follicular counterparts between ovaries influence each other through systemic rather than local routes.

Selection of bovine oocytes for IVM on the basis of visual assessment of morphological features was first examined by Leibfried and First (1979). Since then, many reports have proposed classification schemes based on the compactness and number of layers of cumulus cells surrounding the oocyte (Yang and Lu, 1990; Hazeleger and Stubbings, 1992, Laurincik et al., 1992) and on the appearance of the oocyte itself (Loos et al., 1989; Younis et al., 1989). Oocytes with the highest developmental competence have been reported to have an even, smooth, finely granulated cytoplasm, and are surrounded by a compacted cumulus with > 3 layers of granulosa cells. A positive correlation between COC morphology and blastocyst yield after in vitro culture has been reported (Hazeleger et al., 1995). We expected a greater proportion of oocytes with a compacted cumulus in the phase of the follicular wave that
resulted in the highest blastocyst yield (i.e., Day 5). However, the percentage of compact COC did not change over time during the follicular wave. Conversely, the percentage of denuded oocytes decreased from Day 3 to Days 5 and 7, while the percentage of non compact COC increased from Day 3 to Days 5 and 7. A previous study (Salamone et al., 1999) reported a higher percentage of expanded COC collected from follicles in the regressing phase of the wave. This is in accordance with the results of another report in which oocytes with the highest competence were those surrounded by a less-compact cumulus (Blondin and Sirard, 1995). In the present study, oocytes with expanded cumulus cell layers were assigned to the degenerate group, acknowledging the custom of many in vitro embryo production laboratories of considering only oocytes with compacted cumulus as healthy and viable for IVM. However, the relationship between follicle degeneration, cumulus expansion and oocyte competence shown in this study should serve as a caveat for re-evaluating the current morphologic definition of what is considered a degenerate or healthy oocyte. These results may form a basis for revising the criteria for selecting oocytes for in vitro embryo production in the cow.
2.6 References


3.1 Summary

With the improvement of assisted reproduction techniques, the quality of the female gamete has become paramount for in vitro production procedures. There is a need for an easy to assess, non-invasive, fast and reliable indicator of oocyte competence. The aim of this study was to investigate the relationship between ultrasonographic attributes of a follicle, its stage of development and the competence of the oocyte that it contains. We tested the hypotheses that follicular echotexture characteristics are related to the: 1) phase of development of the follicle, 2) presence of the CL and/or the dominant follicle in the ovary, and 3) developmental competence of cumulus oocyte complexes from the same ovary. Ultrasonographic images of follicles (n = 402) at different stages of development were digitalized and analyzed. Cross-bred beef cows (n = 84), age 4 to 14 years, were given a luteolytic dose of PGF\textsubscript{2α} and 8 Days later
underwent ultrasound-guided ablation of all follicles ≥ 4 mm to induce new follicular wave emergence. Images of dominant follicles and of the 3 largest subordinate follicles were acquired on Days 2, 3, 5 or 7 of the follicular wave (Day 0 = wave emergence), i.e. growing, early static, late static, and regressing phases of subordinate follicle development, respectively. COC from subordinate follicles ≥ 3 mm underwent in vitro maturation, fertilization and culture to the blastocyst stage.

Image analysis revealed differences in echotexture between dominant and subordinate follicles among Days 2 to 7 of the follicular wave. Follicles at Day 7 of the wave displayed consistently lower values (P < 0.05) for image attributes in peripheral antrum, follicular wall and perifollicular stroma than all the other days. All follicles displayed a consistent pattern of variation in echotexture among follicular phases. Data did not support a local effect of the CL or dominant follicle on follicular echotexture.

Categorization of images according to oocyte developmental competence (no embryo development, less than average or more than average) from the same ovary showed that, at the 8 to 16 cell stage, echotexture endpoints of perifollicular stroma were lower in ovaries not producing embryos compared to ovaries producing embryos. Our results showed that changes in follicular image attributes are consistent with changes in follicular status and provide the rationale for further development of image analysis as diagnostic tool in assisted reproduction.
3.2 Introduction

The pattern of periodic emergence of ovarian follicular waves in ruminants is regulated by a series of tightly timed systemic feedback mechanisms between the ovary and the pituitary gland (reviewed in Adams, 1999). The results of extensive ultrasonographic studies have documented that follicular growth during the estrous cycle in the cow is characterized by two or three follicular waves (Pierson and Ginther, 1987, 1988; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a, 1989b, 1989c; Knopf et al., 1989). During each wave, a cohort of follicles begins to grow beyond 4 mm in diameter. From this cohort, one follicle is selected, becomes the dominant follicle and continues to grow while the other follicles in the cohort (subordinates) become committed to regression around Day 5 of the follicular wave and undergo atresia.

Oocyte competence has been defined as the ability of oocytes to develop in vitro to the blastocyst stage (Farin et al., 2001) and many attempts have been made to identify indicators of oocyte competence (Madison et al., 1992; Hazeleger et al., 1994; Gandolfi et al., 1997). Currently, follicle size is the main criterion for selection of oocytes for in vitro embryo production (Lonergan et al., 1994). Oocytes from subordinate follicles are used primarily for in vitro embryo production protocols because they are present in the ovary in significant numbers, display a certain degree of developmental ability and are able to develop into term offspring when transferred into a recipient after in vitro maturation, fertilization and culture. However, follicles of similar diameter may be in very different physiologic phases (i.e., growing, static or regressing) and, therefore, contain oocytes that may vary considerably in maturational state or developmental competence. In this regard, results of recent studies suggest that cumulus oocyte
complexes (COC) collected from subordinate follicles in the late static or early regressing phase (Day 5 of the follicular wave) were more developmentally competent than those of follicles at other phases, irrespective of follicle dimension (Salamone et al., 1999; Vassena et al., submitted).

Ultrasound images are composed of a two-dimensional matrix of picture elements (pixels) that differ in their grey-scale value (Kremkau, 1998; Ginther 1995; Pierson and Adams, 1995). Each pixel, described by one of 256 shades of grey (0 corresponding to black and 255 to white), represents a discrete tissue reflector; i.e., it is the result of an ultrasonic echo produced by an acoustic interface between tissues of differing density (Pierson and Adams, 1995). The ultrasonographic appearance or image pattern of a tissue is referred to as echotexture (Ginther, 1995) and is determined by the histologic structure of the tissue (Ginther 1995; Singh et al., 1997, 1998). Computer algorithms specifically designed for ultrasound image analysis have been developed to overcome the inconsistencies of visual evaluation, and to provide a quantitative approach to grey-scale pixel-value analysis (SYNERGYNE, Version 2.8, WHIRL, Saskatoon, SK, Canada; ©Pierson). These algorithms have been used extensively in studies characterizing the echotexture dynamics of ovarian structures at different phases of the follicular wave (Adams and Pierson, 1995; Pierson and Adams, 1995; Singh et al., 1997, 1998; Tom et al., 1998a, 1998b). Results of these studies have documented that phase-specific changes in dominant and subordinate follicles and the corpus luteum (CL) can be distinguished by computer-assisted examination of ultrasound image attributes. However, no reports were found describing the use of this approach to assess the developmental competence of oocytes contained in the follicles analysed.
This study was designed to test the hypothesis that changes in echotexture of ovarian follicles at different stages of the follicular wave are reflective of the developmental competence of the COC they contain. The specific objectives were to investigate whether echotexture characteristics of the follicles and perifollicular stroma are related to: 1) the phase of development of the follicle, 2) the local presence of the CL and/or the dominant follicle in the ovary (ipsilateral versus contralateral), and 3) the developmental competence of the oocytes collected from the follicles.

3.3 Materials and Methods

3.3.1 Animal grouping and image acquisition

Crossbred beef cows (n = 143), 4 to 14 years of age, were used during July, August and September. A total of 152 cows were kept in 3 outdoor paddocks on a single farm, and maintained on a plane of nutrition (barley and hay) in preparation for slaughter. After initial ultrasound examination of the reproductive system (Aloka SSD-500 with a 5 MHz linear-array transducer; ISM Inc., St. Laurent, PQ, Canada), 9 cows were excluded because of advanced pregnancy. Thirteen of the remaining 143 cows were pregnant ≤ 70 Days and were given prostaglandin to induce abortion (25 mg dinoprost; Lutalyse, Pharmacia & Upjohn Animal Health, Orangeville, ON, Canada). They were included in the last replicate of the study, at least 40 Days after induced abortion. Cows (n = 143) were assigned randomly to 1 of 4 groups to be slaughtered on Day 2 (n=41), 3 (n=42), 5 (n=40) or 7 (n=20; Day 0 = wave emergence). Emergence of
a new follicular wave was synchronized among animals to permit ultrasound image
digitalization and oocyte retrieval on a given day of slaughter. Moreover, the
synchronization scheme allowed for the induction of a follicular wave during a luteal
phase in all animals. Synchronization involved an injection of a luteolytic dose of
PGF2α i.m. (5 ml Lutalyse) followed 8 Days later by transvaginal ultrasound-guided
ablation of all follicles ≥ 4 mm. The emergence of a new follicular wave (Day 0) was
expected to occur one day after follicle ablation (Bergfelt et al, 1994). Cows were
divided into 8 replicates (10 to 20 cows per replicate); each replicate included cows from
at least 2 day-groups. Before slaughter, on days 2, 3, 5 or 7 after wave emergence, the
ovaries were examined ultrasonographically (Aloka SSD-900; ISM Inc.) with a 7.5 MHz
linear-array transducer mounted on an extension for transvaginal use. At any stage of the
follicular wave, the follicle with the largest diameter was considered to be the dominant.
Images of the dominant follicle and the 3 largest subordinate follicles in each ovary were
digitalized directly from the ultrasound machine to a computer using a video capture
board (DTV 2000, Diamond Multimedia Systems Inc., San Jose, Ca, USA) and custom
developed software optimized for ultrasound images under a Linux operating system.
Animals were slaughtered ≤ 2 hours after ultrasound examination and ovaries were
collected (n = 33 cows) within 30 minutes after slaughter to collect COC for in vitro
embryo production.
3.3.2 *In vitro* embryo production

At the abattoir, each ovary was placed in an individual plastic bag filled with phosphate-buffered saline (PBS) + 1% (w/v) antibiotic/antimycotic (10,000 IU penicillin, 10 mg streptomycin and 25 μg amphotericin B/ml; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Each bag was marked with an identification number different from the ear tag number so that the investigator was blinded from Day groupings. The ovaries were maintained at 30°C and transferred to the laboratory within 3 hours of collection. The COC were aspirated into a 15 ml glass vial containing 3 ml of warm collection medium (TCM 199 + 0.75% kanamycin). Aspiration was accomplished with an 18 g 1.5 inch needle and a vacuum pump using a continuous controlled flow-rate of 35 ml/min. The needle was rinsed with warm collection medium before and after aspiration of each ovary. The dominant follicle and, when in doubt, the largest subordinate follicle were aspirated separately from the remaining subordinates. COC from all subordinate follicles ≥3 mm in the same ovary were collected and pooled in the same tube.

After collection, the COC were washed twice in the collection medium and once in the maturation medium. The maturation medium consisted of TCM 199 + 0.1% PVA + 1 μl/ml FSH/LH (Folltropin V, Vetecharm Canada Inc., Belleville, ON, Canada) + 0.75% kanamycin + 10% (v/v) fetal calf serum (FCS, Gibco BRL, Burlington, ON, Canada). COC were matured for 22 hours in a drop of 20 μl of maturation medium in a petri dish previously conditioned under tissue culture oil at 38.5°C, 100% humidity and 5% CO₂.
A few hours before the end of maturation, semen was prepared for IVF. Semen from a Charolais bull known from previous use to be effective for IVF was used in the present study. Two 0.5 ml straws were thawed in a water bath at 37°C for 1 minute and assessed for post-thaw motility. The semen was pooled in a 15 ml polypropylene test tube (VWR Canlab, Mississauga, ON, Canada) previously warmed to 37°C and then dispensed into four 1.7 ml Eppendorf tubes (250 µl of semen each) containing 1 ml of SP-TALP medium. The tubes were placed for 1 hour in the incubator at 39°C to allow selection by swim-up of the sperm. The supernatant (800 µl) containing the motile fraction of sperm was collected from each Eppendorf tube and pooled in a warmed 15 ml Falcon tube. The semen was washed twice by adding 5 ml of FERT-TALP medium followed by centrifugation (500 G for 8 min); the supernatant was discarded and replaced each time. A sample of 5 µl of semen solution was diluted 1:20 with H2O and sperm concentration was estimated using a modified Neubauer chamber (VWR Canlab) under a phase-contrast microscope (Diaphot; Nikon Canada Inc., Missisauga, ON, Canada) at a magnification of 400x. The concentration of spermatozoa in the fertilization solution was adjusted to 3.5 million cells/ml. Heparin was added to the sperm suspension at a final concentration of 10 µg/ml. Fertilization microdroplets of 15 µl were prepared in a petri dish and covered with conditioned tissue culture oil. Matured COC were washed twice in FERT-TALP medium, and then placed in the sperm suspension in an incubator at 38.5°C for 20 hours.

After exposure to the sperm suspension, the presumptive zygotes were washed once in FERT-TALP medium, vortexed for 3 minutes to remove the remaining cumulus cells, and washed again in IVC medium before being transferred to conditioned IVC
microdroplets. The medium used for IVC was CR1 + 10% FCS (Rosenkrantz et al., 1990) under tissue culture oil. The developing zygotes were maintained at 38.5°C, in an atmosphere of 5% CO₂ and 100% humidity, and evaluated for development at the 2- to 4-cell, 8- to 16-cell, morula, and blastocyst stages (i.e., at 36, 84, 120 and 192 hours post-insemination). Regardless of the developmental stage attained, culture was terminated at day 9 (216 hours post-insemination).

3.3.3 Quantitative echotexture analysis of ultrasound images

Analysis of ultrasound images was performed using a series of custom-developed computer algorithms optimized for ultrasonography (SYNERGYNE, Version 2.8 © RA Pierson, University of Saskatchewan, Saskatoon, Canada) on a Sun Sparc Station (Sun Microsystems, Mt View, CA, USA) computer. A total of 746 images from 143 cows were initially digitized. Because of technical problems with ultrasound machine settings and image digitalization, images from 59 cows were not included in the study. Computer-assisted analyses were performed on follicle images (n = 402) from 84 cows; 15 cows on Day 2 (n = 92), 23 cows on Day 3 (n = 95), 27 cows on Day 5 (n = 132), and 19 cows on Day 7 (n = 83). Image analyses were performed by an individual to whom identities of individual images and cows were not disclosed.

3.3.4 Spot-analysis of the follicle antrum
Quantitative echotexture analysis of the follicular antrum was performed by placing a computer-generated circular spot at the centre of the antral region of each follicle. The diameter of the spot was enlarged progressively by increments of 2 pixel units until the follicular wall was encountered, apparent by a sudden increase in mean values at the antrum-follicular wall interface. The spot diameter was then decreased by 2 pixel units and values were recorded. The mean values (average of grey-scale pixel values for all pixels within the measuring spot; black = 0, white = 255), heterogeneity (standard deviation of values of all pixels within the measuring spot), and the diameter of the spot were recorded. Normalization is the process by which 2 or more ultrasonographic images collected in different moments or by different operators are made comparable. The pixel values of different images are adjusted using the grey-scale bar of the ultrasound screen (independent from the machine settings and consistent among ultrasound sessions) as reference. To determine if image data required normalization among days prior to statistical analysis (Singh at al., 1998), normalized and non-normalized data from a subset of 40 images were compared. No differences were detected in mean grey-scale values ($P < 0.05$) or heterogeneity ($P < 0.05$) between normalized and non-normalized data sets; hence, image normalization was not performed in this study. Values for echotexture endpoints (from both spot and line analyses) of the 3 largest subordinate follicles per ovary were averaged to obtain a single value for each endpoint before statistical analysis. This was done to make direct comparisons with oocyte competence data where COC from subordinate follicles were pooled from each ovary into a single group.
Line analysis was used to measure the grey-scale values of pixels along a straight line traversing the area of interest (Pierson and Adams, 1995; Singh et al., 1998). A computer-generated line was traced on each side of the image of the follicular wall, at positions that minimize the confounding effects of enhanced through-transmission and refraction artefacts (Kremkau, 1989; Ginther, 1995) i.e., at approximately 2- and 10-o’clock positions. A two-dimensional graph was generated for each line, corresponding to the grey-scale value of each pixel along the length of the line (i.e., antrum, follicle wall and stroma; Figure 3.1). The linear distance represented by one pixel was calculated by measuring the length of the scale bar on the ultrasound image in number of pixels and estimated to be 0.25 mm. The antrum-wall interface was identified by the first sudden increase in grey-scale value along the computer-generated line (Singh et al., 1998). Based on the results of a morphometric study of bovine follicles (Singh and Adams, 2000), data for only that part of the line extending 2 pixels inward from the antrum-wall interface (peripheral antrum), 2 pixels outward from the interface (follicle wall), and 2 more pixels outward from the interface (perifollicular stroma; Figure 3.1) were used for statistical analysis (Singh et al., 1998). Peak grey-scale values, mean grey-scale values, pixel heterogeneity, and area under the curve were recorded for each 2-pixel segment of the line. The intercept and slope of the best-fit simple linear regression line for each of the three segments (peripheral antrum, follicle wall, perifollicular stroma) were also obtained. The values of the 3 segments for each follicle were obtained by taking the average of 2 values (one line per side, two sides per follicle).
3.3.6 Statistical analysis

Echotexture characteristics of the follicles were compared among the 4 follicular phases (i.e., Days 2, 3, 5, or 7) and between the 2 follicular categories (i.e., dominant or subordinate) by 2-way analysis of variance. For analyses involving the local effects of the CL and dominant follicle, the ovary was considered the experimental unit (n). To determine the local effects of ovarian structures (CL or dominant follicle) on follicular echotexture, and its interaction with follicular stage (Days 2, 3, 5 or 7), 3-way analyses of variance were performed (i.e., ipsilateral or contralateral to the CL, ipsilateral or contralateral to the dominant follicle, and Day). Image data for dominant follicles were not included in the latter analyses. To study the relationship between the developmental competence of oocytes and the image characteristics of the subordinate follicles, a smaller database was prepared, in which only the animals that were used for the *in vitro* embryo production protocol were considered. A value of the developmental ability of oocytes from these animals on a per ovary basis was calculated as average of the proportion of oocytes from subordinate follicles that reached the cleavage stage (0.52), the 8 to 16 cell stage (0.4) and the morula stage (0.33). In this study, these values were used to determine whether oocytes from an ovary had average, less than average or more than average developmental competence. The influence of developmental ability of the oocytes up to cleavage, 8 to 16 cell, or morula stage on follicular echotexture was assessed by 2-way analyses of variance. For all analyses, a probability value of less than 0.05 was considered significant (i.e., less than a 5% probability that the difference detected was due to chance alone).
Figure 3.1. Graphic representation of grey-scale values of each pixel along a computer-generated line traced over an ultrasound image of the ovarian follicle wall. Dotted vertical lines represent the borders of the areas of interest (i.e., peripheral antrum, follicle wall, perifollicular stroma). The antrum-wall interface is identified as a point along the line where grey-scale values suddenly increase. This interface was used as a point of reference (pixel number = 0) for defining the areas of interest (i.e., -2 to 0 pixels = peripheral antrum; 0 to +2 pixels = follicle wall; +2 to +4 pixels = perifollicular stroma). Values (peak and mean grey-scale value, heterogeneity, and area under the curve) of the segments were recorded separately.
3.4 Results

3.4.1 Echotexture analysis and follicular antrum (Spot analysis)

The effect of day of the follicular wave on the mean grey-scale values of the antrum of dominant and subordinate follicles is presented in Table 3.1. The overall mean grey-scale values of the antrum of dominant follicles was lower than in subordinate follicles ($P = 0.02$). Regardless of the type of follicle (dominant or subordinate), Day 7 follicles had a lower mean grey-scale value than those on Days 2, 3 or 5 ($P = 0.001$). The interaction between day of the wave and follicle type was not significant ($P = 0.74$). Heterogeneity of the spot did not reveal any influence of the day of the follicular wave ($P = 0.54$) or of the type of follicle ($P = 0.14$). The size of the measuring spot, which is related to the size of the follicle, was different between follicle types ($P < 0.0001$), Days ($P = 0.02$), and there was a significant follicle-by-day interaction ($P < 0.004$).
Table 3.1 Grey-scale values (mean ± SEM) of the antrum of ovarian follicles on different days of the follicular wave (Day 0 = wave emergence). Values in parentheses indicate the number of observations.

<table>
<thead>
<tr>
<th>Follicle</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant</td>
<td>3.9 ± 0.6</td>
<td>5.0 ± 0.9</td>
<td>5.1 ± 1.0</td>
<td>2.1 ± 0.2</td>
<td>4.2 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(16)</td>
<td>(25)</td>
<td>(15)</td>
<td>(65)</td>
</tr>
<tr>
<td>Subordinate</td>
<td>11.3 ± 0.9</td>
<td>10.0 ± 0.9</td>
<td>10.1 ± 0.7</td>
<td>8.1 ± 0.8</td>
<td>9.8 ± 0.4b</td>
</tr>
<tr>
<td></td>
<td>(25)</td>
<td>(37)</td>
<td>(44)</td>
<td>(30)</td>
<td>(136)</td>
</tr>
<tr>
<td>Overall</td>
<td>9.3 ± 0.9y</td>
<td>8.5 ± 0.8y</td>
<td>8.2 ± 0.6y</td>
<td>6.1 ± 0.7z</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(53)</td>
<td>(69)</td>
<td>(45)</td>
<td></td>
</tr>
</tbody>
</table>

Day (P = 0.019), Follicle (P < 0.0001), Interaction (P = 0.74).

a, b Within columns, values with no common superscripts are different (P < 0.05).
y, z Within rows, values with no common superscripts are different (P < 0.05).

3.4.2 Echotexture analysis of peripheral antrum, follicle wall and perifollicular stroma (Line analysis)

The effect of the phase of the follicular wave on echotexture characteristics of the peripheral antrum, follicular wall and perifollicular stroma of dominant and subordinate follicles is presented in Figures 3.2, 3.3 and 3.4. The pattern of change in grey-scale values (peak grey-scale values, Figure 3.2; area under the curve, Figure 3.3; mean grey-scale values, Figure 3.4) between dominant and subordinate follicles over days was similar among the 3 parts of the follicle. The values for the dominant follicles tended to follow a pattern over days, consistently decreasing from Day 5 to Day 7 (P < 
This pattern was similar in all follicular segments and for all the echotexture endpoints analysed (peak grey-scale values, mean grey-scale values and area under the curve). Similarly, a very consistent pattern was detected among subordinate follicles over days. In the peripheral antrum and in the perifollicular stroma the decreasing trend started at Day 3 of the wave, but it was not significant at this time. There was a significant decrease of echotexture between Days 5 and 7. Mean grey-scale value and area under the curve of the follicular wall showed the same decrease as the other portion of the follicle analysed, but the decrease between Days 3 and 5 (subordinate follicles) was statistically significant ($P < 0.05$). The values increased from Day 2 to Day 3 and decreased from Day 3 to 5 to 7, where it reached the lowest value.
Figure 3.2 Peak grey-scale value (mean ± SEM) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white) and subordinate (black) follicles in cattle (n = 84 cows) on different days of the follicular wave (Day 0 = wave emergence). A,B Values (dominant and subordinate combined) with no common letters are different (P < 0.05). Numbers at the bottom of each bar of the lower chart indicate observations for each follicle type.
Figure 3.3 Area under the curve (pixel²; mean ± SEM) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white) and subordinate (black) follicles in cattle (n = 84 cows) on different days of the follicular wave (Day 0 = wave emergence).

Values (dominant and subordinate combined) with no common letters are different (P < 0.05).

Bars with no common letters are different (P < 0.05).

Numbers at the bottom of each bar of the lower chart indicate observations for each follicle type.
Figure 3.4 Mean grey-scale value (mean ± SEM) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white) and subordinate (black) follicles in cattle (n = 84 cows) on different days of the follicular wave (Day 0 = wave emergence).

A,B Values (dominant and subordinate combined) with no common letters are different (P < 0.05).

a,b,c Bars with no common letters are different (P < 0.05).

Numbers at the bottom of each bar of the lower chart indicate observations for each follicle type.
Figure 3.5 Peak pixel value, mean pixel value, heterogeneity and area under the curve (mean ± SEM) of perifollicular stroma in ovaries that produced no embryos developing to the 8 to 16 cell stage (white bars), less than average development (black bars) or more than average development (grey bars).

^a^ Within parameters, values with no common letters are different (P < 0.05).
Table 3.2 The difference between mean echotexture values of dominant and subordinate follicles at different days of the follicular wave (Day 0 = wave emergence). Negative values indicate that the value was higher for the subordinate follicle than for the dominant.

<table>
<thead>
<tr>
<th>Peak grey-scale</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antrum</td>
<td>+0.34</td>
<td>-7.17</td>
<td>-0.94</td>
<td>-5.91</td>
</tr>
<tr>
<td>Wall</td>
<td>+5.94</td>
<td>-17.53</td>
<td>-3.07</td>
<td>-8.37</td>
</tr>
<tr>
<td>Stroma</td>
<td>+11.67</td>
<td>-11.11</td>
<td>-3.20</td>
<td>-6.77</td>
</tr>
</tbody>
</table>

Mean grey-scale
- Antrum
  -0.46
- Wall
  +3.00
- Stroma
  +9.1

Area under the curve (pixel^2)
- Antrum
  +13.98
- Wall
  +11.70
- Stroma
  +25.24

Antrum = peripheral antrum; Wall = follicle wall; Stroma = perifollicular stroma.

3.4.3 Echotexture analysis and local effect of ovarian structures

There was no local effect of the dominant follicle or of the CL on any of the subordinate follicle echotexture endpoints studied. Consistent with the previous line-analysis, the day effect was significant for peak grey-scale values, heterogeneity and slope of the regression line for the peripheral antrum ($P = 0.001$, $P < 0.0001$ and $P = 0.0001$, respectively), and for peak and mean grey-scale values, and area under the curve for the follicle wall ($P = 0.0001$). There was no local effect of the CL on dominant follicle echotexture endpoints except those involving the peripheral antrum (Table 3.3).
Table 3.3 Local effect of the CL on echotexture of ultrasound images of the peripheral antrum of dominant follicles in cattle on different days of the follicular wave (Day 0 = wave emergence; Ipsilateral = dominant follicle in the ovary ipsilateral to the CL; Contralateral = dominant follicle in the ovary contralateral to the CL).

<table>
<thead>
<tr>
<th>Peak grey-scale</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsilateral</td>
<td>17.4 ± 4.1</td>
<td>19.4 ± 4.0</td>
<td>23.2 ± 3.3</td>
<td>8.9 ± 2.4</td>
<td>18.4 ± 2.0</td>
</tr>
<tr>
<td>Contralateral</td>
<td>*</td>
<td>18.5 ± 2.3</td>
<td>19.3 ± 4.6</td>
<td>15.9 ± 2.5</td>
<td>19.0 ± 2.1</td>
</tr>
<tr>
<td>Overall</td>
<td>20.6 ± 4.9</td>
<td>18.9 ± 2.0</td>
<td>21.5 ± 2.7</td>
<td>12.9 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

Mean grey-scale

| Ipsilateral     | 13.3 ± 4.0  | 14.6 ± 4.3  | 16.3 ± 2.7  | 5.6 ± 1.7   | 13.2 ± 1.7d |
| Contralateral   | *           | 13.7 ± 1.8  | 14.2 ± 4.0  | 10.0 ± 1.7  | 13.6 ± 1.7d |
| Overall         | 15.8 ± 4.3  | 14.0 ± 1.9   | 15.4 ± 2.2  | 8.1 ± 1.3   |             |

Area under the curve (pixel²)

| Ipsilateral     | 55.8 ± 19.1 | 43.8 ± 13.0 | 49.0 ± 8.1  | 16.8 ± 5.1  | 43.7 ± 6.4d |
| Contralateral   | *           | 41.2 ± 5.5  | 42.7 ± 11.8 | 30.0 ± 5.2  | 41.3 ± 5.4d |
| Overall         | 63.1 ± 18.4 | 42.2 ± 5.7   | 46.3 ± 6.7  | 24.4 ± 4.0  |             |

* Only 1 observation available
2-way analyses of variance: Day of the follicular wave (Days 2, 3, 5 or 7); CL ipsilateral or contralateral to the dominant follicle
Peak grey-scale: Day (P = 0.01), CL (P = 0.03), interaction (P = 0.04)
Mean grey-scale: Day (P = 0.01), CL (P = 0.06), interaction (P = 0.1)
Area under the curve: Day (P = 0.007), CL (P = 0.1), interaction (P = 0.21)
a,b Among cells, values with no common superscripts are different (P < 0.05)
d,e Within columns, values with no common superscripts are different (P < 0.05)
y,z Within rows, values with no common superscripts are different (P < 0.05)

3.4.4 Echotexture analysis and oocyte competence

The echotexture data from 33 animals (Day 2, n = 3; Day 3, n = 4; Day 5, n = 11; Day 7, n = 15) were compared with the developmental competence of their oocytes. Results are presented in Figure 3.5. For this analysis, ovaries were categorized into 3
groups irrespective of the day of the follicular wave on which they were collected. The groups were: ovaries whose oocytes did not develop in vitro, ovaries whose oocytes displayed less than average embryonic development in vitro and ovaries whose oocytes displayed more than average embryonic development in vitro. No differences were found among the three groups for the cleavage and the morula stage embryos. For the 8 to 16 cell stage, however, peak grey-scale values, area under the curve, mean grey-scale values and pixel heterogeneity of the perifollicular stroma were different among different categories of the ovaries. Pixel heterogeneity of the perifollicular stroma of the subordinate follicles was lower for the ovaries whose oocytes did not produce embryo development compared to those whose oocytes produced more than average development (P < 0.05). The Subordinate follicles whose COC produced no embryo development had lower peak grey-scale values, area under the curve and mean grey-scale values (P < 0.05) that those that produced 8 to 16 cell stage embryos, either below or above average.

To study the relationship of oocyte competence and day of the follicular wave on echotexture endpoints, data from Day 2 and Day 3 groups were combined (n = 7) in a single group due to the low number of animals (n= 3 for Day 2, n= 4 for Day 3 group). Thus, in this analysis, we distinguished oocytes from follicles in the growing phase (Day 2 to 3), static phase (Day 5), and regressing phase (Day 7) of the follicular wave. For each of the embryonic stages (cleavage, 8 to 16 cell and morula), ovaries within each follicular phase were categorized into the same 3 groups described previously (no development, less than average and more than average development). No effect of the stage of embryonic development was detected, that is, the echotexture endpoints of the subordinate follicles from ovaries whose COC produced embryos or did not produce
embryos, were not different when examined on specific days of the follicular wave. However, the Day effect was statistically significant for the perifollicular stroma at all stages of development (Table 3.4).

**Table 3.4** Image analysis characteristics of perifollicular stroma of subordinate follicles from ovaries that underwent *in vitro* embryo production, in relation to day of the follicular wave, irrespective of embryonic developmental stage.

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Days 2 to 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak grey-scale</td>
<td>80.9±5.3</td>
<td>95.4±4</td>
<td>75.8±3.3</td>
</tr>
<tr>
<td>Mean grey-scale</td>
<td>70.5±4.9</td>
<td>79.9±3.4</td>
<td>64.5±2.8</td>
</tr>
<tr>
<td>Heterogeneity</td>
<td>11.3±1.1</td>
<td>15.8±1</td>
<td>11.4±0.8</td>
</tr>
<tr>
<td>Area Under Curve (pixel²)</td>
<td>215.7±16.1</td>
<td>238.8±10</td>
<td>193.6±8.5</td>
</tr>
</tbody>
</table>

*a,b,c* Within rows, values with no common superscripts are different (*P* < 0.05).

### 3.5 Discussion

The introduction of ultrasonographic imaging to the field of reproduction has led to real time assessment of ovarian activity, and elucidation of the pattern of follicular growth and development (reviewed by Adams, 1999). In addition, quantitative evaluation of changes in the ultrasonographic appearance (echotexture) of the uterus and ovaries has documented the utility of this technique for detecting important physiologic information. Earlier studies used visual examination to score echotexture changes (Pierson and Ginther, 1985; Townson and Ginther, 1989). However, the number of
Shades the human eye is able to distinguish is limited (i.e., approximately 16 to 20 shades of grey; Ginther, 1995) and subjective variation in individual perception of grey-scale data lead to different interpretations of the image among operators. Furthermore, visual examination depends heavily on operator consistency and expertise. Computer-assisted image analysis obviates these limitations. Specific algorithms may be used quantitatively assess ultrasound images and minimize the operator's influence and subjective judgment of the image displayed (Pierson and Adams, 1995). In the present study, we used quantitative computer-assisted echotexture analysis to study the relationship between ultrasound image characteristics, the functional status of a follicle, the developmental competence of the contained oocyte, and the local influence of the CL and dominant follicle.

The results of the spot and line-analyses supported the hypothesis that echotexture characteristics of portions of the follicular wall are reflective of the phase of development of the follicle. We concluded that there is a difference among follicles at different stages of the wave; changes were evident on Day 7 of the follicular wave when the walls of both dominant and subordinate follicles become hypoechogenic.

Changes in the mean grey-scale values of the follicular antrum, studied with the spot analysis technique, were influenced by the day of the follicular wave and follicle type (dominant or subordinate). Both dominant and subordinate follicles collected on Day 7 of the wave had darker antra than on all the other days examined. In an earlier study (Tom et al., 1998), no changes were detected in the mean grey-scale values of the antrum in dominant follicles during the follicular wave; however, present findings are supported by another study from our laboratory (Singh et al., 1998), where both the phase of the follicular wave and the type of follicle (dominant or subordinate) influenced
the mean grey-scale values of the follicular antrum. In the latter and present studies, subordinate follicles had brighter antra than dominant follicles. It is interesting to note that line analysis of the peripheral antrum also indicated significantly lower values on Day 7 than on any other day for mean grey-scale value, peak grey-scale value and area under the curve in both dominant and subordinate follicles. The decrease in these values started at Day 3 of the wave for subordinate follicles and continued to Day 7, while it was delayed by 2 days for the dominant follicles, starting on Day 5 and continuing through Day 7 (Figure 3.2, 3.3 and 3.4). Dominant follicles are in growing phase at Day 3 of the follicular wave, and begin the static phase around Day 5 (Adams, 1999). However, subordinate follicles enter the static phase between Days 3 and 5 (Adams, 1999). Hence, the differences between dominant and subordinate follicles in the timing of changes of echotexture characteristics is temporally related to the functional stage of the follicles at the time of image digitalization.

Line analysis of the follicular wall showed lower peak grey-scale values on Days 2 and 7 than on Days 3 and 5 for both subordinate and dominant follicles. Values of mean grey-scale and the area under the curve decreased significantly between Days 3 and 5 for the subordinate follicles, and between Days 5 and 7 for the dominant follicles. The thickness of the granulosa layer in subordinate follicles decreased between the late-growing or early-static phase (Day 3) to the regressing phase (Day 6; Singh and Adams, 2000). By experimental design, we utilized the same thickness as the region of analysis for the follicular wall among days. It is reasonable to assume that as the follicular wall became thinner, more of the perifollicular stroma was included in the region of analysis of the follicular wall. In a previous study (Singh et al., 1998), the mean grey-scale values of the follicular wall in dominant follicles did not change from Day 3 to Day 6 of
the follicular wave, while the subordinate follicles displayed an increase of mean grey-scale value from Day 3 to Day 6. Our results of the line analysis show that mean grey-scale value, peak grey-scale value and area under the curve of the perifollicular stroma decreased with the onset of the static phase for dominant follicles and regressing phase for subordinate follicles. The echotexture values were lower on Day 7 than on Days 2, 3 or 5. Contrarily, in an earlier study (Singh et al., 1998), mean grey-scale values of perifollicular stroma during the late static and regressing phases of both dominant and subordinate follicles were higher than during earlier phases. Although not significant until Day 7 of the wave, the decreasing trend started at Day 3 for the subordinate follicle stroma, while it was delayed until Day 5 for the perifollicular stroma of dominant follicles.

A reason for the apparent differences in results around Day 7 of the follicular wave between the two studies (present study and Singh et al., 1998) might be due to the difference in analysis technique and/or the quality of images due to difference in the ultrasound equipment used. We used an Aloka 900 machine with 7.5 linear array transducer, while Singh and coworkers (1998) used an ATL Ultramark 9 HDI machine with a broad-band 5-9 MHz convex-array transducer. Another reason for the differences in the results might be that, while in the present study images were captured from ovaries in vivo, Singh and coworkers (1998) used ovaries excised and paced in a water-bath.

Echotexture endpoints of dominant versus subordinate follicles changed between Days 2 and 3 (Table 3.2). This is temporally associated with the time of selection of the dominant follicle (Adams, 1999). We postulate that these echotexture changes occur after the emergence of the follicular wave because, at the beginning of the wave, the
follicle destined to become dominant is not likely to be different from those that will develop into subordinate follicles. If the dominant follicle of a wave is removed (Ko et al., 1991; Adams et al., 1992; Bergfelt et al., 1994) within the first 5 days (Adams et al., 1993) of the follicular wave, the largest subordinate follicle takes over and becomes dominant. Conversely, if the dominant follicle is left in place, the largest subordinate behaves like the rest of the pool of subordinates and regresses.

The local effect of ovarian structures on ovarian function has been investigated previously (Pierson and Ginther, 1987; Ginther et al., 1989c; Sirois and Fortune, 1988). These studies involved monitoring follicle development or ovulation in relation to the relative position of the CL or dominant follicle. In the present study, image data were collected during periods that encompassed dramatic changes in output of luteal and follicular products. Hence, we had the opportunity to investigate the local effect of the CL and the dominant follicle on the echotexture characteristics. Results indicated that the presence of the CL and/or the dominant follicle in the ovary did not influence the echotexture characteristics of the subordinate follicles in the same ovary. This result was supported by other findings obtained in our laboratory (Vassena et al., submitted) and elsewhere (Ginther et al., 1989c; Sirois and Fortune, 1988), in which ovarian structures did not appear to exert a local effect in the ovary.

Oocyte quality is of paramount importance in assisted reproduction techniques, and finding an echotexture parameter that allows us to distinguish between competent and incompetent oocytes could provide an important and non-invasive tool for researchers and clinicians. Results of a previous study showed that oocytes collected from follicles on Day 5 of the follicular wave were more competent than oocytes collected on Days 2, 3 or 7 of the wave (Vassena et al., submitted). Interestingly, the
difference between the values for the dominant and the subordinate follicles for all the
segments of the follicle examined (peripheral antrum, follicle wall and perifollicular
stroma) were lowest at Day 5 (Table 2.2). This observation may provide an important
link between oocyte competence and echotexture characteristics of the follicle.

Oocytes from subordinate follicles collected on Day 7 displayed lower
developmental competence in vitro than those collected on Day 5 (Vassena et al.,
submitted). In addition, both line and spot analyses revealed lower values for
subordinate follicles on Day 7 than on any other Day. Thus, it may become possible to
use image analysis to monitor the development of follicles in during ovarian
superstimulation, or to assess the optimal time for oocyte collection, to minimize the
effect of a pre-mature or post-mature follicular environment.

On a subset of the ovaries, we tested the ability of oocytes from subordinate
follicles to become fertilized (cleavage stage), undergo the first 4 mitotic divisions (use
the maternal products accumulated in their cytoplasm before major genomic activation),
and develop to the morula stage (to test the oocyte ability to switch from maternal to
embryonic control of genomic activity and to successfully develop past the 8 to 16 cell
developmental block; Latham, 1999). Echotexture parameters used in the present study
were not associated with the ability to cleave or the proportion of embryos that
developed into morulae. However, the perifollicular stroma of follicles that produced
oocytes that development to the 8 to 16 cell stage was brighter than the stroma of
follicles that produced oocytes that did not develop. This trend was maintained for all
echotexture endpoints concerning the stroma. Day 7 subordinate follicles (atretic) were
darker, and ovaries producing poor quality oocytes had darker stroma. These
observations suggest that incompetent oocytes tend to be produced by ovaries with a
high percentage of follicles in a state of atresia too advanced to allow for embryonic
development.

In summary, our results support the hypothesis that perifollicular stroma
echotexture is related to the developmental competence of the oocytes contained in the
follicle. Results supported the hypothesis that the effect of the dominant follicle and the
CL are mediated via a systemic route; no local effects were detected. We found a close
relationship between in vivo follicle developmental stage and image analysis. Together
with the observation that oocyte competence is associated with follicular status, this
provides an important rationale for the use of ultrasound image analysis for detecting
competent oocytes. The level of sensitivity of this technique is not yet sufficient for use
in a diagnostic setting; however, the identification of statistically significant endpoints
will be the basis for further improvement of ultrasound image analysis, with the aim of
providing reproductive scientists with a non-invasive, safe and immediate diagnostic
tool.
3.6 References


Chapter 4

GENERAL DISCUSSION

Understanding the complex mechanisms behind diverse reproductive functions and their pathological disruptions allows scientists and clinicians in veterinary and human medicine to perform diagnostic and therapeutic procedures in a faster, safer and more convenient way. The more our knowledge of processes like oocyte maturation, gamete fertilization and early embryonic development increases, the more the importance of the female gamete quality becomes apparent.

The overall objective of this thesis was to study the developmental competence of the oocyte contained in bovine ovarian follicles. More specifically, the aim of this research was to find an indicator of oocyte health/competence with non-invasive methods that would allow us to detect the best oocytes to be used in assisted reproduction. In this regard, ultrasound image analysis was a natural choice, as real-time B mode ultrasonography is safe, fast, non-invasive and relatively affordable as a diagnostic tool. Moreover, the transmission of ultrasonographic images to distant places for specialized analysis and evaluation has previously been accomplished (Pierson and Adams, 1999) and is expected to become available to researchers and clinicians in the near future.
We attempted to find a relationship between oocyte competence, stage of the follicular development from which they were collected and ultrasonographic attributes. To achieve this goal, we proposed to identify the most developmentally competent oocytes in a follicular wave, regardless of other parameters usually considered for oocyte collection, such as follicle diameter. Our assumption was that the degree of competence of an oocyte in a follicle is not, within a certain range (subordinate follicles >2 mm diameter), related to follicle dimension, but rather to follicle developmental stage at the time of collection. The results of this part of the study are presented in Chapter 2. Next, we used ultrasound image analysis in an attempt to link the characteristics of oocytes to the echotexture attributes of the follicles from which they were collected. The results of this part of the study are presented in Chapter 3.

Developmental competence of oocytes is of paramount importance in determining the success of an assisted reproduction protocol involving embryo production in any species. It is defined as the ability of an oocyte to develop in vitro into a blastocyst stage embryo. Increasing evidence suggests that competence is acquired by the oocytes while still in the follicle (Blondin et al., 1997). From the experiments described in Chapter 2, we concluded that oocytes collected on Day 5 of the follicular wave have higher developmental potential in vitro than oocytes collected on Days 2, 3 and 7 of the follicular wave. These data confirm those of a previous study (Salamone et al., 1999) in which a higher percentage of expanded and hatched blastocysts were produced from oocytes collected during the static or regressing phases of the follicular wave, compared to oocytes collected from follicles in the growing phase.
Of the three steps involved in an *in vitro* embryo production protocol, the maturation process is the one that has the most dramatic impact on the outcome of later developmental phases (Leibfried-Rutledge et al., 1987; Blondin et al., 1997). Regardless of the *in vitro* culture system and media employed, oocytes matured *in vivo* have consistently yielded approximately twice as many blastocysts as their counterparts matured *in vitro* (Brackett and Zuelke, 1993; Leibfried-Rutledge et al., 1987). While in the follicle, oocytes accumulate maternal mRNA, transcription factors and other proteins that will be used later in development. Maternal factors drive the early stages of embryonic development and allow for the activation of the embryonic genome (at the 8 to 16 cells stage in cattle; Latham, 1999, Hyttel et al., 2001). However, when the oocyte is removed from the follicle, it loses the ability to produce/store RNA in less than 2 hours (Sirard and Coenen, 1994). Insufficient cytoplasmic maturation of the oocyte may lead to an incorrect switch from maternal to embryonic control of development. This is consistent with the observation that the effects of improper maturation become evident later in development rather than during IVM or IVF (Leibfried-Rutledge et al., 1987). Prolonged metabolic coupling between the oocyte and the granulosa cells of the cumulus oophorus may play an important role in this regard. Gap junctions between the two cellular compartments remain patent until after the preovulatory LH surge *in vivo* (Larsen et al., 1987). However, the process of gap junction breakdown, as assessed by Connexin 43 rearrangement in intracellular clusters, begins immediately upon removal of the COC from the follicular environment and is completed after 6 hours of IVM (Sutowsky et al., 1993). One study found that leaving oocytes in the ovary at 30° C for 4 hours before aspiration and *in vitro* maturation yielded a higher blastocyst rate than the
control group (Blondin et al., 1997). Furthermore, there are indications that oocytes which have a compact cumulus, but with cells in outer layers that are starting to expand, have high developmental competence (Hazeleger et al., 1994). Completely degenerate COC from subordinate follicles in vivo have a very expanded cumulus. It appears that early COC degeneration is beneficial to the oocyte. On Day 5 of the follicular wave in cattle, most of the subordinate follicles in the ovary are in the late static-early regressing stage, and around this time they become committed to regression by atresia (Adams, 1999). Day 5 oocytes may have high developmental competence because they benefit from a longer sojourn in the follicle, during which they accumulate a greater amount of, or more efficient, maternal products in the cytoplasm. When collected, they leave the follicular environment before follicular degeneration becomes too advanced and detrimental to their survival (Day 7). The results of ultrasound image analysis on subordinate follicles (Chapter 3) indicated that, on Day 7 of the wave, follicular echotexture attributes were significantly lower than any other day analyzed (P < 0.05). The decreasing trend of echotexture values, however, started when the follicles were in the static phase of development, i.e., Day 5 of the follicular wave for dominant follicles and Day 3 for subordinate follicles. Subordinate follicles at Day 7 are undergoing atresia, the follicular wall decreases in thickness and the proportion of degenerated cells increases (Singh and Adams, 2000). Image acquisition in this study may have captured this aspect of follicular behavior.

When the dominant follicle of an anovulatory follicular wave is pharmacologically forced to ovulate between Days 5 and 8 (i.e., in its static-early regressing phase), the oocyte contained in the follicle is developmentally competent. Interestingly, the difference between the echotexture values for the dominant and
subordinate follicles for all the segments examined (peripheral antrum, follicle wall and perifollicular stroma; Table 3.2) were minimal on Day 5 for mean grey-scale values, peak grey-scale values and area under the curve. On Day 5 of the wave, the subordinate follicles displayed characteristics similar to those of the dominant follicle.

In this study, the local effect of ovarian structures (CL and dominant follicle) on oocyte competence and on follicle echotexture characteristics was also investigated. Asymmetry in follicular dynamics between the left and right ovaries of the same individual has been the subject of many studies, and has been used to elucidate local versus systemic mechanisms that control ovarian function (reviewed in Adams, 1999). Some have reported greater follicular activity in the right ovary in cattle and a higher incidence of right-side ovulations (ca. 60%; reviewed in Pierson and Ginther, 1987b), whereas others report no such differences (Ginther et al., 1989c; Sirois and Fortune, 1988). Ginther and coworkers (1989c) concluded that the dominant follicle effects subordinate follicle suppression by systemic rather than local channels. Results of the present study are consistent with these findings; no differences in the developmental competence of oocytes collected from follicles in the presence or absence of the CL were found (Chapter 2). These findings have been confirmed by echotexture analysis of the follicles. Our data also did not support the hypothesis that image echotexture of either dominant or subordinate follicles is locally influenced by the presence of ovarian structures. Not only is there a variation in echotexture when there is a functional change in the follicle (follicular stage), but there is no variation in echotexture when there is no functional change (local CL/dominant follicle).

In summary, image analysis proved to be an effective technique in differentiating ovarian follicular stages. Furthermore, we demonstrated that oocyte competence is
related to stage of follicular development. Taken together, the results of the present study offer the rationale for an attempt to use image analysis for predicting oocyte competence. More studies are needed to refine this technique as a diagnostic tool. For example, it is important to identify which echotexture attributes are most highly correlated with oocyte competence and to define the level of sensitivity that is adequate for use in a diagnostic setting. The present study helps achieve this goal and forms the basis for further development of this technique.
Chapter 5

GENERAL REFERENCES


Savio JD, Boland MP, Heynes N, Mattiacci MR and Roche JF (1990). Will the first dominant follicle of the estrous cycle of heifers ovulate following luteolysis on Day 7? *Theriogenology*. 33; 677-687


