REGULATION OF FOLLICULAR WAVE PATTERN IN CATTLE

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ABSTRACT

The wave-like developmental pattern of follicles ≥1 mm in temporal relationship with follicle stimulating hormone (FSH) and the existence of 2- and 3-waves of follicular development during an interovulatory interval (IOI) have been clearly defined in cattle. However, information about the developmental pattern of antral follicles <1 mm and the repeatability of the wave pattern (2- or 3-wave IOI) is lacking. Using approaches such as immunization against GnRH (to suppress circulating concentrations of FSH) and histomorphometric study of ovarian tissues collected from cyclic heifers on different days after ovulation, the developmental pattern of antral follicles <1 mm and the role of FSH in their development were studied in heifers. Ultrasonographically acquired follicular data were used to determine the repeatability of 2- and 3-wave patterns and the effect of season on the wave patterns. The ovulatory follicle in 3-wave IOI is exposed to a shorter term high-progesterone environment than that of 2-wave IOI, and it has been argued that the less-aged ovulatory follicle of 3-wave IOI yields a more fertile oocyte than the 2-wave IOI. The developmental competence of oocytes in preovulatory follicles of 2- versus 3-wave IOI was compared using *in vivo* environments created to mimic short-term low- and high-progesterone environments similar to 2- and 3-wave IOI, respectively. The developmental competence of oocytes in persistent dominant-type follicles was also determined.

The vaccination against GnRH attenuated FSH surges but did not suppress the basal circulating concentrations of FSH. The attenuation of FSH surges suppressed the wave-like emergence of follicles ≥4 mm but not of the antral follicles <4 mm. The study revealed an inverse relationship between the mean and peak circulating concentrations of FSH and the number of follicles recruited into ≥1 mm size category. Histomorphometric study revealed that antral follicles <1 mm developed in a wave-like fashion in response to a rise in the circulating concentrations of FSH. After treatment with exogenous FSH, the growth rate of follicles in GnRH-immunized heifers was similar to controls.

The duration of IOI was predictive of the wave pattern (i.e., 2- or 3-wave IOI), and the pattern was repeatable within individuals throughout the year. The dominant follicle of Wave 1 in 2-wave IOI had a longer duration of dominance than in 3-wave IOI. Hence, the dominant follicle of Wave 1 may have a primary role in the regulation of 2- and 3-wave patterns. Greater

attrition of follicles in 3-wave IOI, due to the emergence of an extra wave compared to 2-wave IOI, may contribute to earlier follicular depletion and onset of reproductive senescence in heifers with primarily a 3-wave pattern. The fertilization capacity of oocytes that were exposed to the short-term low-progesterone environment (i.e., similar to the early growing phase of the ovulatory follicle of 3-wave IOI) was increased, but the developmental competence post-fertilization was not different from oocytes that were exposed to a short-term high-progesterone environment (i.e., similar to the early growing phase of preovulatory follicle of 2-wave IOI). Multiple follicles developed under the prolonged-low progesterone environment, but failed to ovulate.

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This was my father's dream

Dedicated to my

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LIST OF ABBREVIATIONS

CL	Corpus luteum
FSH	Follicle stimulating hormone
FSHr	Follicle stimulating hormone receptor
h	Hour
Kg	Kilogram
IOI	Interovulatory interval
LH	Luteinizing hormone
MHz	Mega Hertz
μg	Microgram
mm	Millimeter
mRNA	messenger Ribonucleic acid
PGC	Primordial germ cells
RIA	Radioimmunoassay

Chapter 1

GENERAL INTRODUCTION

Folliculogenesis is the developmental process in which an activated primordial follicle develops to a preovulatory size following the growth and differentiation of the oocyte and its surrounding granulosa cells (Gougeon, 1996; Senger, 1997; Knight and Glister, 2001). During folliculogenesis, a follicle may be classified as primordial, primary, secondary or tertiary (antral, vesicular) (Pedersen and Peters, 1968). This classification is based on the size of oocyte, the morphology of granulosa cells, and the number of granulosa cell layers surrounding the oocyte (Lussier et al., 1987; Braw-Tal and Yossefi, 1997). The development of an activated primordial follicle to the preovulatory size is considered to be continuous unless a follicle undergoes atresia during the course of its development. The total time required by an activated bovine primordial follicle to reach the preovulatory size has been estimated to be 80-100 days (reviewed in Britt, 1991). During this long developmental period, the exposure of a follicle to different hormonal environments may have an influence on its competence to reach the preovulatory size and release a fertile oocyte. The dynamics of follicles ≥4 mm and their response to different concentrations of circulating gonadotropins and steroid hormones have been clearly demonstrated by a number of studies (reviewed in Adams et al., 1999). In contrast, little is known of the dynamics of ovarian follicles during preceding stages of folliculogenesis which comprises small antral and preantral follicles.

Studies have shown that antral follicles ≥4 mm develop in a wave-like fashion; i.e., synchronous emergence and growth of a group of follicles in response to a surge in the circulating concentrations of follicle stimulating hormone (Pierson and Ginther, 1984; Pierson and Ginther, 1987a; Savio et al., 1988; Sirois and Fortune, 1988; reviewed in Adams, 1999). Recently, the development of follicles 1 to 3 mm has been characterized as wave-like, similar to the pattern described for follicles ≥4 mm (Jaiswal et al., 2004). Cattle exhibit 2 or 3 waves of follicular development during an interovulatory interval (IOI). The duration of the development of an ovulatory follicle in a 3-wave IOI is shorter than the 2-wave IOI (Ginther et al., 1989d). Hence, it has been speculated that the oocyte developing within a preovulatory follicle of 2-wave IOI (longer duration of development) versus 3-wave IOI (shorter duration of development) may

have a different competence for fertilization (Ahmad *et al*., 1997; Townson *et al*., 2002; Bleach *et al*., 2004; Celik *et al*., 2005).

Currently, there is much interest on the molecular pathways during different stages of folliculogenesis (Liu et al., 2001; Park et al., 2005; Yoon et al., 2006). Hence, an understanding of the developmental dynamics of follicles <1 mm and their relationship with gonadotropic hormones and growth factors would provide a functional link between various molecular pathways, molecular markers, developmental stages of follicles, and the underlying ovarian physiology. Additionally, knowledge about the dynamics of follicles <1 mm would help to develop protocols to optimize reproductive efficiency of cattle and to develop therapeutic approaches for treating ovarian anomalies in cattle and females of other mammalian species.

To characterize the developmental pattern of antral follicles <1 mm, and to understand folliculogenesis in general, it is imperative to understand the embryogenesis of ovarian follicles. The following review is focused on embryogenesis of follicles and current knowledge about folliculogenesis in cattle. Information from other species has been incorporated to bridge missing links in cattle studies.

1.1 Formation of oocytes

1.1.1 Origin of oocytes as primordial germ cells

Oocytes originate extragonadally as primordial germ cells (PGC) from the endoderm of the embryonic yolk sac (Byskov and Hoyer, 1994; Senger, 1997). The PGC migrate by amoeboid movement from the epithelium of the yolk sac via the dorsal mesentery of the hindgut and arrive at the gonadal ridges (Senger, 1997; Smitz and Cortvrindt, 2002) by Day 35 to 36 of gestation in cattle (Erickson, 1966b). The gonadal ridges are undifferentiated gonads which are established by Day 32 of gestation in cattle (Erickson, 1966b). The factors that guide the PGC to the gonadal ridge are not clearly understood. However, *in vivo* (Ffrench-Constant et al., 1991) and *in vitro* studies (Alvarez and Merchant, 1986) have implicated fibronectin, which is present along the route to the gonadal ridge (Fujimoto et al., 1985) for the active migration of PGC (Ffrench-Constant et al., 1991). *In vitro* studies in mice have indicated that Transforming Growth Factor β1 (TGF β1) secreted by the gonads exerts a long-range chemotropic effect on PGC (Godin and

Wylie, 1991; De Felici and Pesce, 1994) for their unidirectional migration to the gonadal ridge (Godin and Wylie, 1991). Studies in mice (De Felici and Pesce, 1994; Zama et al., 2005) have shown that Kit Ligand (KitL, also known as stem cell factor [scf] or mast cell factor) is required for the survival and proliferation of migrating PGCs. The KitL is expressed in somatic tissue of mouse embryos along the pathway of PGC migration and at the gonadal ridges. It interacts with Kit, which is expressed by the migratory PGCs and increases the proliferation of PGCs by suppressing the apoptosis (Matsui et al., 1991). At the gonadal ridge, the PGC are internalized possibly by processes present on the epithelium. Since PGC are internalized through the epithelium of the gonadal ridge, the gonadal epithelium was mistakenly termed the germinal epithelium (Merchant and Alvarez, 1986).

1.1.2 Transformation of primordial germ cells into oogonia

Primordial germ cells undergo a limited number of mitotic divisions (Russe, 1983) during their passage as well as upon arrival at the gonadal ridge (Smitz and Cortvrindt, 2002). Mitotic divisions occur more often at the gonadal ridge compared to the divisions en route (Russe, 1983). In cattle, a sudden increase in the number of mitotic figures per ovary (304 mitotic figures at Day 60 versus 13 at Day 50) begins at Day 60 of gestation (Erickson, 1966b) which is indicative of intense oogonial mitotic activity. When the PGC cease to divide after settling in the gonadal ridge, they are termed as the oogonia (Russe, 1983; Smitz and Cortvrindt, 2002). Oogonia can be differentiated from the PGC at the cellular level due to abundance of organelles and in particular, the endoplasmic reticulum and tubular type mitochondria (Russe, 1983).

The germ-cells arrive at the gonadal ridge over a period of time, hence they are found in different mitotic cycles within clusters of dividing germ cells (Russe, 1983). During development of the ovary, the PGC that arrive first become mature earlier than the late arriving PGC and occupy the deep part of the growing gonad (Stein and Anderson, 1979). The peripherally located primordial germ cells act as stem cells for the generation of new oogonia (Russe, 1983).

1.1.3 Transformation of oogonia into oocytes

Oogonial mitosis ceases at or near Day 150 of gestation (Erickson, 1966b), fixing the number of germ cells available to the bovine female (Erickson, 1966b; Smitz and Cortvrindt, 2002). There is only about a 10% difference in the number of germ cells between right and left ovaries; hence, the population of follicles in one ovary is a good indicator of the number of follicles present in the other ovary (Erickson, 1966a).

Meiosis of oogonia begins by Day 75 to 80 of gestation in cattle (Erickson, 1966b; Byskov and Hoyer, 1994). Oogonial germ cells enlarge following the initiation of the meiotic process and are defined as primary oocytes (Byskov and Hoyer, 1994; Smitz and Cortvrindt, 2002). The first meiotic division of primary oocytes does not proceed beyond the pachytene stage of prophase-I (Erickson, 1965). At the pachytene stage of prophase-I, the chromosomes are decondensed and packed within the oocyte nucleus, which is then called the germinal vesicle (Smitz and Cortvrindt, 2002). The oocytes are comparatively bright and are easily recognizable due to an increase in cell size owing to an increase in the cytoplasm and swelling of the nucleus (Russe, 1983).

1.2 Formation of primordial follicles and follicular reserve

Oogonia that are undergoing meiotic prophase are extremely vulnerable to degeneration (Beaumont and Mandl, 1962) and many are lost during this process (Russe, 1983). For example, in the fetal human ovary, only about 5% of the total oogonia produced reach the resting diplotene stage of the meiotic prophase-I (Baker, 1963). A single layer of flattened epithelial cells condense around the vast majority of surviving oocytes and enclose them to form primordial follicles (Erickson, 1966b; Russe, 1983; Hirshfield, 1989; Byskov and Hoyer, 1994; Eppig, 2001). Primordial follicles are delineated from surrounding stromal tissue by a basal lamina (Russe, 1983; Hirshfield, 1989; Byskov and Hoyer, 1994; Eppig, 2001). Oocytes that fail to be surrounded by epithelial cells degenerate (Wagenen and Simpson, 1973; Smitz and Cortvrindt, 2002).

A bovine female is born with a pool of approximately 133,000 primordial follicles (Erickson, 1966a) which is not renewable and is gradually depleted (Erickson, 1966a; Gosden et

al., 1983) until it reaches near zero when the cow is 15 to 20 years of age (Erickson, 1966a). A recent study claimed that there was renewal of oocytes in post-natal mouse ovaries and using germ cell specific protein markers demonstrated the presence of germ-line stem cells in the ovaries (Johnson et al., 2004). However, a year later, the same group of researchers contradicted their previous finding and demonstrated that the bone marrow contains germ-line stem cells which are transported cyclically to the ovaries via systemic circulation and brings about follicular renewal in adult mice and human ovaries (Johnson et al., 2005). Although, one more study provided a new insight on the regeneration of female germ cells from the mesenchymal cells of the tunica albuginea (Bukovsky et al., 2004), strict experimentation is required before changing the belief that female germ cells are non-renewable.

1.3 Formation of ovaries and origin of different ovarian cell types

Gonadal ridges develop as a thickening of the coelomic epithelium on the medial aspect of the mesonephric kidneys (Dyce et al., 1996) and make connections with mesonephric tissue by streams of cells which are termed rete-ovarii (Byskov and Hoyer, 1994). At the gonadal ridge, oogonia become enclosed in germ-cell (ovigerous) cords (Russe, 1983; Byskov and Hoyer, 1994) which are composed of epithelial cells and oogonia (Russe, 1983; Hirshfield and DeSanti, 1995; Dyce et al., 1996) and are delineated from surrounding mesenchymal cells by a basal lamina (Byskov and Hoyer, 1994). The epithelial cells or the somatic cells i) originate from the coelomic epithelium; ii) have cuboidal or spherical nuclei; and iii) are precursors to the granulosa or follicular cells (Hirshfield and DeSanti, 1995). The mesenchymal or stromal cells i) originate from the stratified medial aspect of the mesonephric kidney; ii) have elongated nuclei and an overall appearance of fibroblasts; and iii) give rise to theca cells (Hirshfield and DeSanti, 1995). At the basal part, the germ cell cords are connected to mesonephric tissue (Byskov, 1975), and at the surface, they are connected to gonadal epithelium (Jeppesen, 1975). In cattle, the gonadal ridge is transformed to a definitive ovary by Day 40 of gestation (Erickson, 1966b). With the disruption of the germ cell cords, the ovary divides into cortical and medullary parts (Erickson, 1966b; Smitz and Cortvrindt, 2002).

1.4 Classification of developmental stages of follicles

The development of a follicle begins with the transformation of the flattened pre-granulosa cells of the primordial follicle to cuboidal granulosa (follicular) cells, after which a follicle with a single layer of granulosa cells is termed a primary follicle (Braw-Tal and Yossefi, 1997; Eppig, 2001). The proliferation of granulosa cells lead to an increase in their layers around the oocyte. A follicle with 2-6 layers of granulosa cells is called secondary follicle and a follicle with more than 6 layers of granulosa cells and a fluid-filled antrum is called a tertiary follicle (Appendix 1). A tertiary follicle is also referred as a vesicular or antral follicle. Its diameter at the time of antrum formation is 0.25 mm and reaches up to >14 mm which is referred as a pre-ovulatory or Graafian follicle. Following a preovulatory gonadotropin surge, the Graafian follicle is referred to as an ovulatory follicle (Braw-Tal and Yossefi, 1997).

1.5 Development at different stages of folliculogenesis

The growth of an oocyte begins immediately after primordial follicle activation and the differentiation of pre-granulosa cells in to granulosa cells (Braw-Tal and Yossefi, 1997; Eppig, 2001). The initial growth of an oocyte is characterized by the development of a Golgi area from vesicles around the nucleus and the progressive spread of Golgi towards the periphery. The growth of a follicle is characterized by an increase in the number of granulosa cell layers around the oocyte concurrent with an increase in the size of the oocyte; and formation of the zona pellucida (Russe, 1983; Adashi, 1996; Braw-Tal and Yossefi, 1997; Lundy et al., 1999; McNatty et al., 1999) which is a translucent halo of mucopolysaccharides (Adashi, 1996). The accumulation of Golgi areas in the oocyte indicates the contribution of the oocyte to the formation of zona pellucida (Russe, 1983). However, the contribution of granulosa cells is also being postulated (reviewed in Roy, 1994). During the formation of zona pellucida, the surrounding granulosa cells make attachment with it through desmosomes. These granulosa cells are termed the corona radiata cells. Concurrent with an increase in the thickness of the zona pellucida, the corona radiata cells develop long processes which finally attach to the oocyte through the zona pellucida (Russe, 1983).

The blood capillary network outside the follicular basement membrane increases with the progression of follicular growth. Blood capillaries are present in primary stage follicles and a few venules appear when a follicle reaches the secondary stage (Tanaka et al., 2001). In primary stage follicles, blood capillaries are detected around the follicle along with the concentric alignment and differentiation of stromal cells to the theca interna cells. The follicular growth up to this stage is referred as the preantral growth and includes primary and secondary stage follicles. With further growth of a follicle, fluid-filled pockets of follicular fluid appear in the granulosa cell layers. When 5 to 8 layers of granulosa cells have formed, the fluid-filled pockets fuse to form an antral cavity, and the follicular growth thereafter is referred as an antral follicular growth (Lundy et al., 1999) and includes tertiary and Graafian stage follicles.

In cattle, the growth rate of follicles at different stages of development were calculated (Marion and Gier, 1971; Lussier et al., 1987) according to the time-period required to double the number of granulosa cells (termed the generation interval) visible in the largest cross section through a follicle (Appendix 2). Mathematical calculations based on the generation intervals at different stages of follicular development revealed that the growth of follicles during the initial stages of development (follicle diameter up to 0.5 mm) proceed slower (Scaramuzzi et al., 1980) than later stages of development (Lussier et al., 1987; Scaramuzzi et al., 1980). An activated primordial follicle takes about 80 to 100 days to reach the preovulatory follicle diameter (reviewed in Britt, 1991), whereas a late-preantral follicle takes a period equivalent to 2 estrous cycles to reach the preovulatory size (Lussier et al., 1987).

1.6 Factors regulating primordial follicle activation

Some of the primordial follicles start to grow as soon as they are formed in the developing fetus (Russe, 1983). However, most of the primordial follicles remain in the quiescent state for a variable time periods and are depleted either by entry into the growth phase or by atresia (Erickson, 1966b; Gougeon, 1996). After activation from the quiescent state, growth of the primordial follicle is continuous and sequential until it ovulates or becomes atretic (Peters and Levy, 1966). The majority of the primordial follicles (>99%) do not mature to the ovulatory stage and undergo atresia (Ireland, 1987).

The mechanism which controls the activation of primordial follicles is not known. Indirect evidence from the following studies indicates that the mechanism of activation of the primordial follicles is hierarchical in nature and is also controlled.

1.6.1 Role of ovarian stroma

In one study, a high percentage of primordial follicles dissected from the same ovarian tissue initiated growth when cultured *in vitro* (Wandji et al., 1996). This finding led the authors to conclude that the ovarian stroma exerts inhibitory control over initiation of primordial follicle growth *in vivo*.

1.6.2 Role of rete ovarii

Rete ovarii consists of rete tubules which originate from the mesonephric duct. During ovarian development, the rete tubules enters through the ovarian hilus and branches out to form the medulla (Byskov and Moore, 1973; Byskov et al., 1977; and Moore) and also reaches within the groups of germ cells. Hence, it was hypothesized that the rete ovarii is important in the formation of follicles and early follicular development (Peter and Pedersen, 1967; Byskov, 1974). A morphological study in cat, mink and ferret (Byskov, 1975) and a transplantation study in mice (Byskov, 1977) revealed that rete ovarii cells make connexions with the granulosa cells and thereby plays an important role in the formation and growth of follicles.

1.6.3 Differences in the constitution of primordial follicles

During the development of ovary, germ cell cords form a link between the surface epithelium and mesonephric tissue (Byskov, 1975; Jeppesen, 1975) which led to the hypothesis that granulosa cells have a dual origin, i.e., one from the surface epithelial cells and another one from the mesonephric tissue cells (Byskov, 1978; Hirshfield, 1992). Each cell type has an inherent threshold for entering into the cell cycle and the proportion of each cell type in the constitution of a primordial follicle determines the length of its inactive phase (reviewed in Hirshfield, 1992).

1.6.4 Size of the primordial follicle pool

Studies are contradictory with respect to the relationship between the stock of primordial follicles and their activation into the growing phase. Studies in mice (Cran and Moor, 1980) and humans (Gougeon et al., 1994) have reported a direct relationship between the stock of primordial follicles and their activation, i.e., a decrease in the number of follicles entering the growing phase was related to a decrease in the stock of primordial follicles. However, the age-related increase in the number of primary and early growing follicles in women (Gougeon and Chainy, 1987) and mice (Krarup et al., 1969) is indicative of a short supply of putative inhibitory substance being secreted by the primordial follicles due to the reduction in their number (reviewed in Gougeon, 1996). Other studies have reported an inverse relationship between the pool of primordial follicles and the rate of their entry into the growing phase (Krarup et al., 1969; Hirshfield, 1994).

1.6.5 Role of follicular fluid

The possibility was raised in an early study that some factors, especially those present in the follicular fluid of antral follicles, played a role in the controlled activation of primordial follicles (Peters et al., 1973a). In a study on mice that examined the relationship between the pool of primordial follicles and the rate of their entry into the growth phase, it was found that the number of large antral follicles remained constant during estrous cycles until the primordial follicle reserve was completely exhausted (Hirshfield, 1994). The finding provides rationale for the role of follicular fluid in the controlled activation of primordial follicles.

1.6.6 Hierarchy

Early embryonic studies in mice have revealed that the first oogonia to begin meiosis are localized at the inner part of the cortex within close proximity to the mesonephric cells of the rete ovarii (reviewed by Byskov and Hoyer, 1994). Meiosis was prevented when ovarian tissue was stripped apart from the underlying mesonephric tissue (Byskov, 1974). However, when fetal mice

ovarian tissue was cultured with the mesonephric tissue, meiosis was resumed (Byskov and Grinsted, 1981). Hence, it was hypothesized that the mesonephric tissue influences the induction of meiosis in oogonia (reviewed by Byskov and Hoyer, 1994). It is also speculated that the pattern of localization of oogonia in relation to mesonephric tissue establishes a hierarchy among oocytes in the ovary after follicular formation. Experiments designed to test the theory of "first in first out, last in last out" (Henderson and Edwards, 1968; Polani and Crolla, 1991; Hirshfield, 1992) support the idea that the primordial follicles whose oocytes entered in meiosis first are also the first to be activated. Perhaps the hierarchy in the formation of the oocytes may be manifest in the form of successive follicular waves.

1.7 Role of follicle stimulating hormone on follicular development

Follicle stimulating hormone (FSH), a glycoprotein hormone produced and secreted by the anterior pituitary gland, plays a primary role in the regulation of follicle growth (Ulloa-Aguirre et al., 1995). FSH acts by binding to specific receptors, localized exclusively on the granulosa cells of follicles (Simoni et al., 1997).

1.7.1 Role of FSH in the development of follicles <1 mm

The role of FSH in the regulation of small follicle growth has been debated. It has been argued that the FSH receptors (FSHr) are not coupled to the adenylate cyclase second messenger system and hence FSHr are non-functional until a follicle reaches the secondary stage of development in cattle (Wandji et al., 1992) as well as in other mammalian species (Sokka and Huhtaniemi, 1990; O'Shaughnessy et al., 1997; Oktay et al., 1997). These inferences were made because of the lack of noticeable effects of exogenous FSH on small follicles *in vitro* (Wandji et al., 1992; Sokka and Huhtaniemi, 1990) which may be attributed to a minimal requirement of FSH during the early stages of follicular development (Govan and Black, 1975). The lower requirement of FSH may be further attributed to extremely slow rate of growth of small follicles (Scaramuzzi et al., 1980; Lussier et al., 1987) due to the longer time required for granulosa cells to double in number called the "doubling time" (Lussier et al., 1987; reviewed in Fortune, 1994).

However, a large amount of evidence from *in vivo* and *in vitro* studies supports the notion that FSH is the primary regulator of follicular growth at all stages of folliculogenesis.

The granulosa cells in the ovary are the only target site for FSH action (Simoni et al., 1997). A recent study detected functional FSHr in the precursor cells of the granulosa cells in the hamster's ovary (Roy and Albee, 2005). The functionality of FSHr was tested by measuring cAMP production following *in vitro* culture of the fetal ovaries with FSH. Suppression of fetal concentrations of circulating FSH using anti-FSH antibody resulted in a dose-dependent manner decrease in the primordial follicle formation.

Immediately after the activation of primordial follicles in cattle, FSH receptors are expressed on the granulosa cell membranes (Xu et al., 1995; Bao and Garverick 1998). Studies in rodents have indicated that FSH is required for the maturation of flattened pre-granulosa cells into cuboidal granulosa cells (Lintern, 1977; Arendsen, 1982) which marks the transition of an activated primordial follicle to the primary stage follicle. Suppression of FSH inhibits transition of an activated primordial follicle into the growing primary follicle (Gougeon et al., 1992). A significantly high number of primordial follicles retain in the hypophysectomized mice than in their age-matched controls (Jones and Krohn, 1961b; Edwards et al., 1977).

A study to determine the relationship between the appearance of follicles during the early stages of gestation and the serum concentrations of FSH in the female bovine fetus revealed that in fetus, as well as in adult cows, the number of follicles and stages of follicular development were associated with changes in the circulating concentration of FSH (Tanaka et al., 2001). In rats (Dahl et al., 1988) and mouse (Lintern, 1977), the activation of primordial follicles into the growth phase was increased in response to exogenous FSH, whereas the suppression of endogenous FSH following treatment with GnRH antagonists resulted in a decreased activation of primordial follicles. The morphometric examinations of the ovaries of new-born rats that were isografted to the kidney capsules of ovariectomized (i.e., gonadotropin-rich) or ovariectomized-hypophysectomized (gonadotropin-poor) adult hosts revealed the presence of more growing follicles in the grafts in the gonadotropin-rich environment (Arendsen, 1982).

Studies involving hypophysectomy indicated a retarded growth of preantral follicles in fetal monkeys (Gulyas et al., 1977) and rats (Hirshfield, 1985). However, the administration of exogenous FSH in hypophysectomized mice (Wang and Greenwald, 1993a; 1993b), sheep (Cecconi et al., 1999) or women (Abir et al., 1997) resulted in an increased number of small pre-

antral follicles. *In vitro* studies (Hulshof et al., 1995; Gutierrez et al., 2000; Ralph et al., 1995; 1996) in which bovine preantral follicles were cultured with FSH indicated the involvement of FSH in the growth and development of preantral follicles. FSH has also been reported to suppress apoptosis in serum-free cultures of rat preantral (Mcgee et al., 1997) and antral (Tilly and Tilly, 1995) follicles.

FSH is required at all stages of follicular development. Studies have indicated a regulatory role of FSH in i) the transition of flattened pregranulosa cells to cuboidal granulosa cells (Jones and Krohn, 1961; Edwards et al., 1977; Lintern, 1977; Arendsen, 1982; Dahl et al., 1988; Gougeon et al., 1992); ii) the growth of preantral follicles (Abir et al., 1997; Gulyas et al., 1977; Hirshfield, 1985; Wang and Greenwald, 1993a; 1993b; Hulshof et al., 1995; Ralph et al., 1995; 1996; Cecconi et al., 1999; Gutierrez et al., 2000); and iii) the transition of a follicle from the preantral to the antral stage through its direct effect on antrum formation (Roy and Treacy, 1993).

1.7.2 Role of FSH in the development of follicles >1 mm

Follicles in their later stages of development (4 to 9 mm: Adams et al., 1992b; 1 to 3 mm, Jaiswal et al., 2004) develop in response to the surges in the circulating concentrations of FSH. During an interovulatory interval, the circulating concentrations of FSH surge in a recurrent fashion and stimulate the wave-like recruitment of 4 to 5 mm follicles (Adams et al., 1992b; Gong et al., 1995). FSH concentrations begin to decline following the recruitment of 4 to 5 mm follicles. The progressive increase in the secretion of estradiol and inhibin from the growing 4 to 5 mm follicles exert a negative feedback action for FSH at the hypothalamo-pituitary axis (Adams et al., 1992a; 1993a; Gibbons et al., 1997). Suppression of FSH to the basal concentrations coincides with the selection of a dominant follicle. The dominant follicle acquires receptors for luteinizing hormone (LH) on the granulosa cells earlier than its subordinates (Campbell et al., 1995) and therefore survives under the basal concentrations of FSH using the support from LH (reviewed in Adams, 1999). In heifers, the sustained higher concentrations of FSH during superstimulatory treatment overrides the process of follicular selection and results in the development of multiple follicles (Mihm et al., 1997). In ewes follicles can grow up to the preovulatory size under the elevated concentrations of circulating concentrations (Picton et al., 1990).

The actions of FSH and LH depend on the size of the follicle. In experiments where the circulating concentrations of FSH in heifers were suppressed with gonadotropin releasing hormone (GnRH) agonist (Driancourt et al., 1991; Gong et al., 1996; Webb and Armstrong, 1998; Garverick et al., 2002;) or by an active immunization against GnRH (Prendiville et al., 1995) follicles did not grow beyond 4 mm size. The late antral stage follicles were stimulated to grow only when the GnRH-agonist treated heifers (Garverick et al., 2002) or ewes (Picton and McNeilly, 1991) were challenged with exogenous FSH equivalent to the endogenous peak concentrations of FSH (Picton and McNeilly, 1991). The follicle attains dominance at a critical size of ≥8 mm (Adams et al., 1993a, 1993b), after which its development is dependent on the pulsatile secretion of LH. Hence, when the pulsatile secretion of LH was suppressed initially following the treatment of heifers with GnRH-agonist, follicles did not grow beyond 7 to 9 mm (Gong et al., 1996).

The action potential of FSH on follicles depends on the threshold concentrations of FSH. In hypogonadotropic women, the recruitment of late antral follicles required a threshold concentration of FSH (Brown, 1978). The minimal concentration of FSH required to recruit antral follicles varies among individuals as well as among similar-sized follicles within the same individuals. The dissected follicles of similar size from the same individual that were cultured with FSH expressed variable amounts of aromatase activity, which is indicative of the differential response of similar-sized follicles to the available FSH (Fry and Driancourt, 1996).

1.8 Regulation of follicular dynamics during the interovulatory interval

In cattle and several other mammalian species, the developmental pattern of follicles during the terminal stages of folliculogenesis (follicles ≥4 mm) has been characterized as wave-like, which refers to the periodic and synchronous growth of a group of follicles (reviewed in Adams, 1999). A recent study demonstrated that follicles 1 to 3 mm in diameter also develop in a wave-like fashion (Jaiswal et al., 2004). The wave-like developmental pattern of follicles in heifers was first proposed more than 40 years ago (Rajakoski, 1960) using the histological data of ovaries collected from different heifers on different days of the estrous cycle. However, the proposed wave-like developmental pattern of follicles was confirmed using ultrasonographic real-time

follicular images (Figure 1.1) collected over a time-period on the same follicles from the same set of animals (Pierson and Ginther, 1984; Pierson and Ginther, 1987a; Savio et al., 1988; Sirois and Fortune, 1988).

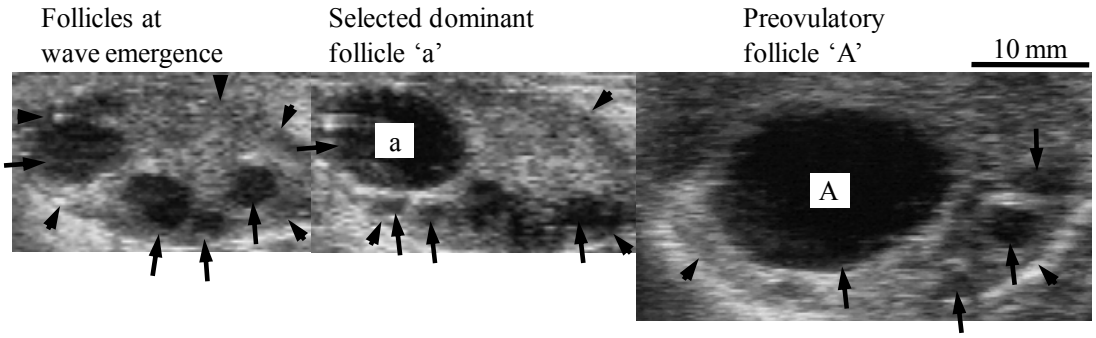

Figure 1.1 Ultrasonic images of antral follicles ≥1 mm in the bovine ovary. Arrow heads delineate the border of the ovary, arrow indicate follicles of various sizes.

Cattle exhibit 2 or 3 waves of follicular development during an interovulatory interval. During each wave, a single follicle outgrows the others in the cohort and is thereby selected to become the dominant follicle. Although the dominant follicle of each wave has the capability to ovulate (Savio et al., 1990a), only the dominant follicle of the last wave ovulates (Bergfelt et al., 1991; Lucy et al., 1992), i.e., the wave that produces the dominant follicle during progesterone decline consequent to the luteolysis is ovulatory. In the presence of the luteal phase progesterone, the dominant follicle undergoes atresia and a new follicular wave emerges (Savio et al., 1993a).

Lengthening the luteal phase of the estrous cycle using exogenous progestogens lengthens the estrous cycle and results in dominant follicle turnover. Higher progesterone concentration suppresses LH pulse frequency which in turn results in a short lived dominant follicle. For example, energy restriction in beef cows results in low frequency of LH and consequent reduction in the size of the dominant follicle. The short dominance is accompanied by the reduced inter-wave interval and the preponderance of 3-wave cycles (Grimard et al., 1995).

Progesterone has a suppressive effect on the growing phase of the dominant follicle (Adams et al., 1992a). Hence, the normal luteal levels of progesterone promote regression of the dominant follicles and recurrence of follicular waves (Sirois and Fortune, 1990; Stock and Fortune, 1993). The subluteal levels of progesterone (1 to 2 ng/ml) which are lower than the luteal levels (3.5 to 6

ng/ml) but higher than the basal follicular phase levels (<1 ng/ml) (Stock and Fortune, 1993) increases the LH pulse frequency, but not the basal LH concentrations (Ireland and Roche, 1982; Roberson et al., 1989). An increase in the LH pulse frequency prolongs the growth of the dominant follicle (Sirois and Fortune, 1990) and by making the substrates available for estradiol synthesis, increases the secretion of circulating concentrations of estradiol by the dominant follicle (Sirois and Fortune, 1990). Higher circulating concentrations of estradiol inhibits FSH surges and results in the complete absence of growing follicles 5 mm or larger (Sirois and Fortune, 1990) and formation of new dominant follicles (Sirois and Fortune, 1990; Stock and Fortune, 1993).

In 2-wave cycles, the ovulatory follicle develops under a high-progesterone milieu for 7 days compared to 3 days in 3-wave cycles. Additionally, the ovulatory follicle in 2-wave cycles grows for a longer period (prolonged dominance) than in 3-wave cycles (11 versus 7 days, respectively; Ginther et al., 1989d) and hence its size at ovulation is larger (17 versus 14 mm, respectively; Ginther et al., 1989d). Similarly, long-term progestogens treatment used for estrus synchronization results in the development of a persistent oversized follicle (Hansel et al., 1961; Hill et al., 1971; Henricks et al., 1973; Roche, 1974; Beal et al., 1988; Jochle, 1993; Savio et al., 1993a; Stock and Fortune, 1993). For estrus synchronization, the progestagen treatment given after induced luteolysis creates a sub-luteal phase progestational environment (Savio et al., 1993a). As described earlier, this subluteal phase progesterone concentration allows a higher than normal pulse frequency of circulating luteinizing hormone (Ireland and Roche, 1982; Roberson et al., 1989; Stock and Fortune, 1993; Kojima et al., 2003) and consequent development of a persistent, over-sized dominant follicle (Adams et al., 1992a; Sirois and Fortune, 1990; Stock and Fortune, 1993; Ahmad et al., 1995; Revah and Butler, 1996; Kojima et al., 2003). The persistent follicle releases an aged oocyte and results in poor fertility (Hansel et al., 1961; Hill et al., 1971; Henricks et al., 1973; Roche, 1974; Beal et al., 1988; Jochle, 1993; Savio et al., 1993a; Stock and Fortune, 1993).

Pregnancy rates in cattle with 2- versus 3-wave patterns were compared (Ahmad et al., 1997; Townson et al., 2002; Bleach et al., 2004) based on the notion that the preovulatory follicle in the 2-wave pattern grows for a longer period and may contain a relatively aged oocyte (Ginther et al., 1989d). However, the outcome of various studies is contradictory. Some studies have reported similar pregnancy rates in 2- versus 3-wave IOI (Ahmad et al., 1997; Bleach et al.,

2004), whereas another reported a significantly lower pregnancy rate following ovulation at the end of the 2-wave pattern (Townson et al., 2002).

The temporal characteristics of 2- and 3-wave IOI have been examined (Noseir, 2003; Ginther et al., 1989d; Adams, 1994) but factors predictive of a specific pattern have not been identified and the repeatability of a given wave pattern within individuals is not known. Although the subject has not been studied extensively, there appears to be no clear seasonal-, breed-, or age-specific predilection for a given wave pattern (Zeitoun et al., 1996; Driancourt, 2001). An increased proportion of 3-wave patterns have been associated with a low plane of nutrition (Murphy et al., 1991; Rhodes et al., 1995) and heat stress (Badinga et al., 1993; Wolfenson et al., 1995).

1.9 General objectives

The objectives of the work reported in this thesis were to characterize the developmental pattern of antral follicles < 1 mm and their temporal relationship with the circulating concentrations of FSH, to determine the regulatory mechanism of 2- versus 3-wave interovulatory intervals, and to determine the effect of different exposure to progesterone as in the 2- versus 3-wave interovulatory intervals on the competence of oocytes.

The outcome of the studies will have important implications on the breeding management of cattle. The understanding of the fundamental developmental pattern of antral follicles <1 mm along with the known pattern of development of antral follicles ≥1 mm will update current knowledge about folliculogenesis and would lead to the development of effective protocols for ovarian manipulation for the purpose of estrus/ovulation/follicular-wave synchronization, superstimulation of ovaries, fixed-time artificial insemination and therapeutic interventions for disrupted ovarian function. The understanding of the mechanism by which 2- and 3-wave interovulatory intervals are regulated would be helpful for the uniform selection of herd and efficient reproductive management. The effect of variable growth period of follicles and exposure to progesterone in 2- versus 3-wave interovulatory intervals on the competence of the oocyte would enable to refine the estrus synchronization and fixed-time artificial insemination protocols.

1.9.1 Specific objectives

Objectives 1 to 3 (Chapters 2 to 4): To characterize the developmental pattern of antral follicles <1 mm in diameter and to elucidate the role of FSH on the developmental dynamics of follicles.

Hypothesis: Antral follicles <1 mm develop in a wave-like fashion in temporal relationship with changes in circulating concentrations of FSH.

Chapter 2: To determine the effect of prolonged suppression of FSH by actively immunizing heifers against GnRH on the follicular wave dynamics of follicles ≥1 mm.

Hypotheses: Follicles <1 mm are responsive to FSH. Vaccination against GnRH suppresses the circulating concentrations of FSH and recruitment of follicles into the ultrasonographically detectable waves of follicles ≥1 mm.

Chapter 3: To determine the effect of exogenous FSH on the responsiveness of follicles developed under the prolonged suppression of FSH.

Hypothesis: Suppression of endogenous FSH followed by the replacement with exogenous FSH alters the number of follicles ≥1 emerging into a detectable follicular wave.

Chapter 4: To characterize the developmental pattern of antral follicles <1 mm in cattle using histological approach.

Hypothesis: Antral follicles <1 mm develop in a wave-like fashion in temporal association with surges in the circulating concentrations of FSH.

Objective 4: (Chapter 5): To determine the repeatability of the 2- and 3-wave patterns within individuals, and to identify follicular and luteal characteristics predictive of the 2- versus 3-wave pattern of follicular development during the interovulatory interval (IOI) in cattle.

Hypotheses: A wave-pattern (i.e., 2- or 3-wave) is repeatable within individuals. The dominant follicle of Wave 1 regulates the wave pattern.

Objective 5 (Chapter 6): To determine the effect of progesterone (and hence, LH pulsatility) during the final stages of oocyte maturation on the competence of the oocyte to develop into an embryo.

Hypothesis: Long-term low but not the short-term low- or high- progesterone environments affect the developmental competence of fertilized oocytes.

Chapter 2

EFFECT OF ATTENUATION OF FSH SURGES AND LH-PULSE FREQUENCY ON THE RECRUITMENT OF ANTRAL FOLLICES ≥1 mm IN CATTLE

R.S. Jaiswal, J. Singh, S. Robbins, M. Dominguez and G.P. Adams

2.1 Abstract

The study was designed to test the hypothesis that suppression of FSH ablates the wave-like pattern of follicular development. Heifers were assigned randomly to vaccinated (n=12) and control (n=12) groups. Heifers in the vaccinated group were given anti-GnRH vaccine on Days 0, 28 and 56. Hormonal data were acquired from all heifers, whereas the follicular data were acquired only from a subgroup of heifers (n=6/group) that was subjected to daily ultrasonography (Day -6 to Day 107) to monitor the dynamics of follicles ≥1mm. Vaccinated heifers developed maximum titers (69±3% binding at 1:5000) by Day 71. In the vaccinated group, ovulations ceased on Day 21.7 ± 2.7, follicular wave emergence was not detectable after Day 32.3 ± 6.3. Basal and mean plasma concentrations of LH tended to decrease over time (P=0.07), and LH pulse frequency and amplitude decreased (P≤0.03) after the second vaccination. No difference in mean plasma concentration of FSH was detected between groups (P=0.69). In both groups, periodic surges in FSH concentrations were apparent, but the peaks were attenuated after the second vaccination in the vaccinated group (P ≤0.05). The mean number of 1 to 3 mm follicles did not differ between groups (P≥0.29). The peaks in the number of follicles, an indication of wave pattern, was evident in both groups (P<0.01), however, the peak numbers of 1 to 3 mm follicles increased progressively in the vaccinated group (P=0.03) than in the control group (P=0.13). Peaks in the number of 1 to 3mm follicles were inversely correlated with the peak (r=-0.99, P=0.04) and mean concentrations (r=-0.35, P=0.01) of FSH. In conclusion, vaccination against GnRH attenuated FSH surges but did not suppress mean basal concentrations of FSH concentrations. The study also revealed an inverse relationship between the circulating concentrations of FSH and the number of 1 to 3 mm follicles recruited into a wave.

Immunization against GnRH was associated with a loss of wave-eliciting FSH surges and the loss of the wave pattern of development of follicles ≥4 mm.

2.2 Introduction

The use of ultrasonography to monitor daily changes in individual follicles has revealed that follicles ≥1 develop in a synchronous wave-like manner (reviewed in Adams, 1999; Jaiswal et al., 2004) in response to the surges in circulating concentrations of FSH (Adams et al., 1992b; Gong et al., 1995). However, the pattern of development of follicles <1 mm remained obscured due to lack of a non-invasive tool like ultrasonography.

Follicles <1 mm include primordial, primary, secondary and tertiary (antral) stage follicles (Braw-Tal and Yossefi, 1997; Lundy et al., 1999). When a quiescent primordial follicle is activated, its early granulosa cells express receptors for FSH (Camp et al., 1991; Xu et al., 1995; Bao et al., 1997). The functionality of the FSH receptors in the hamster ovary has been tested in the precursor cells of the granulosa cells (Roy and Albee, 2005) and in the preantral and small antral follicles (Roy et al., 1987). The growth promoting effect of FSH on the early stages of follicular development has been demonstrated in cattle (Tanaka et al., 2001; Itoh et al., 2002) sheep (Cecconi et al., 1999), rodents (Hirshfield, 1985; Dahl et al., 1988; Arendsen, 1982; Lintern, 1977; Wang and Greenwald, 1993a; 1993b), monkeys (Gulyas et al., 1977), and humans (Abir et al., 1997).

Growth rates of follicles from the primary to the ovulatory stages of development have been estimated (Marion and Gier, 1971; Lussier et al., 1987), but these estimations do not reveal the developmental dynamics of follicles <1 mm relative to changes in circulating concentrations of FSH. The understanding of the developmental pattern of ovarian follicles <1 mm and their temporal relationship with circulating FSH would provide a precise approach to manipulate ovarian functions for clinical and subclinical applications.

The experiment was designed as part of a larger study to test the hypothesis that antral follicles of all size categories (<1 mm and ≥1 mm) develop in a wave-like manner in response to endogenous surges in circulating FSH. The specific objective was to determine the effect of prolonged suppression of FSH, by active immunization against GnRH, on the dynamics of follicles ≥1 mm. It was hypothesized that follicles <1 mm are responsive to FSH and that

vaccination against GnRH suppresses the circulating concentrations of FSH and recruitment of follicles into the ultrasonographically detectable waves of follicles ≥1 mm.

2.3 Materials and Methods

2.3.1 Animals and treatments

The experiment was carried out between August and January using sexually-mature Hereford-crossbred heifers (n = 34), 18 to 24 months of age and weighing 450 to 550 kg, maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52^{o}N and 106^{o}W). Heifers were given two luteolytic doses of prostaglandin (500 µg Cloprostenol i.m; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) 12 h apart to create a more uniform physiologic status among heifers at the time of immunization against GnRH. Seven days later (Day 0), heifers assigned randomly to the respective groups were given an anti-GnRH vaccine (n = 18), a sham vaccine (n = 6), or left untreated (n = 10). Heifers were vaccinated on Days 0, 28 and 56 (Day 0 = day of first vaccination).

The anti-GnRH vaccine consisted of eight copies of a peptide GnRH bound to both the carboxy and amino ends of eight copies of the 52KD carrier protein leukotoxin, produced by *Pasturella hemolytica* (Cook et al., 2000). This immunogenic GnRH fusion protein (IPS-21) was formulated in a water-in-oil adjuvant (Montanide® ISA-70, Seppic Inc., Lyons, France), and was given subcutaneously as a 1 mL dose (200 µg antigen) at one site in the neck region (Biostar Inc., Saskatoon, Canada). The sham-vaccine consisted of 1 mL adjuvant and was given at one site in the neck region. The formulation was expected to produce an immunological response (>20% binding at 1:5000 dilution) in all treated animals, lasting for >80 days (Cook et al., 2000). Maximal anti-GnRH titres were expected after 70 days of initial anti-GnRH vaccination (Cook et al., 2000).

As part of the design of a larger study on the effects of anti-GnRH, a subset of vaccinated heifers (n = 12), sham-vaccinated heifers (n = 6), and untreated control heifers (n = 6) was chosen randomly from the larger group for the purposes of the present study. From the subset, 6 of the 12 heifers vaccinated against GnRH, and all 6 heifers from the untreated control group were slaughtered on Day 85 to collect ovarian tissue for the purposes of a separate study. Hence, data

involving end points after Day 85 are from six vaccinated heifers and six sham-vaccinated heifers.

2.3.2 Ovarian ultrasonography

Ovarian follicular development was monitored in six heifers chosen randomly from the vaccinated group and all six heifers in the sham-vaccinated group. Transrectal ultrasonography was done daily using a 7.5 MHz linear-array transducer (Aloka SDD-900, Tokyo Japan; lateral and axial resolution: 1 mm.) to characterize the dynamics of follicles ≥1 mm (Jaiswal et al., 2004) from Day -7 (prostaglandin treatment) to Day 107. Day 107 was chosen as the end of the observational period based on an estimated 20-day interval required for a follicle to develop from an early antral stage (i.e. ~250 μ) to the ultrasonically detectable stage (1 mm; Lussier et al., 1987; Fortune, 1994). Hence, if gonadotropin concentrations are minimal when anti-GnRH titres are maximal (i.e., ~84 days), and early antral growth is gonadotropin-dependent, we expected maximal follicular suppression (minimal ultrasonographically detectable follicles) at ~ Day 104 (i.e., 20 days after maximal GnRH titres). To minimize variation and bias in data acquisition, all ultrasonographic examinations were performed by one investigator (RJ) who was not aware of animal-treatment identity.

2.3.3 Anti-GnRH titers

Blood samples (n = 12 vaccinated; n = 6 sham-vaccinated) were collected from the jugular vein into non-heparinized tubes (10 mL; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) on Days 0, 14, 28, 42, 56, 70, 84, 98 and 111 (Day 0 = day of first vaccination). Serum anti-GnRH antibody binding activity was estimated by a modified radioimmunoassay procedure (Meloen et al., 1994). Briefly, synthetic GnRH (Gonadorelin, Catalogue# H4005, Bachem California Inc., Torrance, CA) was iodinated with ^{125}I (Amersham, Oakville, ON, Canada) by the chloramine-T method (Greenwood and Hunter, 1962). A standard amount (12,000 cpm; approximately 1 pg) of ^{125}I-labeled GnRH was added to serial dilutions of serum, providing a final incubation volume of 0.7 mL. Following a 24 h incubation period at 2º to 6ºC, a suspension of charcoal and buffer was added to adsorb the non-antibody bound ^{125}I-GnRH. The radioactivity

in the charcoal fraction was measured following centrifugation. Data were expressed as the percentage of a standard dose of ^{125}I-GnRH bound to an antibody at specified serum dilutions. The samples were analyzed in duplicate at serum dilutions of 1:100 and 1:5000. Samples with <10% binding were considered negative for anti-GnRH. Nonspecific binding at 1:5000 was 4.7%, based on sera from pre-immunized and sham-vaccinated heifers. The intra- and inter-assay coefficients of variation for the samples analyzed at a dilution of 1:5000 were 7.1 and 11.0%, respectively.

2.3.4 FSH and LH concentrations

Jugular blood samples were collected daily from all the heifers (n = 6/group) that were used in the study beyond Day 85, whereas blood samples from remaining heifers (n = 6/group) were collected on Days 0, 7, 14, 21 to 35, and 42 to 84. Blood samples were collected in heparinized tubes (10 mL; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and were centrifuged for 15 minutes at 1500 X g within 30 to 60 min of collection. Plasma was aspirated and stored at –20^{o}C until measurement of FSH using a double antibody radioimmunoassay (Rawlings et al., 1984). The primary antibody was NIDDK-anti-ovine FSH, and concentrations were expressed using standards prepared from USDA-bovine FSH-I-l. The minimum detectable limit of the assay was 0.13 ng/mL. The range of the standard curve was 0.13 to 16 ng/mL. The intra- and inter-assay coefficients of variation were 4% and 3% for the low reference sample (mean 1.28 ng/mL), and 7% and 3% for the high reference sample (mean 3.09 ng/mL), respectively.

To determine LH pulse frequency and amplitude, blood samples were collected at 15 min intervals for 8 h on Days 24, 53, 82 and 109 (Day 0 = day of first vaccination). To minimize the potential effects of stress on follicle dynamics in heifers examined daily by ultrasonography, frequent blood samples on all but the final sampling period (Day 109) were taken from heifers in the vaccinated and control groups (n = 6/group) that were not subjected to daily ultrasonography. On Day 109, heifers (n = 6/group) that were subjected to daily ultrasound scanning were used because the others were sent to slaughter on Day 85. Blood samples (5 mL) were collected via catheters (vinyl tubing; 1.0 mm inner diameter × 1.5 mm outer diameter; Product code SV70; Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia) that were inserted into the

jugular vein on the day before frequent blood sampling (Evans et al., 1994). Catheters were filled with heparinized saline between samplings (1000 U.S.P. units of heparin sodium per liter of saline; Hepalean, Organon Teknika Inc., Toronto, ON, Canada). Plasma LH concentrations were measured using a double antibody radioimmunoassay and were expressed as NIDDK-bLH4 units (Evans et al., 1994; Honaramooz et al., 1998). The range of the standard curve was 0.06 to 8 ng/mL. All samples were analyzed in a single assay; the intra-assay coefficients of variation were 8% and 2% for the low (mean, 0.45 ng/mL) and high (mean, 0.96 ng/mL) reference samples, respectively. Mean and basal concentrations and LH pulse frequency and amplitude were calculated using Pulsar software (Gitzen and Ramirez, University of Illinois, IL) and illustrated (Evans et al., 1994; Honaramooz et al., 1998).

2.3.5 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System software package (SAS learning edition 2.0; SAS Institute Inc., Cary, NC) and the data were reported as mean ± SEM.

Peaks in the plasma concentrations of FSH were determined using the cycle-detection program (Clifton and Steiner, 1983) and were reconfirmed manually using the definition of a peak as a rise in FSH for at least two consecutive days followed by a decline for at least two consecutive days (McCorkell et al., 2006). Peaks in the number of 1 to 3 mm follicles were also determined similarly. Mean and peak plasma FSH concentrations in the sham-vaccinated ($n = 6$) and control ($n = 6$) groups were compared by student's t-test. Since, there was no difference between groups ($P \geq 0.31$), data were combined for further analyses.

The effects of day and group were analyzed by analysis of variance for repeated measures using the mixed procedure (Littell et al., 2000) for i) anti-GnRH titers of samples collected on Days 0, 14, 28, 42, 56, 70, 84, 98 and 111, ii) FSH profile (mean basal and peak concentrations) for samples collected from Day -6 to Day 107, and iii) follicle numbers in size categories 1 to 3 mm, and ≥4 mm. If main effects or their interactions were statistically significant ($P < 0.05$), multiple comparisons were made using Tukey's post-hoc test. The pulsatile characteristics of LH secretion (mean and basal concentrations, pulse frequency and amplitude) on Days 24, 53, 82, and 109 were compared between vaccinated and control (sham- and untreated) groups by

student's t-tests. Changes in the pulsatile characteristics of plasma LH concentration over time in the vaccinated group were analyzed by analysis of variance for repeated measures in heifers that were sampled repeatedly (i.e., Days 24 to 82; n = 6).

The associations between i) anti-GnRH titres and LH profile (basal and mean concentrations, pulse frequency and amplitude), ii) anti-GnRH titres and FSH profile (mean and peak concentrations), iii) peak numbers of 1 to 3 mm and ≥4 mm follicles and FSH profile, and v) peak number of follicles ≥4 mm and peak numbers of follicles 1 to 3 mm, were estimated using Pearson's correlation analysis.

All procedures were approved by the University of Saskatchewan Protocol Review Committee, under the umbrella of the University Committee on Animal Care and Supply, and were performed in accordance with the principles outlined by the Canadian Council on Animal Care.

2.4 Results

Serum anti-GnRH titers (mean ± SEM) increased sharply after the second vaccination (Figure 2.1). Titres reached 54 ± 5% binding at a serum dilution of 1:5000 by 42 days. Heifers developed maximum titers of 69 ± 3% binding 70 days after the first vaccination. No tissue reactions at the site of injection were detected in any of the heifers.

Only one interovulatory interval (20.7 ± 1.2 days) was detected approximately 22 days after the first vaccination (Day 0) in 5 of 6 heifers in the vaccinated group; the remaining heifer exhibited two interovulatory intervals, each of 19 days duration (Table 2.1). Of the 7 interovulatory intervals detected in 6 heifers, 4 consisted of 2 follicular waves and 3 consisted of 3 waves. The emergence of new waves of follicles ≥4 mm stopped within 1 week (Day 32.3 ± 6.3) after the second vaccination (Day 28).

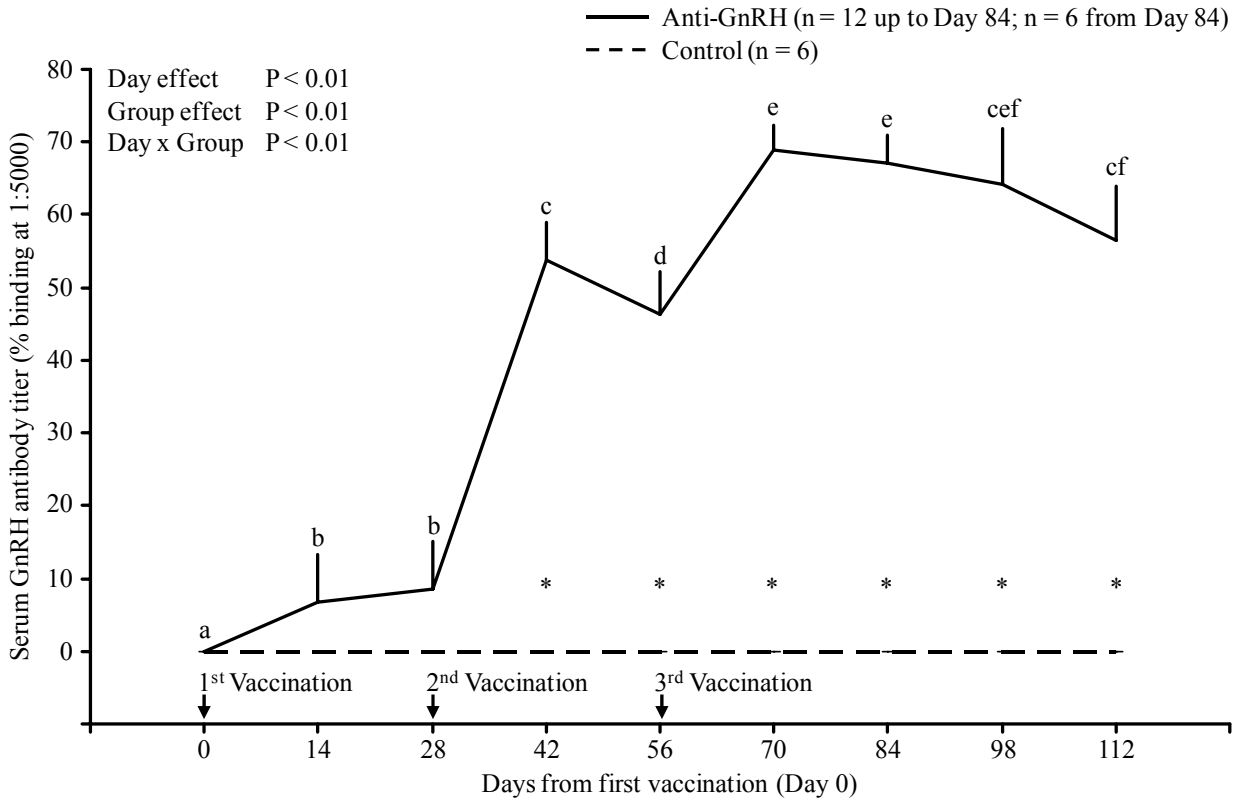

Figure 2.1 Anti-GnRH titres in heifers vaccinated or sham-vaccinated on Days 0, 28 and 56 (Day 0 = day of first immunization). Serum anti-GnRH titres were measured at 14 days intervals from the day of first vaccination (Day 0). [abcdef] In the vaccinated group, values with no common superscripts among days are different ($P < 0.05$). *Values differed between groups ($P < 0.01$).

Table 2.1 Ovarian response to immunization against GnRH in heifers (Day 0 = day of first vaccination).

Heifer identification	Interovulatory interval (days)	Number of waves per interovulatory interval	Day of last ovulation	Day of emergence of last detectable wave
161	27	3 waves	24	30
167	21	3 waves	18	27
168	19	2 waves	18	35
175	22	3 waves	19	26
180*	19	2 waves	--	--
	19	2 waves	33	61
189	18	2 waves	15	15
mean ±SEM	20.7± 1.2		21.7 ± 2.7	32.3 ± 6.3

*Heifer #180 ovulated twice (on Days 13 and 33) after the first vaccination on Day 0.

The profile of LH (basal and mean concentrations, pulse frequency and amplitude) was negatively correlated with anti-GnRH titers (r= -0.58; -0.54; -0.61 and -0.65 respectively; $P < 0.01$). In vaccinated group, the comparison of LH profile over time revealed a tendency ($P = 0.07$) for lower basal and mean concentration of LH. The LH pulse frequency and amplitude ($P < 0.03$) were obliterated in the vaccinated group. Mean and basal concentrations of LH differed between groups by Day 82 ($P < 0.01$; Figure 2.2), whereas, LH pulse frequency and amplitude differed between groups by Day 53 ($P < 0.01$).

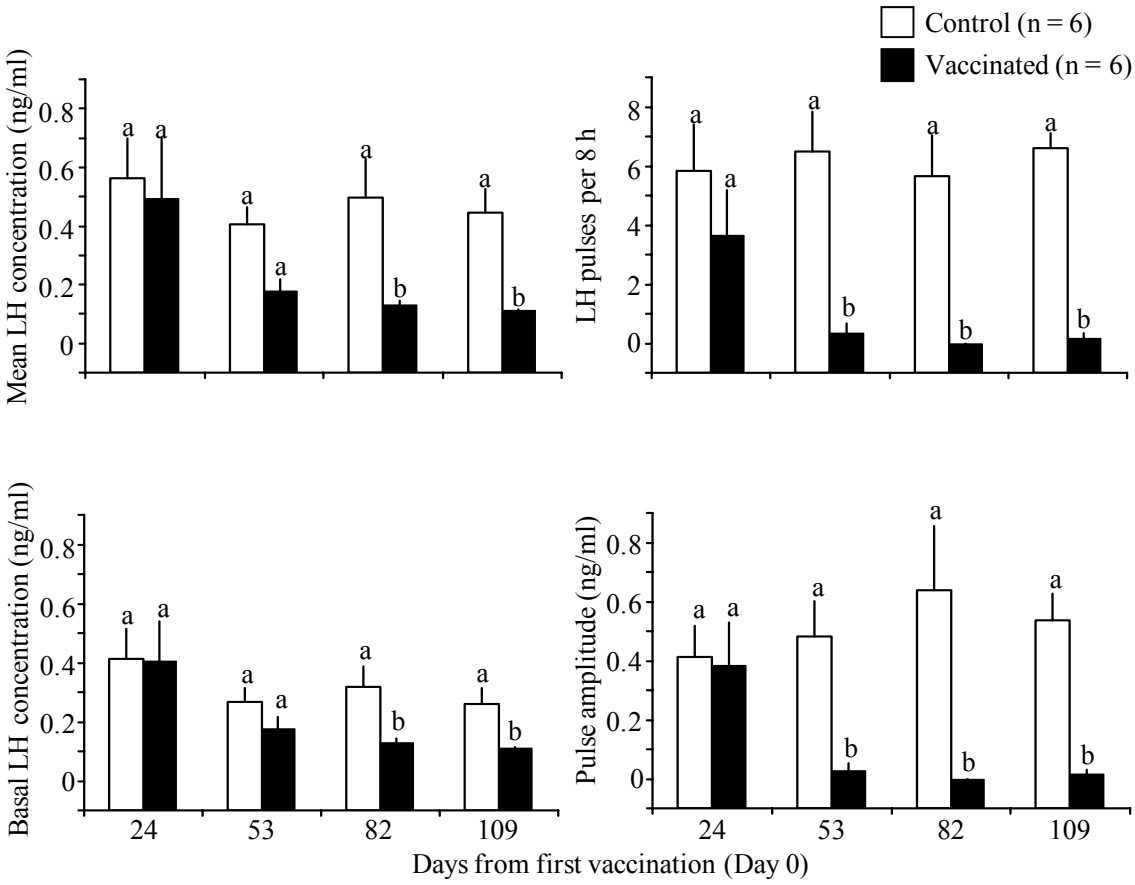

Figure 2.2 Effect of vaccination against GnRH on the pulsatile characteristics of plasma LH concentrations in heifers (mean ± SEM; n = 6 per group). Heifers in the anti-GnRH group were vaccinated on Days 0 (day of first vaccination), 28 and 56. [ab]Values with no common superscripts are different ($P \leq 0.02$).

Peak concentrations of FSH were inversely correlated with anti-GnRH titers ($r = -0.60$, $P < 0.01$); however, the mean concentrations of FSH were not affected by an increase in anti-GnRH titer ($r = 0.02$, $P = 0.89$). In GnRH-immunized heifers, mean plasma concentrations of FSH increased sharply after the first vaccination ($P = 0.02$), but declined after the second vaccination to the concentrations similar to control heifers (Figure 2.3). In the vaccinated group, the FSH peaks were evident throughout the study period (Day effect, $P < 0.01$; Figure 2.4) and there was no difference in the number of peaks of FSH between vaccinated and sham-vaccinated groups (11.5 ± 1.38 versus 13.2 ± 1.07; $P = 0.52$). However, FSH peaks were attenuated after second vaccination in the vaccinated group (Day x Vaccination number, $P < 0.01$).

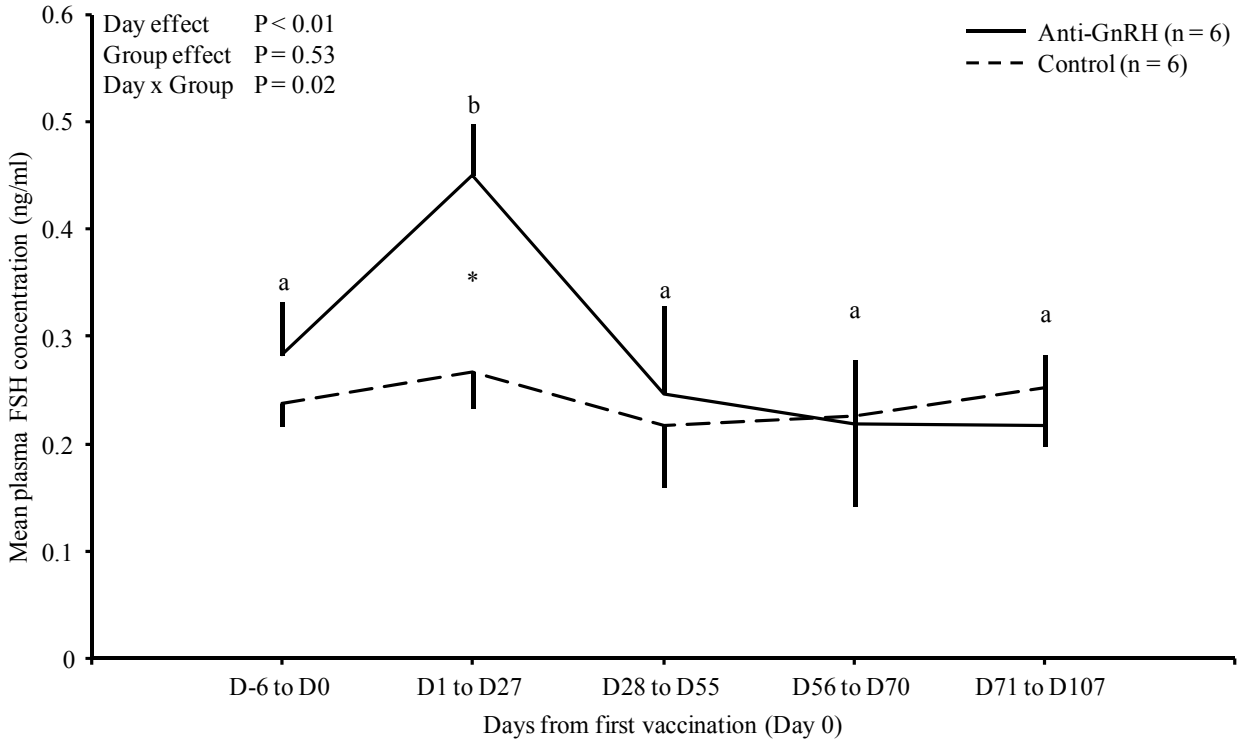

Figure 2.3 Plasma FSH concentrations (mean ± SEM) in vaccinated (n = 6) and control (n = 6) heifers at different intervals after first vaccination against GnRH. For data analysis and illustration, means were tabulated for the period i) before first vaccination (Day -6 to Day 0), ii) between two successive vaccinations (Day 1 to Day 27, and Day 28 to Day 55), iii) after 3rd vaccination and until the day of maximum titre (Day 56 to Day 70), and iv) from the day of maximum titre until the end of the study period (Day 71 to Day 107). [ab]Values with no common superscripts are different ($P \leq 0.02$); *difference in the peak concentration of FSH between the vaccinated and control groups.

The first detected peak in plasma FSH concentrations (defined as a rise in circulating concentrations of FSH for at least two consecutive days followed by a decline for at least two consecutive days) after the first vaccination was significant in the anti-GnRH group and at all periods in the control group (Figure 2.4). However, the peak in FSH after subsequent vaccinations in the anti-GnRH group was not significant (Figure 2.4).

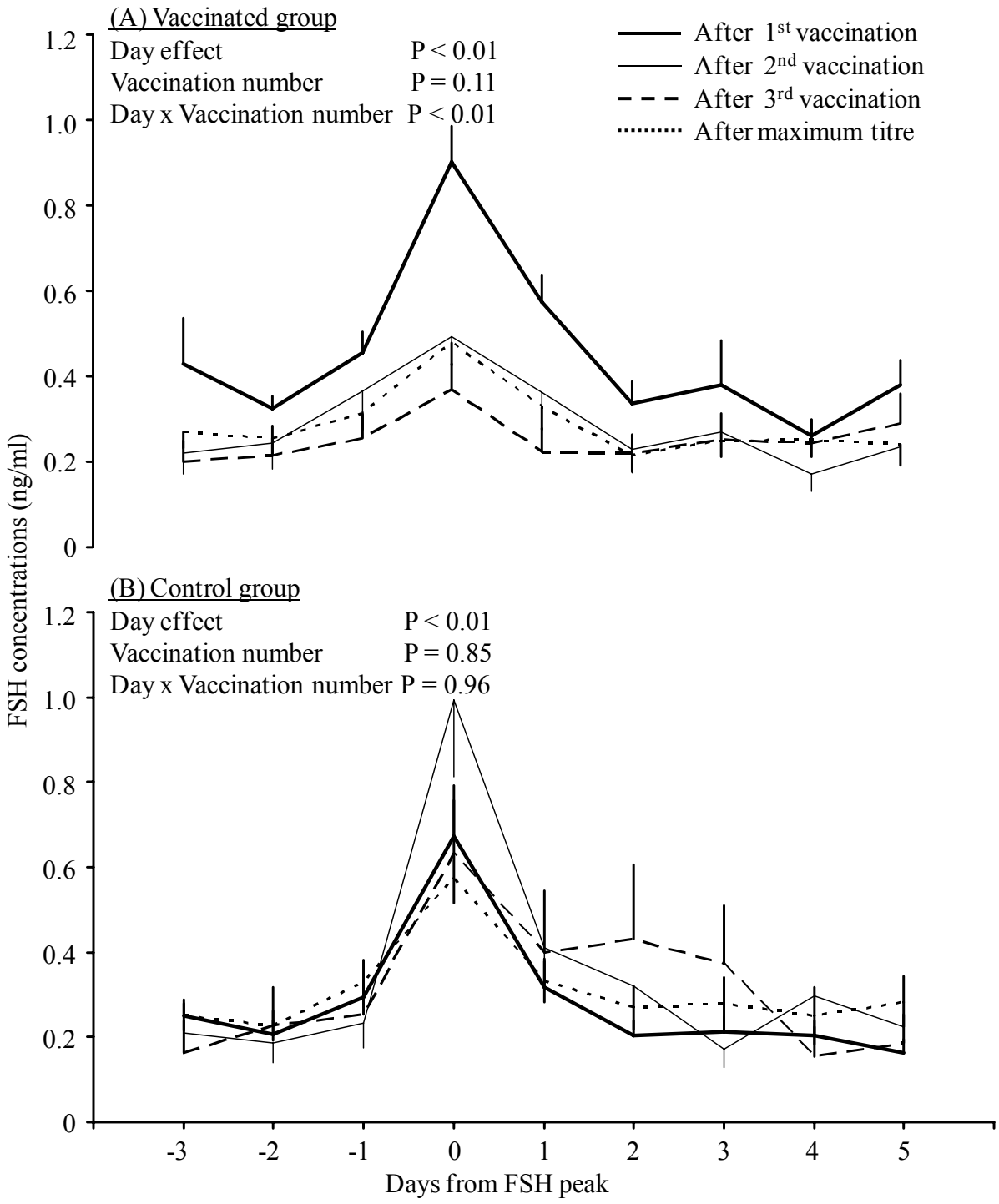

Figure 2.4 Circulating concentrations of FSH (mean ± SEM) in heifers after successive vaccinations against GnRH. For statistical and illustrative purposes, FSH data from each heifer were centralized to the day of the first peak in FSH detected after each vaccination.

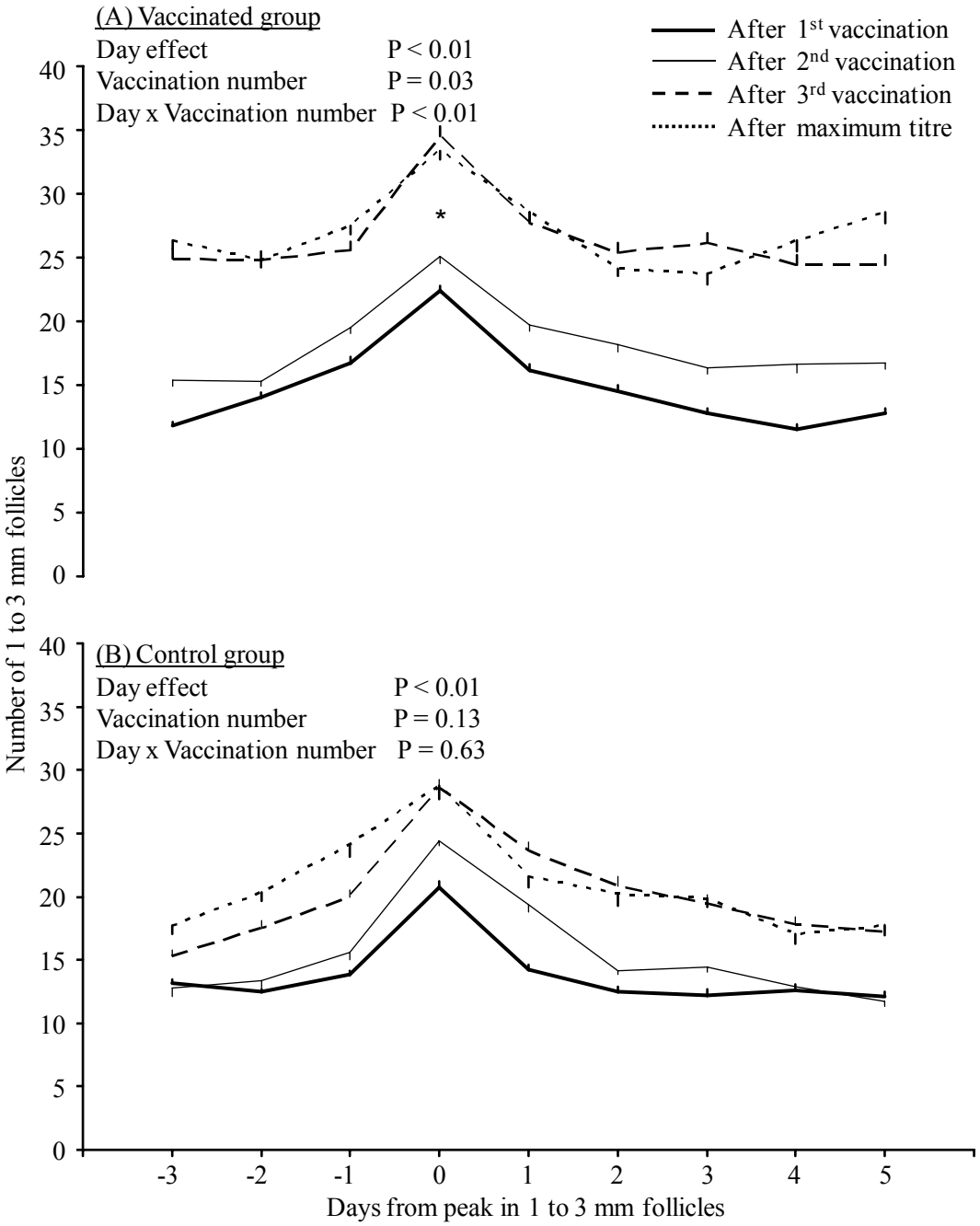

Figure 2.5 The number of 1 to 3 mm follicles (mean ± SEM) in heifers after successive vaccination against GnRH. For statistical and illustrative purposes, data from each heifer were centralized to the day of the first peak in the number of follicles detected after each vaccination (defined as a rise in follicle numbers for at least two consecutive days followed by a decline for at least two consecutive days). *difference in the peak number of follicles between vaccination numbers 1 and 2 versus 3 and 4.

Peaks in the number of 1 to 3 mm follicles were evident in both vaccinated and control groups (Day effect, P < 0.01; Figure 2.5). Though, an increase in the peaks over time was evident in both groups, the difference was significant in the vaccinated group (vaccination number effect, P = 0.03). Overall, there was a progressive increase in the number of follicles 1 to 3 mm from Days -3 to 107 (P < 0.01; Figure 2.6). This trend of increase in the number of follicles did not differ between the groups (P = 0.29). After second vaccination, the number of follicles ≥4 (Figure 2.7) decreased in the vaccinated group than in the sham-vaccinated group (P < 0.01).

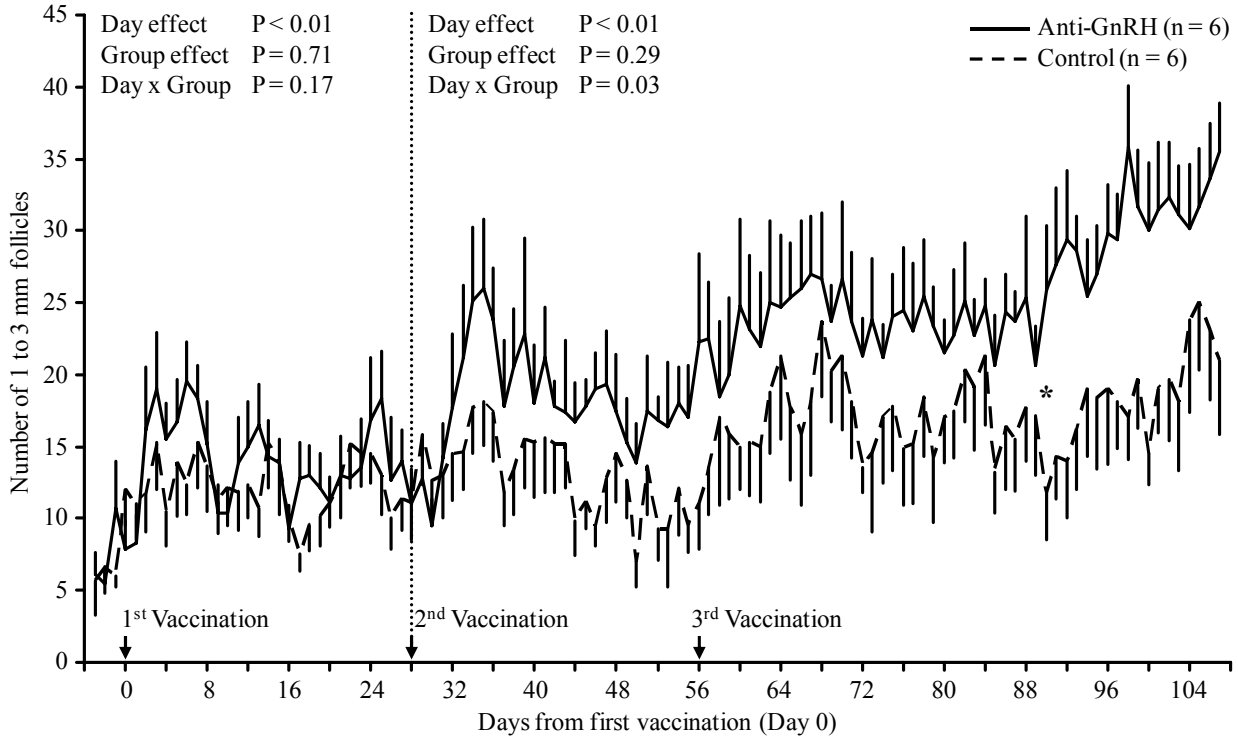

Figure 2.6 Number of follicles 1 to 3 mm in vaccinated (n = 6) and control (n = 6) heifers from Day -3 to Day 107 (Day 0 = day of first vaccination against GnRH). Data were analyzed separately for the periods from Days -3 to 27 (period of detectable wave emergence in the anti-GnRH group) and Days 28 to 107 (absence of detectable wave emergence in the anti-GnRH group).

*First day of difference in the number of follicles between vaccinated and control groups (P = 0.02).

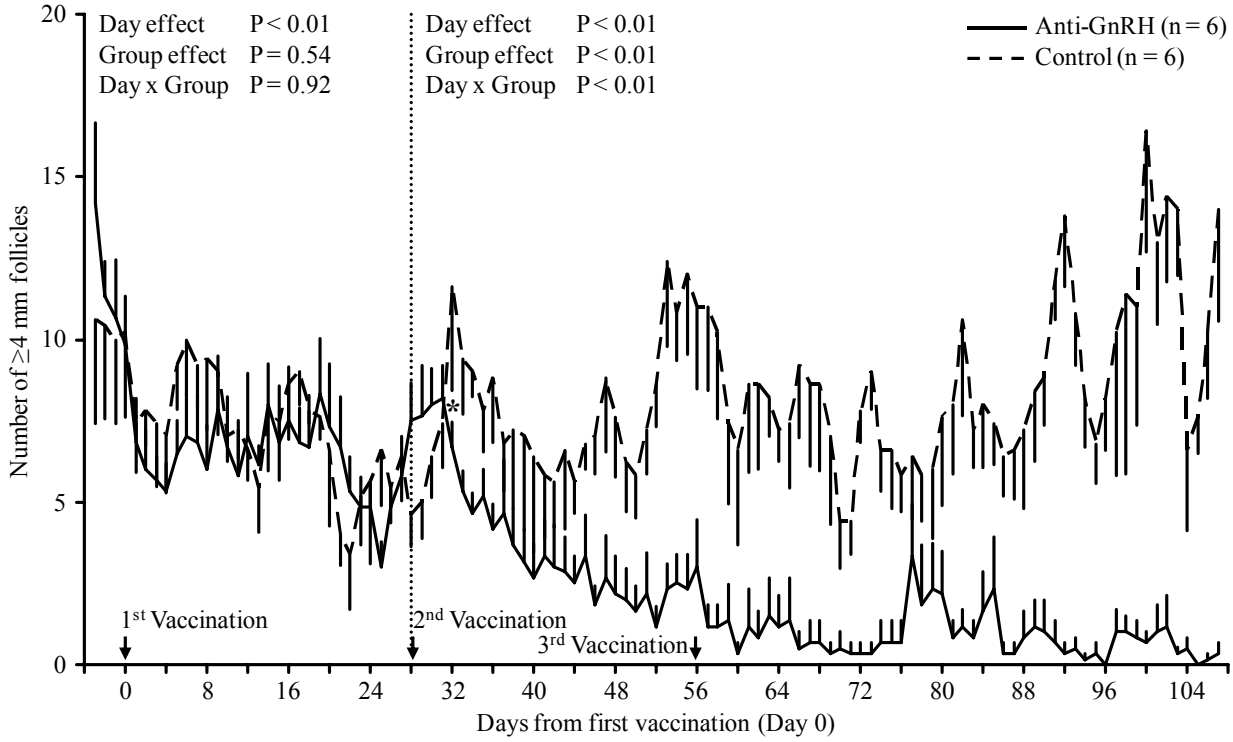

Figure 2.7 Number of follicles ≥4 mm in vaccinated (n = 6) and control (n = 6) heifers from Day -3 to Day 107 (Day 0 = day of first vaccination against GnRH). Data were centralized to the day of first vaccination (Day 0) and were analyzed separately for the periods from Days -3 to 27 (period of detectable wave emergence in the anti-GnRH group) and Days 28 to 107 (absence of detectable wave emergence in the anti-GnRH group).
*First day of difference in the number of follicles between vaccinated and control groups (P ≤ 0.03).

An inverse relationship was detected between the number of 1 to 3 mm follicles and the mean (r = -0.35, P = 0.01) and peak concentrations of FSH (r = -0.99, P = 0.04; Figure 2.8). An inverse relationship was also detected between the number of 1 to 3 mm follicles and the number of ≥4 mm follicles (r = -0.17, P = 0.04).

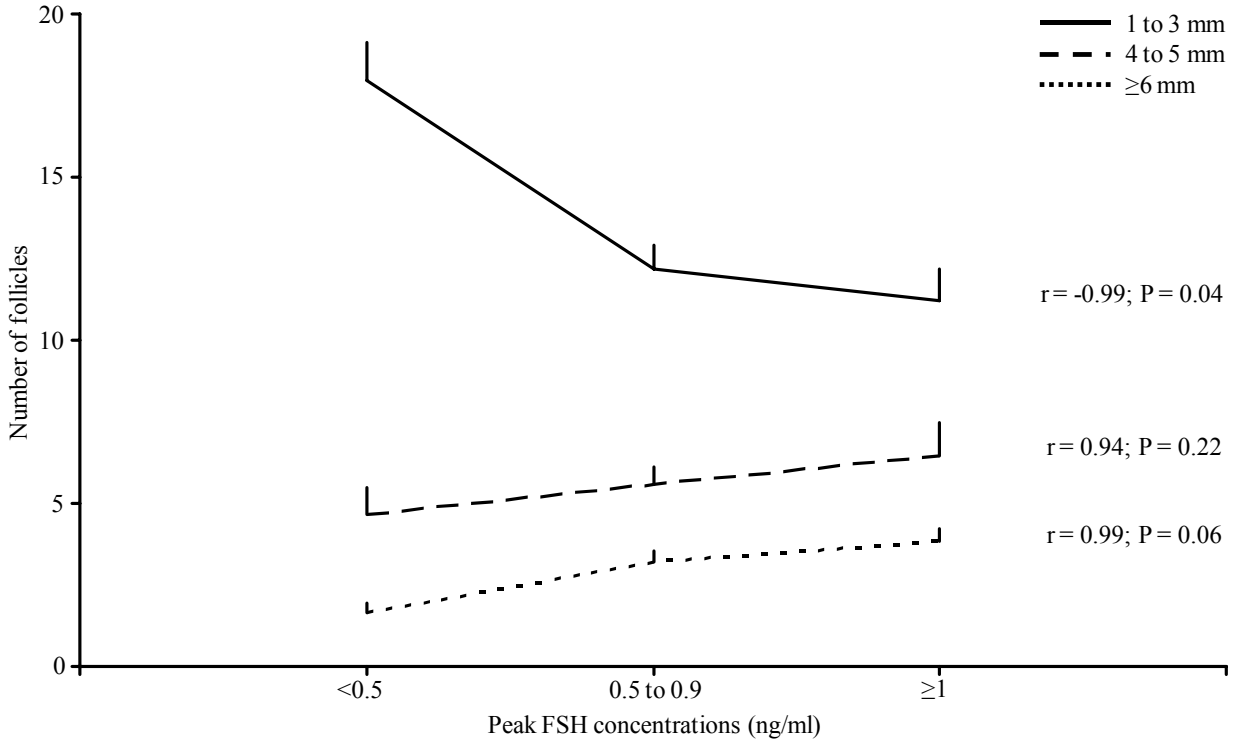

Figure 2.8 Changes in the number of follicles in different size categories in relation with the peak in circulating concentrations of FSH. Data of vaccinated (n = 12) and control (n = 12) heifers were combined for analysis and illustration.

2.5 Discussion

The secretion of LH is controlled by GnRH, which is therefore called as the LHRH hormone (McCann et al., 1998). In contrast, the secretion of FSH is regulated by the FSH-releasing factor in species like sheep (Dhariwal et al., 1965; 1967; McCann et al., 1983), pigs (Vale et al., 1967) and rats (Dees et al., 1985; Lumpkin and McCann, 1984; Yu et al., 1997). The differential regulation of gonadotropins is evident from an inverse relation between increase in anti-GnRH titres and mean and basal concentrations of LH but not that of FSH. The peaks in FSH were attenuated but not suppressed in response to anti-GnRH vaccinations.

Vaccination against GnRH was effective in suppressing the LH pulse amplitude and frequency but not its mean and basal concentrations. Within an increase in anti-GnRH titres, the

peaks in the concentrations of FSH were attenuated but not ablated. The lack of complete suppression of FSH confounded the outcome of the study. Mean concentrations of FSH increased following first vaccination and then returned to the levels similar to the control group. In the vaccinated group, the rise in FSH concentrations after first vaccination may have occurred due to a decrease in the population of large follicles and their FSH-suppressive products such as estradiol and inhibin (Adams et al., 1992a; 1993b; Gibbons et al., 1997). Alternatively, the suppression of GnRH pulsatility and associated pulsatility of LH (Desaulniers et al., 1995) may have resulted in lowered estradiol synthesis due to lowered availability of aromatizable substrate for the synthesis of estradiol (Lucy et al., 1992). The finding confirms earlier reports that the secretion of LH and not FSH is tightly regulated by GnRH (reviewed in McCann et al., 1998). The interovulatory interval after the first vaccination was of the normal duration. However, the waves of follicles ≥4 mm ceased to occur shortly after the second vaccination. There were distinct peaks of 1 to 3 mm follicles but these follicles failed to grow beyond 4 mm after the second vaccination. Peaks in the number of 1 to 3 mm follicles were negatively correlated with the mean as well as peak concentrations of FSH. This finding is in agreement with previous studies (Singh et al., 2004; Burns et al., 2005; Malhi et al., 2005) which reported a high concentration of FSH in heifers that had low numbers of ultrasonographically visible follicles. Peaks in the number of 1 to 3 mm follicles were inversely correlated with the number of follicles ≥4 mm present during the time of peak.

The inverse relationship between the circulating concentrations of FSH and the number of follicles 1 to 3 mm can be explained from two hypotheses proposed in previous studies: i) a minimal suppression of FSH consequent to a low number of follicles permits high circulating concentrations of FSH and vice versa (Singh et al., 2004); ii) low levels of FSH prevent desensitization of FSH receptors on the small preantral follicles and permits their steady growth (Gougeon, 1996). An *in vitro* study on mouse follicles revealed that though multi-layered secondary follicles are dependent on FSH for their growth, follicle survival was compromised with greater doses of FSH (Kreeger et al., 2005). An inverse relationship between peaks in the number of 1 to 3 mm follicles and the number of follicles ≥4 mm present at that time reveals about the existence of some paracrine regulation from large antral follicles on the recruitment of small follicles. A previous study (Peters et al., 1973) found such relationship between the antral and primordial follicles.

The present study was conducted over a span of about 5 months comprising of mid-Fall and peak Winter seasons. There was a slight decline in the mean and peak concentrations of FSH towards the winter months (Figures 2.3 and 2.4) with a concurrent rise in the mean and peak numbers of 1 to 3 mm follicles (Figures 2.5 and 2.6). The information on seasonal variation in hormonal levels is limited in cattle (Harrison et al, 1982; Critser et al., 1983; Day et al., 1986; Stumpf et al., 1988; Kinder et al., 1997). In cattle, the circulating concentration of LH is highest during winter months (Critser et al., 1983; Kinder et al., 1997). The decline in FSH concentration (ergo, increase in the number of follicles - Singh et al., 2004; Burns et al., 2005; Malhi et al., 2005) may be due to an increase in the estradiol concentration following a season related increase in LH concentration. LH makes aromatizable substrate available for the production of estradiol by the granulosa cells of developing follicles (Lucy et al., 1992).

Vaccination against GnRH was effective in suppressing LH rather than FSH concentrations. This may be due to: i) a differential regulatory factor for FSH in cattle as reported earlier for sheep (Dhariwal et al., 1965; 1967; McCann et al., 1983), pigs (Vale et al., 1967) and rats (Dees et al., 1985; Lumpkin and McCann, 1984; Yu et al., 1997); ii) separate regions in the hypothalamus for the controlled release of LH (Samson et al., 1980) and FSH (Mizunuma et al., 1983) as was confirmed by the use of several drugs (Dees et al., 1985; Wenger et al., 1987) and surgical manipulations (McCann, 1982; Kalra et al., 1971; Ojeda et al., 1977, Bishop et al., 1972; Ojeda et al., 1977). Alternatively, there may be a possibility of differential regulation of LH and FSH by the pulsatile secretion of GnRH. High pulsatile secretion of GnRH stimulates the secretion of LH more than that of FSH (McCann et al., 1998). On the contrary, the high pulsatility of GnRH may have an adverse effect on the secretion of FSH. At higher GnRH pulsatility, follistatin is expressed in gonadotrophs and controls the stimulatory effects of activin on the expression of FSHβ gene that in turn results in the low expression of FSHβ gene and vice versa (Counis et al., 2005).

The attenuation of FSH surges may have occurred due to the suppressed secretion of FSH from the bihormonal gonadotrophs. The gonadotrophs in the pituitary are monohormonal and bihormonal (Tougard and Tixier, 1994). The LH-releasing hormone (LHRH) which is also called GnRH (McCann et al., 1998) may be involved in the secretion of both FSH and LH from bihormonal gonadotrophs (Yu et al., 1997) as well as LH release from LH-producing monohormonal gonadotrophs (Lumpkin et al., 1987). In contrast, the FSH-releasing factor

(FSHRF), which is specific for FSH release and acts on the gonadotrophs in a dose-related manner, may be involved in the secretion of FSH from the FSH-producing monohormonal gonadotrophs (Yu et al., 1997). The ratio of monohormonal and bihormonal gonadotrophs as well as LH and FSH monohormonal gonadotrophs needs to be determined to understand the differential regulation of LH and FSH secretion.

The continuous recruitment of follicles in the vaccinated group is indicative of a functional intactness of follicles being exposed to suppressed concentrations of FSH. Suppression of gonadotropins using GnRH agonist suppresses the development of follicles but the expression of FSHr on follicles remains unaffected (Hampton et al., 2004). The threshold concentrations of FSH required to recruit follicles of different size categories are specific to the individual subject (human data, Brown, 1978), follicle size category (ewe data, Picton and McNeilly, 1991), and individual follicle (ewe data, Fry and Driancourt, 1996). A previous study (Gong et al., 1996) monitored follicular dynamics in cattle following suppression of gonadotropins using GnRH agonist. The reduction in the developmental size of the follicles was found to be dependent on FSH concentrations. After suppression of FSH, follicles developed up to 3 mm in size but not beyond, therefore, the study concluded that follicles ≤3 mm are not dependent on FSH for their support. However, close monitoring of the data in that study revealed that the concentrations of FSH were decreased but not completely suppressed and normalization of data to a reference point, e.g., peak in FSH or number of follicles was not employed.

In conclusion, vaccination against GnRH attenuated FSH surges but did not suppress basal FSH concentrations. Follicles 1 to 3 mm continued to be recruited in response to the attenuated surges of FSH. The study provides rationale for the hypothesis that the follicles <1mm develop in a temporal relationship with FSH. Extensive experimentation is suggested to determine the relationship between circulating concentrations of FSH and the number of follicles recruited at wave emergence. This relationship may be used as a marker for the selection of high-fecundity herd.

Acknowledgements: Authors are thankful to Biostar Inc., Saskatoon, Saskatchewan, Canada for supplying the GnRH antigen, Ms. Charlotte Hampton of (Dr. Bernard Laarveld's lab, Department of Animal and Poultry Science) for conducting the anti-GnRH assay and Ms. Susan Cook for her assistance with radio-immunoassays.

Chapter 3

EFFECT OF FSH ON FOLLICULAR RECUITMENT IN HEIFERS ACTIVELY IMMUNIZED AGAINST GnRH

R. S. Jaiswal, J. Singh, R. J. Mapletoft and G. P. Adams

3.1 Abstract

To test the hypothesis that growth of follicles <1 mm is dependent on circulating FSH, heifers actively immunized against GnRH were treated with exogenous FSH and the profile of follicles ≥1 mm was compared with controls. Heifers (n=8) were vaccinated against GnRH three times at 28-day intervals. Between 80 and 107 days after the first vaccination, heifers (n=8 vaccinated; n=8 controls) were treated with a luteolytic dose of prostaglandin followed by follicular ablation to induce a new follicular wave. Beginning 24 h post-ablation, heifers were treated with 200 mg NIH-FSH-P1 divided bid over five treatments at 12 h intervals. Mean basal FSH concentrations were lower in the vaccinated than the control group (P=0.03), primarily as a result of the absence of a post-ablation FSH surge. Although, no follicles ≥4 mm were detected before FSH treatment (P<0.01) in the vaccinated group, the number of follicles ≥4 mm did not differ between groups 48 h after FSH treatment (P=0.23). The proportion of follicles 1 to 3 mm that grew to ≥4 mm in response to FSH treatment was lower in the vaccinated than in the control group (56% versus 82%; P=0.05). The diameters of the three largest follicles were smaller (P<0.01) in vaccinated group, however, their pooled growth rate did not differ between groups. In summary, the anti-GnRH vaccine did not suppress FSH concentrations beyond low-basal levels. Although, follicles in the vaccinated group did not grow beyond 3 mm, their growth rate in response to exogenous FSH was similar to controls.

3.2 Introduction

The role of follicle stimulating hormone (FSH) in regulating the wave-like development of follicles ≥4 mm (Adams et al., 1992b; Sunderland et al., 1994) or ≥1 mm (Jaiswal et al., 2004) has been clearly defined. However, the developmental dynamics and the role of FSH in early folliculogenesis have been debated. Although, receptors for FSH are expressed on granulosa cells of follicles immediately after their activation from the resting primordial stage (Camp et al., 1991; Xu et al., 1995; Bao and Garverick, 1997), it has been suggested that FSH receptors may not be coupled to the adenylate cyclase-second messenger system during early stages of folliculogenesis, and hence, may be non-functional (bovine, Wandji et al., 1992; other species, Sokka and Huhtaniemi, 1990; Oktay et al., 1997). These inferences were made mainly because of the lack of noticeable effects of exogenous FSH on small follicles in vitro (Wandji et al., 1992; Sokka and Huhtaniemi, 1990) which may have been due to minimal requirement for FSH during early stages of follicular development (Govan and Black, 1975), coupled with the extremely slow rate of growth of small follicles (Scaramuzzi et al., 1980; Lussier et al., 1987). The slow rate of growth of small follicles (< 0.68 mm) has been attributed the longer time required for the number of granulosa cells in the largest cross-section through the follicle to double in number called the "doubling time" (Lussier et al., 1987; reviewed in Fortune, 1994).

Conversely, evidence of the growth-promoting effects of FSH on preantral and small antral follicles produced *in vitro* (Itoh et al., 2002) and *in vivo* (Fricke et al., 1997; Tanaka et al., 2001) in cattle suggests a role for FSH in the development of early stage preantral and antral follicles. The functionality of the FSH receptors was also demonstrated in an early study in hamsters in which DNA synthesis in preantral and small antral follicles increased in response to periovulatory changes in circulating concentrations of FSH (Roy et al., 1987), an observation that was supported by recent results from the same laboratory in which functional FSH receptors were detected in granulosa precursor cells of hamster ovaries, (Roy and Albee, 2005).

When FSH concentrations were suppressed using an anti-GnRH vaccine (Crowe et al., 1993; Prendiville et al., 1995) or a GnRH agonist (Gong et al., 1996; Garverick et al., 2002), follicles grew to 3 mm but not beyond. The growth of follicles to a larger size resumed upon treatment with exogenous FSH (Crowe et al., 1993; 2001; Garverick et al., 2002). However, an *in vitro* study in hamsters (Roy and Greenwald, 1989) revealed that the response of follicles to

gonadotropins depended on their exposure to endogenous peaks in gonadotropins. The preantral follicles collected on the day of estrus were more responsive to FSH than the follicles collected on the day of proestrus.

The experiment was designed as part of a larger study to test the hypothesis that antral follicles <1 mm develop in a wave-like fashion in temporal association with the circulating concentration of FSH similar to follicles ≥1 mm. The specific objective was to determine the effect of exogenous FSH on the responsiveness of follicles developed under the prolonged suppression of FSH. It was hypothesized that the suppression of endogenous FSH followed by replacement with exogenous FSH alters the number of follicles ≥1 emerging into a detectable wave. The stated hypothesis was tested by actively immunizing heifers against GnRH and then providing exogenous FSH during the time of maximal anti-GnRH titres.

3.3 Materials and Methods

3.3.1 Animals

As part of the design of a larger study on the effects of anti-GnRH, this experiment was conducted between August and January using sexually-mature, Hereford-cross heifers (n = 34), 18 to 24 months of age and weighing 450 to 550 kg. Heifers were maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52^{o}N and 106^{o}W).

All procedures were approved by the University of Saskatchewan Protocol Review Committee, under the umbrella of the University Committee on Animal Care and Supply, and were performed in accordance with the principles outlined by the Canadian Council on Animal Care.

Heifers were treated intramuscularly at random stages of the estrous cycle with two luteolytic doses of PGF2α (500 μg Cloprostenol per dose; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) 12 h apart to induce luteal regression. Seven days later, heifers were assigned to a vaccinated group (n = 18), a sham-vaccinated group (n = 6), or an untreated control group (n = 10). Heifers in the vaccinated and sham-vaccinated groups were given three doses of anti-GnRH vaccine or a sham vaccine at 28-day intervals, whereas heifers in the control group were left untreated. For the purposes of the present study, a subset of

vaccinated heifers (n = 8), sham-vaccinated heifers (n = 3), and untreated control heifers (n = 5) was chosen randomly from the larger group.

3.3.2 Immunization against GnRH

The anti-GnRH vaccine consisted of eight copies of the GnRH peptide bound to both the carboxy and amino ends of eight copies of the 52 KD carrier protein leukotoxin, produced by *Pasturella hemolytica* (Cook et al., 2000). This immunogenic GnRH fusion protein (IPS-21) was formulated in a water-in-oil adjuvant (Montanide® ISA-70, Seppic Inc., Lyons, France), and was given subcutaneously as a 1 mL dose (200 μg antigen) at one site in the neck region (Biostar Inc., Saskatoon, Canada). The sham-vaccine consisted of 1 mL of adjuvant and was given at one site in the neck region. The formulation was expected to produce an immunological response (>20% binding at 1:5000 dilution) in all treated animals, lasting for >80 days (Cook et al., 2000). Maximal anti-GnRH titres were expected after 70 days of initial anti-GnRH vaccination (Cook et al., 2000).

3.3.3 FSH treatment & ultrasonography

Between 80 and 107 days after the first vaccination (period of maximal titres), heifers in all groups were given two luteolytic doses of PGF2α 12 h apart. One day later, transvaginal ultrasound-guided follicular ablation of all follicles ≥5 mm was done to synchronize follicular wave emergence (Bergfelt et al., 1994). As there were no follicles ≥5 mm in the vaccinated group, a sham ablation was performed which included needle puncture of the ovaries (Bergfelt et al., 1994). The day of follicular ablation was designated Day 0. Beginning on Day 1, heifers were treated intramuscularly with FSH (40 mg per treatment of NIH-FSH-P1 every 12 hours for five treatments; Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada).

Daily transrectal ultrasonography, using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan), was initiated 2 days before follicular ablation to monitor the dynamics of ovarian follicles 4 to 5 mm (Knopf et al., 1989). In addition, the ovaries of a subset of heifers (n = 3 vaccinated; n = 3 sham-vaccinated) were

ultrasonographically examined more critically to characterize the development of follicles ≥1 mm (Jaiswal et al., 2004).

3.3.4 Blood sampling and radioimmunoassay for anti-GnRH titers

Blood samples were collected from the jugular vein into non-heparinized tubes (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) starting with a pre-immune sample on the day of the first vaccination, and at 14-day intervals after the first vaccination until the end of study. Serum anti-GnRH antibody binding activity was estimated by a modified radioimmunoassay procedure (Meloen et al., 1994). Briefly, synthetic GnRH (Gonadorelin, Catalogue# H4005, Bachem California Inc., Torrance, CA) was iodinated with ^{125}I (Amersham, Oakville, ON, Canada) by the chloramine-T method (Greenwood and Hunter, 1962). A standard amount (12,000 cpm; approximately 1 pg) of ^{125}I-labeled GnRH was added to serial dilutions of serum, providing a final incubation volume of 0.7 mL. Following incubation for 24 h at 2 to 6^{0}C, a suspension of 0.5% charcoal and assay buffer was added to adsorb the non-antibody-bound ^{125}I-GnRH. The tubes were centrifuged at 2000 X g using rotor JR-3.2 (Beckman, Mississauga, ON) and counted on a 10-well Micromedic Apex^{TM} ICN gamma counter (TitreTek, Huntsville, Alabama). Data were expressed as the percentage of a standard dose of ^{125}I-GnRH bound to an antibody at specified serum dilutions. The samples were analyzed in duplicate at serum dilutions of 1:100 and 1:5000. Samples with <10% binding were considered negative for anti-GnRH antibody. Nonspecific binding at 1:5000 was 4.7% based on sera from pre-immunized and sham-vaccinated heifers. The intra- and inter-assay coefficients of variation for the samples analyzed at a dilution of 1:5000 were 7.1 and 11.0%, respectively.

3.3.5 Blood sampling and radioimmunoassay for FSH

Jugular blood samples were collected once daily in heparinized tubes (10 mL; Becton Dickinson Vacutainer Systems, Franklin Lakes NJ, USA) and centrifuged for 15 minutes at 1500 X g within 60 min of collection. Blood plasma was aspirated and stored at –20^{o}C until measurement of FSH using a double antibody radioimmunoassay (Rawlings et al., 1984). The primary antibody was NIDDK-anti-ovine FSH, and concentrations were determined using

standards prepared from USDA-bovine FSH-I-1. The minimum detectable limit of the assay was 0.13 ng/mL, and the range of the standard curve was 0.13 to 16.0 ng/mL. The intra- and inter-assay coefficients of variation were 4% and 3% for the low reference sample (mean 1.28 ng/mL), and 7 and 3% for the high reference sample (mean 3.09 ng/mL), respectively.

3.3.6 Blood sampling and radioimmunoassay for LH

LH characteristics (basal and mean concentrations, pulse frequency and amplitude) were determined using frequent blood sampling for the purposes of the larger study (refer section 3.1). Briefly, frequent sampling sessions were done 24, 53, 82 and 109 days after initial vaccination from a randomly selected set of heifers in the vaccinated and control groups (n = 6/group). Blood samples (5 mL) were collected at 15 min intervals for 8 h via indwelling catheters (vinyl tubing; 1.0 mm inner diameter X 1.5 mm outer diameter; Product code SV70; Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia) that were inserted into the jugular vein on the day before frequent sampling (Evans et al., 1994). Catheters were filled with heparinized saline between samplings (1000 U.S.P. units of heparin sodium per liter of saline; Hepalean, Organon Teknika Inc., Toronto, ON, Canada).

Plasma LH concentrations were measured using a double antibody radioimmunoassay and were expressed as NIDDK-bLH4 units (Evans et al., 1994; Honaramooz et al., 1998). The range of the standard curve was 0.06 to 8 ng/mL. All samples were analyzed in a single assay; the intra-assay coefficients of variation were 8 and 2% for the low (mean, 0.45 ng/mL) and high (mean, 0.96 ng/mL) reference samples, respectively. Mean and basal concentrations and LH pulse frequency and amplitude were calculated using Pulsar software (Gitzen and Ramirez, University of Illinois, IL) and illustrated (Evans et al., 1994; Honaramooz et al., 1998).

3.3.7 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System software package (SAS Learning Edition 2.0; SAS Institute Inc., Cary, NC). For statistical analysis and preparation of figures, individual follicle and hormone data were centralized to the day of ablation (Day 0) and expressed as mean ± SEM. Circulating concentrations of FSH and the profile of follicles ≥4

to 5 mm were compared between sham-vaccinated and untreated control heifers using student's t-test. No differences between the sham-vaccinated and untreated control heifers were detected in circulating concentrations of FSH ($P = 0.31$) or in the number profile of follicles ≥4 mm ($P = 0.66$); hence, data were combined and the group was referred as the control group.

Analysis of variance for repeated measures using the mixed procedure (Littell et al., 2000) was used to determine the main effects of day and vaccination and their interaction on: i) anti-GnRH titers, ii) plasma LH concentrations, iii) plasma FSH concentrations, iv) follicle numbers in different size categories, v) diameter profiles of the three largest follicles (Days 1 to 3), and vi) the pooled growth rate of three largest follicles (Days 1 to 3). If main effects or their interactions were statistically significant at $P < 0.05$, multiple comparisons were made using Tukey's post-hoc test.

The difference in the number of 1 to 3 mm follicles on the day before ablation (Day -1) and follicles ≥4 mm on the last day (Day 3) of treatment with FSH was used to determine the proportion of FSH responsive follicles, which were compared among groups using chi-square analysis. The association between anti-GnRH titres and LH profiles (basal and mean concentrations, pulse frequency and amplitude) was estimated using Pearson's correlation analysis.

3.4 Results

Serum anti-GnRH titers (mean ± SEM) increased sharply after the second vaccination. Titres reached 54 ± 5% binding at a serum dilution of 1:5000 by 42 days. Heifers developed maximum titers of 69 ± 3% binding 71 days after the first vaccination. No tissue reactions at the site of injection were detected in any of the heifers.

Mean and basal plasma LH concentrations were not different between groups ($P \geq 0.09$), but LH pulse frequency (number of pulses/8 hour) and amplitude were lower in the vaccinated group than in the control group ($P \leq 0.01$). Higher plasma FSH concentrations in the anti-GnRH group before and after the day of follicular ablation resulted in a significant day-by-group interaction (Figure 3.1). Plasma FSH concentrations increased subsequent to FSH treatment in both groups ($P < 0.01$; Figure 3.1).

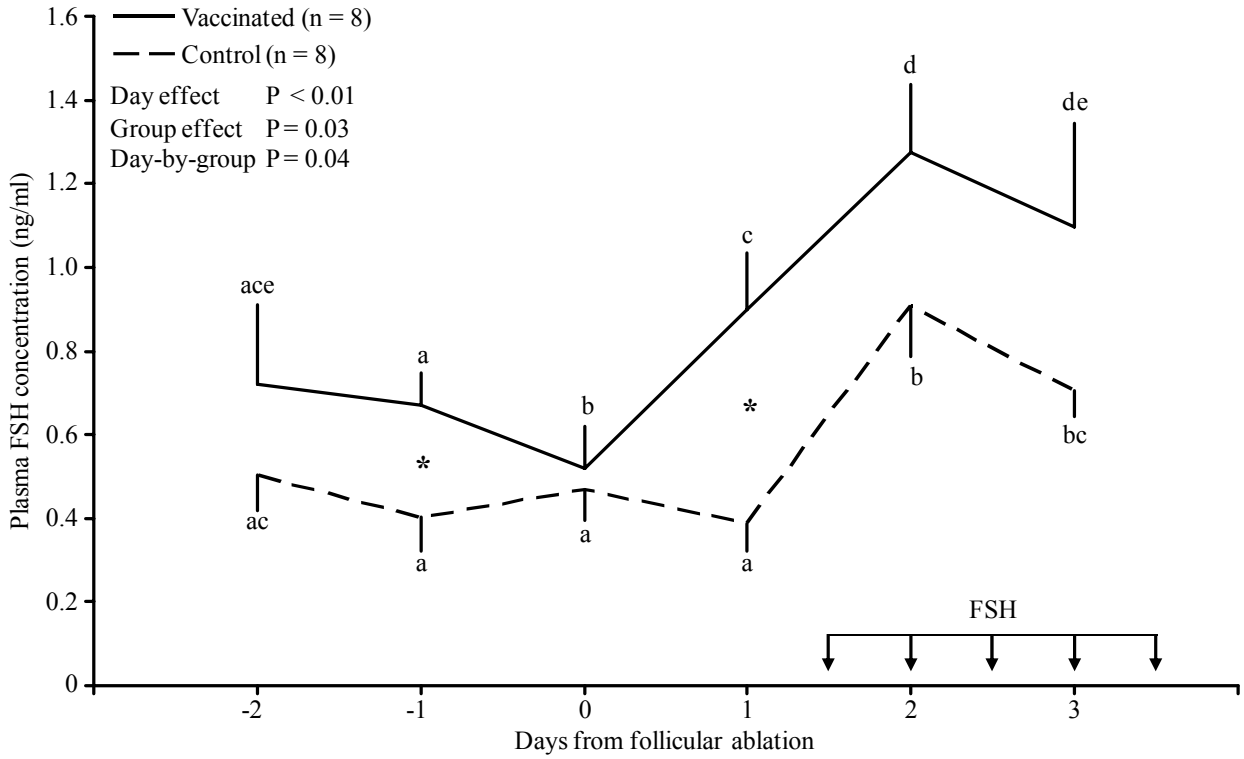

Figure 3.1 Plasma FSH concentrations in heifers immunized against GnRH and in control heifers before and after follicular ablation and treatment with exogenous FSH. [abcde] Within groups, values with no common superscripts were different ($P < 0.05$); *Values differed between the vaccinated and control groups ($P \leq 0.02$).

The number of 1 to 3 mm follicles tended to be higher in the vaccinated group before FSH treatment, but decreased after treatment began (Figure 3.2). Fewer 4 to 5 mm follicles were detected in the vaccinated group than in the control group ($P = 0.02$; Figure 3.2) before the start of FSH treatment, but follicle numbers were similar between groups ($P = 0.63$) within 1 day after the start of treatment.. The proportion of follicles that developed from the 1 to 3 mm category to ≥4 mm category was lower in the vaccinated group (56%; 18.33 ± 3.84 out of 32.33 ± 3.38) than the control (82%; 18.0 ± 2.52 out of 21.67 ± 6.38) group ($P = 0.05$).

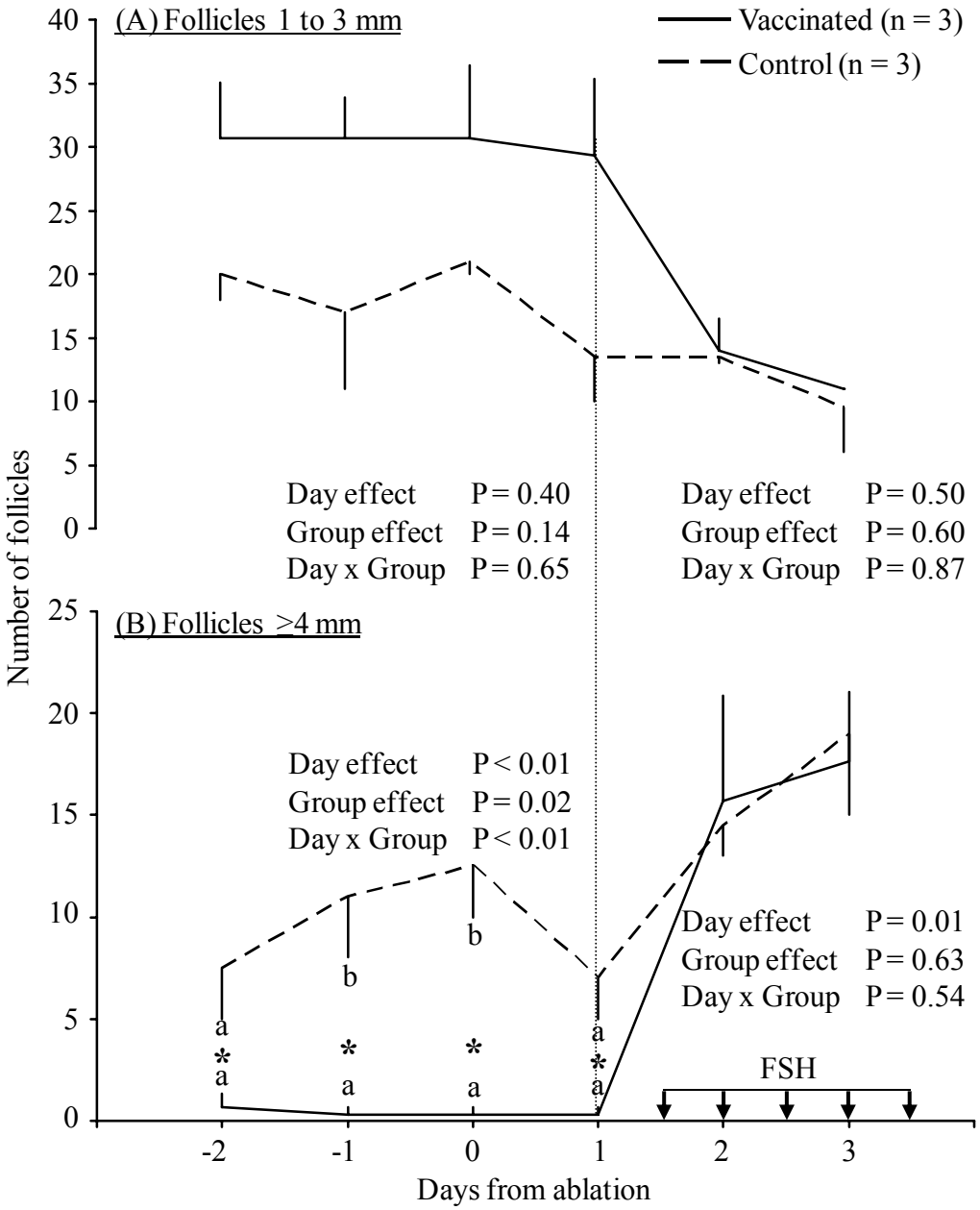

Figure 3.2 Number of 1 to 3 mm follicles (A) and ≥4 mm follicles (B) in anti-GnRH-vaccinated and control heifers before and after treatment with exogenous FSH on Days 1 to 3 (Day 0 = day of follicular ablation; Day 1.5 = day of first FSH treatment). [abcd]Within group values with no common superscript were different ($P < 0.05$); *Values were different between vaccinated and control groups ($P < 0.05$).

On the final day of treatment with exogenous FSH (Day 3), diameters of the largest, second largest, and the third largest follicles were smaller in the vaccinated group than in the control group ($P < 0.01$; Figure 3.3). The diameter profiles of the three largest follicles differed from one another in the control group ($P < 0.01$), however, in the vaccinated group, the diameter profile of first and the third largest follicles tended to differ ($P = 0.09$). No difference was detected in the pooled growth rate of three largest follicles between vaccinated and control groups ($P = 0.92$; Figure 3.4).

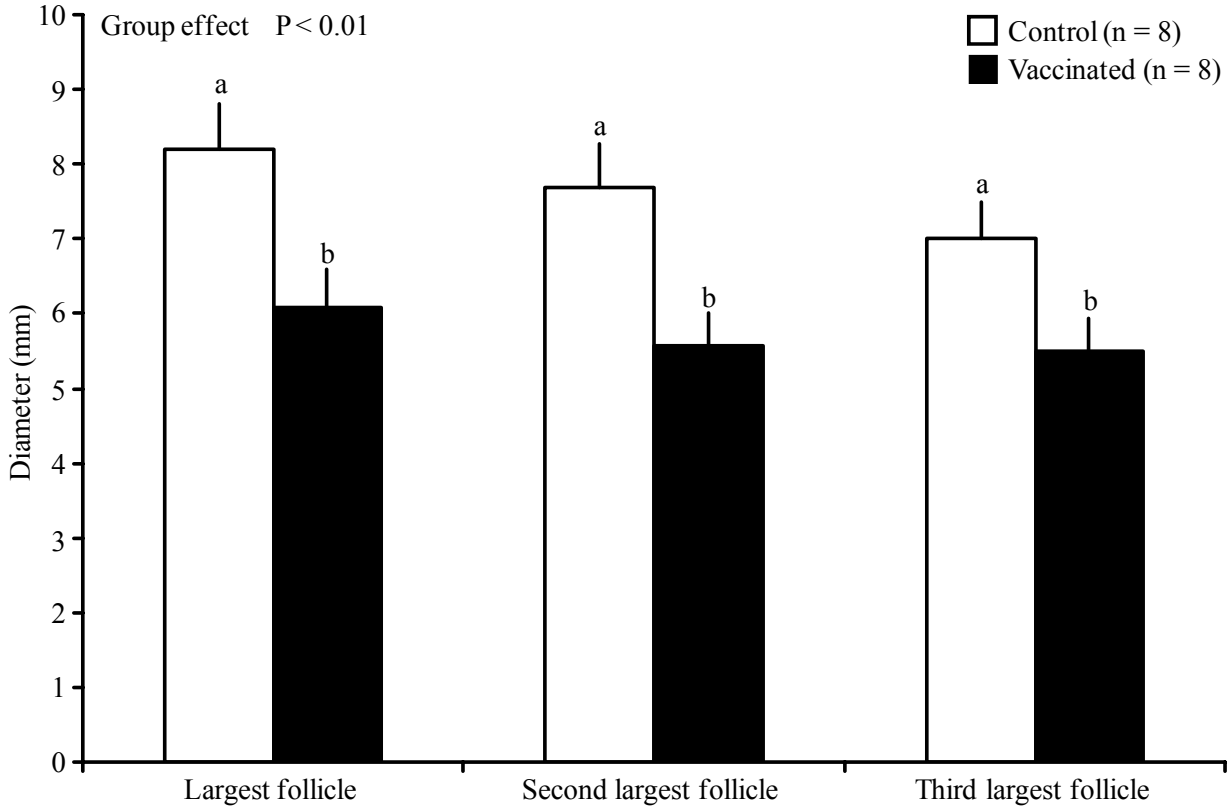

Figure 3.3 Diameter of three largest follicles (largest, second largest, and third largest) on Day 3 (last day of FSH treatment) in anti-GnRH-vaccinated (n = 8) and control (n = 8) groups after treatment with exogenous FSH on Days 1 to 3 (Day 0 = day of follicular ablation; Day 1.5 = day of first FSH treatment). [ab]Within group values with no common superscripts were different ($P < 0.01$)

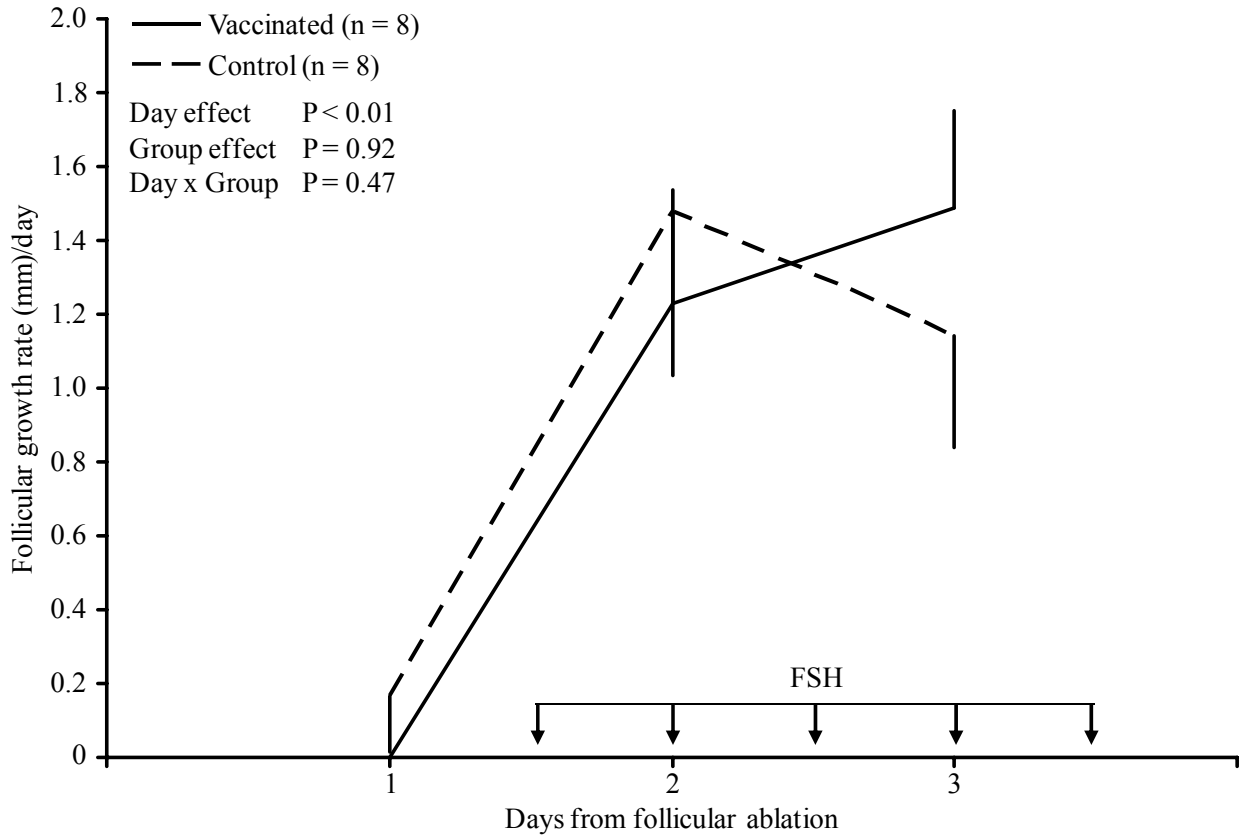

Figure 3.4 Pooled growth rate of the three largest follicles (largest, second largest, and third largest) in anti-GnRH-vaccinated and control groups after treatment with exogenous FSH on Days 1 to 3 (Day 0 = day of follicular ablation; Day 1.5 = day of first FSH treatment).

3.5 Discussion

This study demonstrated the effect of exogenous FSH on the growth of follicles that developed under GnRH antibody-induced suppressed circulating concentrations of FSH. The immunization against GnRH suppressed circulating concentrations of FSH to levels which permitted the growth of follicles up to 3 mm but not beyond. There were no follicles 4 to 5 mm in the vaccinated group before the start of FSH treatment; however, their number equaled that of the control group within 48 h of FSH treatment. Although, the maximum diameter of the three largest follicles remained smaller in the vaccinated group compared to the control group, the growth rate of three largest follicles did not differ between groups. The lack of complete suppression of circulating concentrations of FSH confounded the outcome of study, however, the

study revealed that follicles developed under the lower basal concentrations of FSH were as responsive to exogenous FSH as the follicles that were developed under the physiological concentrations of FSH. The study is in agreement with a recent finding which indicated that suppression of gonadotropins does not affect the expression of FSH-receptors on follicles (Hampton et al., 2004).

The anti-GnRH vaccine used in the present study was formulated differently than the vaccines used in previous studies (Prendiville et al., 1995; Crowe et al., 2001). A study have shown that the formulation is more immunogenic (Cook et al, 2000) than those used in previous studies in which GnRH was conjugated to human serum albumin along with diethylaminoethyl-dextran adjuvant (Prendiville et al., 1995, Crowe et al., 1993; 2001) or non-ulcerative Fruend's adjuvant (Prendiville et al., 1995). The vaccine generated antibodies against GnRH in 100% of the heifers and the efficacy of the vaccine was evident from the high titres (69%) at a serum dilution of 1:5000 than in previous studies in which the maximum titer reached up to 60% at 1:640 dilution (Prendiville et al., 1995) or 33% at 1:2560 dilution (Crowe et al., 2001). The vaccination against GnRH was effective in suppressing LH rather than FSH concentrations. The finding supports previous studies which documented differential regulation of LH and FSH secretions. GnRH controls the secretion of LH, hence, it is also called as LH-releasing hormone (McCann et al., 1998). On the other hand, the secretion of FSH is regulated by the FSH-releasing factor in species like sheep (Dhariwal et al., 1965; 1967; McCann et al., 1983), pigs (Vale et al., 1967) and rats (Dees et al., 1985; Lumpkin and McCann, 1984; Yu et al., 1997).

The difference in the mean plasma concentrations of FSH in the vaccinated and control groups was attributed to the absence of the pre-wave surge in FSH. The pre-wave surge in circulating concentrations of FSH in the control group was in response to follicular ablation (Bergfelt et al., 1994); but no such surge in FSH occurred in the vaccinated group. It appears, therefore, that the pre-wave surge in circulating FSH is mediated by GnRH, but that active immunization against GnRH does not influence constitutive basal secretion of FSH.

In a previous study (Crowe et al., 2001), the anti-GnRH vaccinated group yielded fewer follicles ≥5 mm than in the control group in response to treatment with exogenous FSH. However, the FSH treatment was given for only 2 days and the follicles were counted 6 days after initiating FSH treatment, by which time many follicles might have undergone atresia because of the absence of gonadotropin support for 4 days. The lower amounts of estradiol

secreted by follicles in the vaccinated group than in the control group (Crowe et al., 2001), is indicative of atresia in follicles (Badinga et al., 1992; Price et al., 1995; Stewart et al., 1996).

Following treatment with exogenous FSH, the diameters of the three largest follicles in vaccinated group remained smaller than in the control group, which may be attributed to the smaller diameters of follicles in the vaccinated group at the start of the FSH treatment. Additionally, the circulating concentrations of FSH remained lower in the vaccinated group than in the control group, in which the concentration of endogenous FSH rose in response to follicular ablation (Bergfelt et al., 1994). However, in spite of a 24 h delay in the rise of FSH in the vaccinated group, number of follicles that developed to the ≥4 mm size category and the growth rates of the three largest follicles did not differ between vaccinated and the control groups. This suggests that follicles developed under the suppressed FSH milieu do not lose their ability to respond to exogenous FSH.

In cattle, the growth of follicles 1 to 3 mm to ≥4 mm occurs in response to a surge in the concentrations of FSH (Adams et al., 1992b) and the recruitment of follicles in the ≥4 size category in heifers have been reported to be FSH dose-dependent (Crowe et al., 1993; Crowe et al., 2001). Conversely, the suppression of different sizes of follicles may be dependent on the magnitude of the suppression of FSH, which varies among individuals (human data, Brown, 1978; ewe data, Picton and McNeilly, 1991), follicle size categories (ewe data, Picton and McNeilly, 1991), and individual follicles (ewe data, Fry and Driancourt, 1996). Addionally, the circulating concentrations of FSH act differently at different stages of follicular growth. Low levels of FSH prevented desensitization of FSH receptors in small preantral follicles and permitted their steady growth (Gougeon, 1996). Data analyses indicated that the circulating concentrations of FSH in the vaccinated group were sufficient to sustain the growth of follicles up to 3 mm but not beyond. In a previous study (Gong et al., 1996) in which FSH concentrations were suppressed with a GnRH agonist, follicles did not develop beyond 3 mm. Based on this finding, the authors concluded that follicles ≤3 mm are not dependent on FSH for their support. However, in this study (Gong et al., 1996), concentrations of FSH were not completely suppressed and the normalization of the data to a known reference point, e.g., a peak in the number of 1 to 3mm follicles, was not used. However, a recent study (Chapter 4) in which heifers were actively immunized against GnRH and the normalization of data to the peaks in the number of 1 to 3 mm follicles was employed, revealed a distinct wave-like developmental pattern of

follicles 1 to 3 mm under the attenuated surges of FSH concentrations. A previous study by the same authors (Jaiswal et al., 2004) also revealed a wave-like development of follicles 1 to 3 mm in response to endogenous rise in FSH. Hence, the hypothesis that follicles ≤3 mm are independent on FSH support (Driancourt, 2001; Gong et al., 1996) was not supported.

The development of follicles ≥4 mm (Adams et al., 1992b) or ≥1 mm in response to surges in FSH imparts a wave-like developmental pattern. In comparison to follicles <1 mm, the wave-pattern in follicles ≥1 mm may be evident due to higher granulose cell mitotic index and a decrease in mitotic time (duration required to complete one mitotic cycle) in latter (Lussier et al., 1987) consequent to the increase in estradiol production (reviewed in Lussier et al., 1987). Although, the mitotic index and mitotic time were similar in follicles categorized in the present study, i.e., 1 to 3 mm and ≥4 mm, the doubling-time (time required to double the number of granulosa cells in a follicle) was higher in latter (Lussier et al., 1987). Hence, it may be possible that the growth (expansion) of follicles from 1 to 3 mm to ≥4 not only requires a threshold concentration of FSH to double the number of granulosa cells, but also a threshold concentration of estradiol to maintain a higher mitotic index and mitotic time.

In conclusion, circulating concentrations of FSH were not suppressed beyond basal levels by anti-GnRH vaccination. However, the study demonstrated that follicles can grow up to 3 mm despite the GnRH antibody-induced suppression of FSH surge concentrations; however, surge-like concentrations of FSH are required to mobilize these follicles into larger sized categories. The present study also revealed that follicles that develop under the GnRH antibody-induced FSH surge suppression were as responsive to exogenous FSH as follicles that develop under the normal circulating concentrations of FSH which is indicative of the dependence of small follicles on FSH just like larger follicles and supports the stated hypothesis that small follicles develop in a wave-like fashion similar to larger follicles.

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Chapter 4

DEVELOPMENTAL PATTERN OF ANTRAL FOLLICLES <1 MM IN THE BOVINE OVARY

R.S. Jaiswal, J. Singh and G.P. Adams

4.1 Abstract

The objective of the study was to characterize the developmental pattern of bovine ovarian follicles <1 mm in diameter using histological approach. Ovarian ultrasonography for monitoring follicles ≥1 mm was performed once daily from the day of ovulation until the day of slaughter. Blood plasma samples were collected daily from Day -4 or -5 (Day 0 = ovulation) until the day of slaughter (Days 0, 3 and 5) to measure circulating concentrations of FSH. Ovaries collected after slaughter on Days 0 (day of second ovulation), 3 and 5 (n=6 heifers per day) were fixed by vascular perfusion using Karnovsky's fixative. Before sectioning, each ovary was divided transverse to its long axis into four equal blocks by 3 cuts. Tissue sections (n = 11/block) of 10 μm thickness from each of the three blocks with cut surfaces of the contralateral ovary were stained with Periodic Acid Schiff reaction. Images of the sections were obtained using a Trestle Med Micro system and morphometric analysis was performed using a WCIF version of Image J for Windows. Although, a few follicles as small as 0.15 mm had an antrum, all follicles ≥ 0.31 mm had a well developed antrum. Follicles were grouped into <0.3 mm, 0.3 to 0.5 mm and 0.5 to 1 mm size categories to characterize their number profile on different days after ovulation. There was a peak in circulating concentrations of FSH one day before ovulation ($P < 0.01$). The number of follicles in different size categories differed from each other on different days after ovulation (follicle type x day effect, $P = 0.02$). The number of follicles < 0.3 mm differed among days ($P = 0.02$) primarily due to a peak on Day 3. There was no difference in the number of follicles 0.3 to 0.5mm among days ($P = 0.46$). The number of follicles 0.5 to 1 mm showed tendency for a difference among days ($P = 0.08$). Although, the trend of change in follicle number profile with respect to a surge in FSH was similar for follicles 0.5 to 1 mm (histological data) and 1 to 3 mm (ultrasonographical data), the relationship was not detected. In conclusion,

change in the number of follicles in different size categories over days is indicative a wave-like developmental pattern of follicles. The similarity in the trend of change in the number of follicles 0.5 to 1 mm and 1 to 3 mm indicates a buffering of follicles 0.5 to 1 mm for recruitment at the next wave emergence.

4.2 Introduction

With the advent of ultrasonography, the development of follicles ≥4mm (reviewed in Adams, 1999; Ireland et al., 2000) and ≥1 mm (Pierson and Ginther, 1987a; Jaiswal et al., 2004) have been characterized as wave-like, which refers to a synchronized growth of a group of follicles on specific days of an estrous cycle. Follicle stimulating hormone plays a key role in the regulation of wave-like developmental pattern of follicles ≥4 mm (Adams et al., 1992b) or ≥1 mm (Jaiswal et al., 2004). Receptors for FSH are expressed on granulosa cells of follicles immediately after their activation from the resting primordial stage (Camp et al., 1991; Xu et al., 1995; Bao and Garverick, 1997) and evidences suggest a growth-promoting effects of FSH on follicles <1 mm produced *in vitro* (Itoh et al., 2002) and *in vivo* (Fricke et al., 1997; Tanaka et al., 2001). However, the developmental pattern of follicles <1 mm relative to the periodic surges of FSH is not known.

Antral follicles <1 and ≥1 mm are similar morphologically (Lussier et al., 1987; Braw-Tal and Yossefi, 1997), and also in terms of level of expression of FSH receptors (reviewed in Bao and Garverick, 1998). The periodic emergence of waves of follicles ≥1 mm in response to periodic surges in the circulating concentrations of FSH (Jaiswal et al., 2004), and the consistency in the number of follicles ≥2 mm (Boni et al., 1997; Singh et al., 2004), or ≥3 mm (Burns et al., 2005) recruited into successive waves indicates that the follicular development may get organized into waves at an early stage of development.

The objective of the present study was to characterize the developmental dynamics of follicles <1 mm using histological approach to test the hypothesis that small antral follicles <1 mm develop in a wave-like fashion in temporal association with surges in circulating concentrations of FSH. A clear understanding of the developmental pattern of all stages of ovarian follicles and the underlying regulatory mechanisms of follicular development would be

helpful for developing new pharmaceuticals and protocols for an efficient reproductive management of farm.

4.3 Materials and Methods

4.3.1 Animals

Sexually mature Hereford-cross heifers (n = 18), 18 to 24 months of age and weighing 450 to 550 kg, were selected at random from a group of 28 reproductively sound heifers, as judged by two ultrasonographic examinations 10 days apart. The heifers had not been treated during the previous six months with anything that may be expected to influence ovarian function (e.g., growth promotants or hormones used for ovarian synchronization or superovulation). The experiment was done between January and April, and heifers were maintained in a single outdoor corral at the University of Saskatchewan Goodale Research Farm (52^{o} North and 106^{o} West) and fed alfalfa/grass hay and grain to gain approximately 1.3 Kg in weight per day.

4.3.2 Ovarian ultrasound examinations, tissue collection and processing

Daily transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SDD-900, Tokyo, Japan) was initiated on the day of ovulation to profile the dynamics of follicles ≥1 mm relative to circulating concentrations of FSH. The ovaries were collected at the time of slaughter on Day 0 (day of 2^{nd} ovulation), 3, and 5 (n=6 per group).

Ovaries were fixed by vascular perfusion (Singh and Adams, 2000) within 30 minutes of collection. Briefly, the ovarian artery was cannulated to flush out blood from ovarian tissue using 15-20 mL of phosphate buffered saline (0.1M phosphate buffer, 0.9% sodium chloride; pH 7.2 to 7.4) and then to perfuse ovarian tissue with 30-40 mL of refrigerated (4^{0}C) Karnovsky's fixative (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M phosphate buffer; pH 7.4) at a flow rate of 2 mL per minute.

To minimize the error in measuring the volume of the ovarian tissue, all follicles ≥2 mm were aspirated using a 20G needle and a 5 mL syringe, whereas follicles ≥5 mm were sliced out. The luteal tissue was carefully removed from the ovary and ovarian volume was estimated based

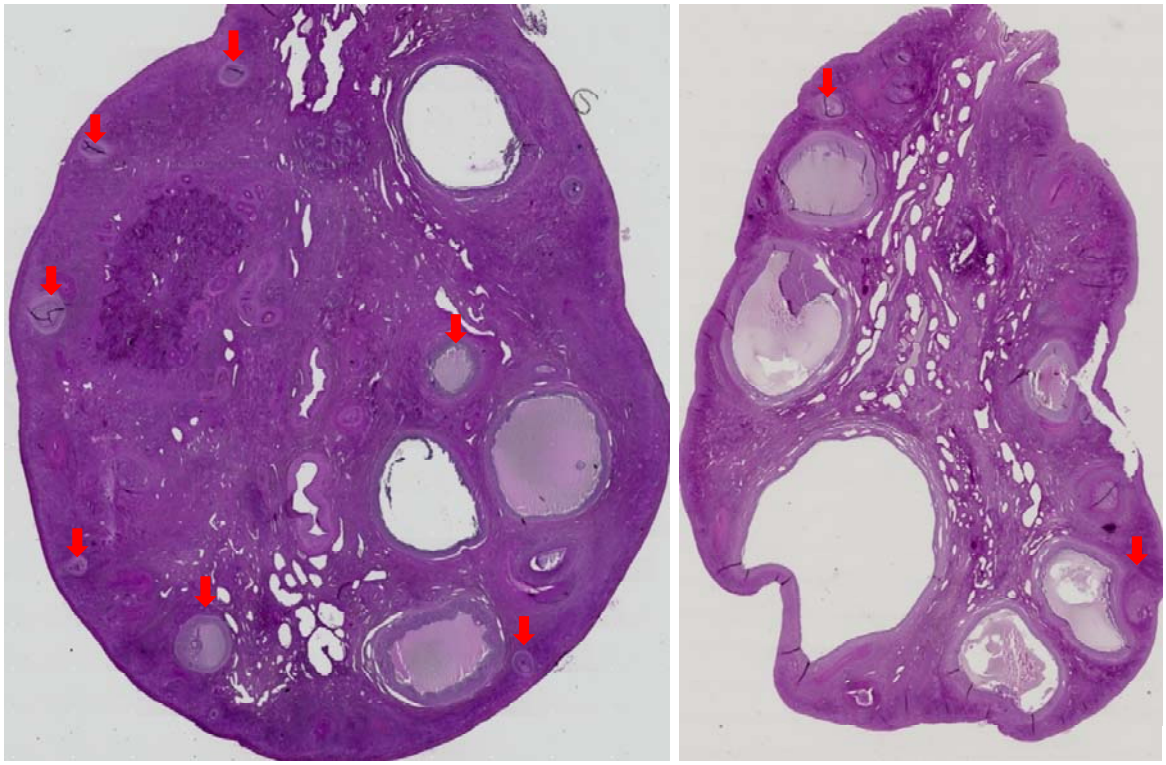
on the amount of water displaced. The fixed ovaries were transported to the lab in Karnovsky's fixative maintained at 4^0C within 3 hours of the tissue collection. At the laboratory, the ovarian pedicle and extra tissue were dissected out and the volume of the trimmed ovarian tissue was measured. Each ovary was sectioned in a transverse plane (perpendicular to both poles of the ovary) to have four equal blocks. The block containing the pole of an ovary related to the utero-ovarian (proper) ligament was referenced as block 1. Ovarian blocks were fixed for another 6 hours in aqueous Bouins fixative and then washed three times each with 50% and 70% alcohol. Ovarian tissues were processed and blocked in paraffin (Luna, 1968). Blocks 2, 3, and 4 of the contralateral ovary were sectioned at 10 μm. From each block, a reference section was selected at random from the first five complete sections. Eleven sections comprising every fifth section from the reference sample were collected on poly-L-lysine coated glass slides. The sections were stained with a Periodic Acid Schiff (PAS) reaction in order to localize the basement membrane of follicles (Singh and Adams, 2000).

4.3.3 Digital image acquisition and follicular morphometry

Digital images were created from the ovarian sections using a Trestle Med Micro system (www.trestlecorp.com) and the accompanying Digital Slide Module software (Figure 4.1). The equipment includes a CCD camera mounted on a microscope assembly, three microscope objectives, a light source, computer and software. The scanner's robotic slide bed moves the slide beneath the microscope objective during image capture, allowing multiple field-of-view images to be obtained. The scanner software stitches the multiple images together to create a single image of the entire tissue sample. Images with a resolution of 0.5 microns per pixel were obtained in JPEG format using lossless compression. All images used 24-bit color and were created using a 4x objective.

Ovarian images were processed for counting follicles ≤1 mm and measuring the volume of ovarian sections used for counting the follicles (Appendix 4). The processing was facilitated using the free online WCIF (Wright Cell Imaging Facility, University Health Network, Toronto, ON Canada) version of Image J for Windows optimized for Confocal Microscopy (Rasband, W.S., Image J, National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2004) that was acquired from http://www.uhnresearch.ca/facilities/wcif/. The sections

wherein a given follicle was visible were evaluated and the section which contained follicle in its maximum diameter was identified. Using the basement membrane as the outer limit of a follicle, two measurements were made at right angle to the long axis of follicle. All follicles that had antrum were counted and atretic follicles were identified as described previously (Lussier et al., 1987). Total number of follicles per whole ovarian volume was counted (Appendix 4)



Day 3-140-4R-11 Day 5-141-2L-9

Figure 4.1 Digital images of ovarian tissue captured using Trestle Med Micro imaging system. Day 3-140-4R-11 indicate that the ovarian tissue was taken on Day 3 (Day 0 = ovulation) from heifer#140, ovarian block number 4 of right ovary and sections number 11. Day 5-141-2L-9 indicate that the ovarian tissue was taken on Day 5 (Day 0 = ovulation) from heifer#141, ovarian block number 2 of left ovary and sections number 9.

4.3.4 Plasma sampling and radioimmunoassay for FSH

Jugular blood samples were collected in heparinized tubes (10 mL; Becton Dickinson Vacutainer Systems, Franklin Lakes NJ, USA) twice daily from Day -4 or -5 (Day 0 = ovulation) until the day of tissue collection on Days 0, 3 and 5. Within 30 to 60 min of collection, blood samples were centrifuged for 15 minutes at 1500 g, and plasma was aspirated and stored at –20^{o}C. Plasma concentration of FSH was measured using a double antibody radioimmunoassay (Rawlings et al., 1984). The primary antibody was NIDDK-anti-ovine FSH, and the concentrations were expressed using standards prepared from USDA-bovine FSH-I-l. The minimum detectable limit of the assay was 0.13 ng/mL. The range of the standard curve was 0.13 to 16 ng/mL. The intra- and inter-assay coefficients of variation were both 8% for the low reference sample (mean 0.89 ng/mL), and 11% and 9% for the high reference sample (mean 2.15 ng/mL), respectively.

4.3.5 Statistical analyses

In cattle, all follicles >0.29 mm had antrum (Lussier et al., 1987). Hence, follicles were grouped as <0.3 mm, 0.3 to 0.5 mm and 0.5 to 1 mm. The number of follicles in each size category were calculated as the volume of whole ovary **x** the number of follicles in a given size category ÷ the volume of the ovary used for counting the follicles.

Statistical analyses were performed using the Statistical Analysis System software package (SAS learning edition 2.0; SAS Institute Inc., Cary, NC). The effect of day and follicle type (<0.3 mm, 0.3 to 0.5 mm and 0.5 to 1 mm) for follicle numbers in each size category was determined by analysis of variance using the mixed procedure (proc mixed). The effect of day on the daily profile of FSH was determined by analysis of variance for repeated measures using the mixed procedure. The effect of day for the follicle numbers in 0.5 to 1 mm and 1 to 3 mm size category was determined by analysis of variance using the mixed procedure. Correlations between the number of follicles in 0.5 to 1 mm and 1 to 3 mm size categories was estimated using Pearson correlation analysis.

All procedures described within the present study were performed in accordance with the principles outlined by the Canadian Council on Animal Care. The study protocols were reviewed

and approved by the University of Saskatchewan Protocol Review Committee which works under the umbrella of the University Committee on Animal Care and Supply.

4.4 Results

Follicles as small as 0.15 mm had an antrum and all follicles ≥0.31 mm had a well developed antrum. Atresia was higher in follicle size category 0.5 to 1 mm (29%) than in the preceding size categories (< 0.3 mm, 7%; 0.3 to 0.5mm, 13%) The number of follicles in different size categories differed from each other on different days after ovulation (follicle type x day effect, $P = 0.02$; Figure 4.2). The number of follicles < 0.3 mm differed among days ($P = 0.02$) primarily due to a peak on Day 3. There was no difference in the number of follicles 0.3 to 0.5mm among days ($P = 0.46$). The number of follicles 0.5 to 1 mm showed tendency for a difference among days ($P = 0.08$). There was a peak in circulating concentrations of FSH one day before ovulation (day effect, $P < 0.01$; Figure 4.2).

The number profile of follicles 0.5 to 1 mm ($P = 0.08$) and 1 to 3 mm ($P < 0.01$) differed among days (Figure 4.3). Although, the trend of change in the number profile of follicles with reference to a surge in FSH (Day -1) was similar among both follicle size categories, the relationship was not detected ($r = 0.05$; $P = 0.97$).

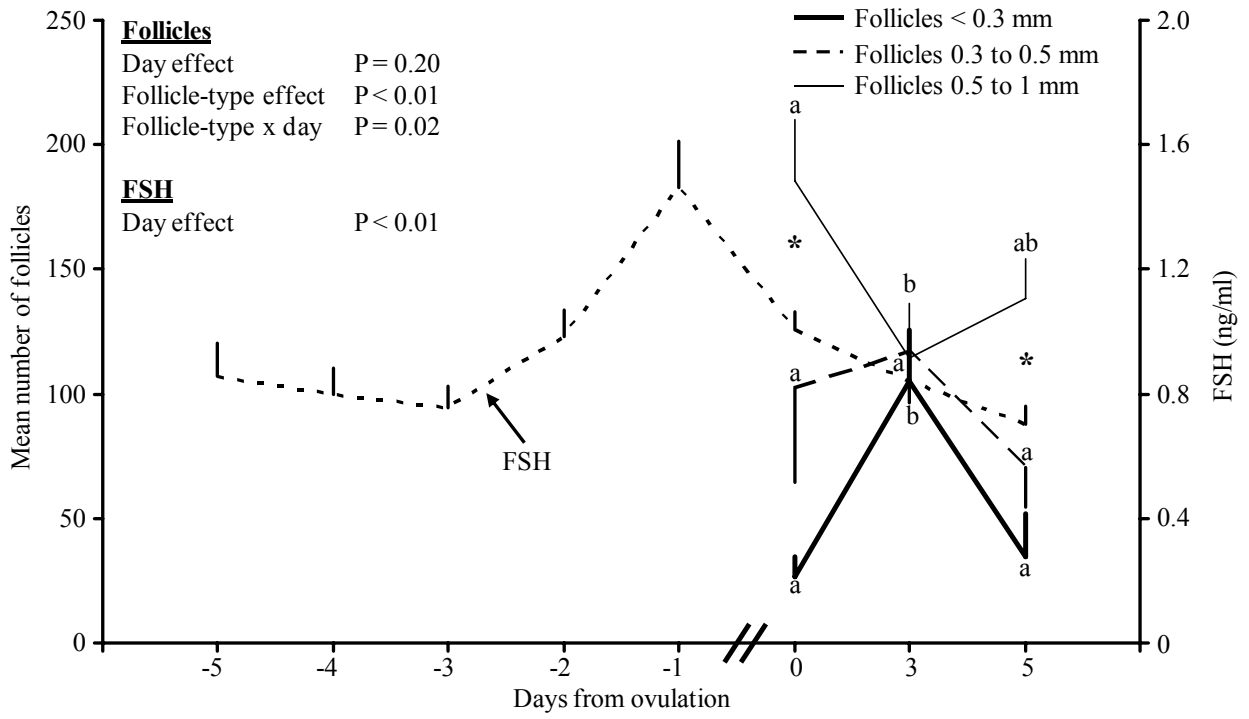


Figure 4.2 Comparative changes (mean ± SEM) in the number of follicles <0.3 mm, 0.3 to 0.5 mm and 0.5 to 1 mm in relation to the circulating concentrations of FSH. Data of FSH profile (n = 18 up to Day 0; n = 12 on Day 3; n = 6 on Day 5) were centralized to the Day of ovulation (Day 0). Ovaries (n = 6 pairs for each day; N = 18) were collected on Days 0, 3 and 5 of ovulation. [ab] Within follicle size category values with no common superscripts were different (P < .05); *Values were different between the follicle size categories (P < 0.05).

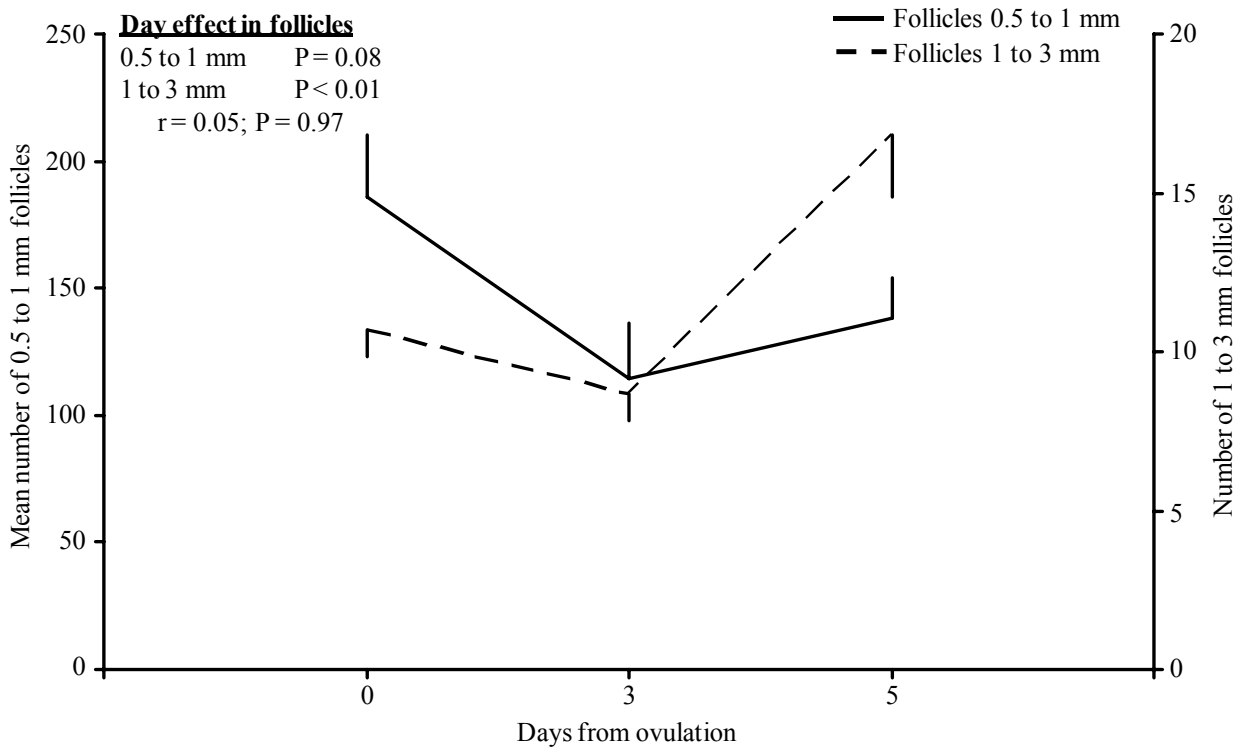


Figure 4.3 Relationship in the follicular number profile (mean ± SEM) of follicles 0.5 to 1 and 1 to 3 mm. Data (n = 6 heifers per day) were centralized to the Day of ovulation (Day 0).

4.5 Discussion

The present study revealed a wave-like developmental dynamics of antral follicles <1 mm using a histological approach. Similar approach was used in a novel study (Rajakoski, 1960) to characterize the "wave-like" developmental pattern of antral follicles >1 mm. A significant difference in the number profile of different size categories of follicles on different days after ovulation is indicative of a wave-like pattern. Further, the peak in circulating concentrations of FSH one day before ovulation followed by changes in the number profile of different size categories of follicles is indicative of a temporal relationship between follicles and FSH. The presence of antrum in all follicles ≥0.31 mm is in agreement with a previous study (Lussier et al., 1987; Braw-Tal and Yossefi, 1997). The similarity and not the difference in the trend of change in the number profile of follicles 0.5 to 1 mm and 1 to 3 mm indicates a buffering of follicles 0.5 to 1 mm for their recruitment during next follicular wave. The number of atretic follicles in the

present study was slightly higher compared to rate of atresia reported for similar size categories in a previous study (Lussier et al., 1987). The number of follicles in different size categories observed in the present study could not be compared with a previous study (Lussier et al., 1987) in which follicular data were obtained on Day 14 of the estrous cycle and without reference to FSH surge or ovulation (expected time of wave emergence; Adams et al., 1992b) as in the present study.

The shifts in the peak number of follicles <0.3 mm and 0.5 to 1 mm is indicative of a wave-like developmental pattern (Pierson and Ginther, 1987a). The distinct peaks in different size categories of follicles on different days may have resulted due to the synchronous development of follicles <0.3 mm to a higher size category of follicles and lack of immediate replacement of follicles <0.3 mm by another set. Although daily changes in the number of follicles 0.3 to 0.5 mm was not significant, their profile appeared to be regulated by the other two categories (<0.3 mm and 0.5 to 1 mm) of follicles. The lack of correlation between follicles 0.5 to 1 mm and 1 to 3 mm may be attributed to the origin of the these categories of follicles from different waves of follicles and also to the higher rate of atresia in 0.5 to 1 mm categories of follicles. Our finding about the wave-like development of antral follicles <1 mm is in agreement with a theoretical model (Gougeon, 1986) that illustrated a wave-like development of preantral and antral follicles.

The development of follicles <1 mm seemed to be regulated by circulating concentrations of FSH. Growth promoting effects of FSH on the preantral and small antral follicles in cattle have been demonstrated by *in vitro* (Itoh et al., 2002) and *in vivo* studies (Fricke et al., 1997; Tanaka et al., 2001). In hamsters, the evidence for the expression of functional FSH receptors on small preantral and antral follicles and the regulatory role of FSH was demonstrated on the basis of an increased synthesis of DNA in these follicles in response to periovulatory changes in FSH (Roy et al., 1987). Failure to notice the effect of endogenous or exogenous FSH on the early stages of follicular development may be attributed to their slow rate of growth (Scaramuzzi et al., 1980) in comparison with larger follicles (Scaramuzzi et al., 1980; Lussier et al., 1987). The delay in the peak number of follicles <0.3 mm compared to that of follicles ≥0.5 mm in response to a surge in the circulating concentrations of FSH may be attributed to their slower rate of growth.

In conclusion, the findings of the present study illustrate a wave-like development of antral follicles <1 mm in response to a surge in the circulating concentrations of FSH.

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Chapter 5

REPEATABILITY OF 2- AND 3-WAVE PATTERNS OF FOLLICULAR DEVELOPMENT DURING THE BOVINE ESTROUS CYCLE

Rajesh S. Jaiswal, Jaswant Singh, Laura Marshall and Gregg P. Adams

5.1 Abstract

Ultrasonographic data from 91 complete interovulatory intervals (IOI) of 31 beef heifers were used to identify characteristics predictive of the 2- versus 3-wave pattern of follicular development. The repeatability of 2- and 3-wave patterns within individuals was determined using a subset of 75 IOI from 15 heifers examined during multiple cycles. From this subset, data of 56 IOI from 13 heifers were used to determine the effect of season on follicular wave patterns. The majority of IOI ≤21 days (88%) were of the 2-wave pattern ($P<0.05$). Conversely, the majority of IOI ≥22 days (78%) were of the 3-wave pattern ($P<0.05$). The proportion of non-alternating patterns (repeatability) was greater than the proportion of alternating patterns (70% versus 30%; $P<0.01$) and was not influenced by the season (74% versus 26%; $P<0.01$). Emergence and follicular dominance of Wave 2 were delayed ($P<0.01$), and the onset of CL regression was earlier ($P<0.01$) in 2- versus 3-wave IOI. In conclusion, the duration of IOI was predictive of the wave pattern, and the 2- or 3-wave pattern was repeatable within individuals. The longer period of dominance of the dominant follicle of Wave 1 in 2- versus 3-wave patterns suggests that factors influencing the dominant follicle of Wave 1 are responsible for regulating the IOI pattern. Greater follicular attrition recorded in 3- versus 2-wave patterns, due to the emergence of an additional wave during the IOI, provides rationale for the hypothesis that depletion of the follicular reserve and onset of reproductive senescence may occur earlier in individuals exhibiting predominantly 3- versus 2-wave patterns.

5.2 Introduction

A wave pattern of ovarian follicular development refers to periodic, synchronous growth of a group of antral follicles (Adams, 1999). In cattle, follicle wave emergence is characterized by the sudden (within 2 to 3 days) growth of 8 to 41 (average, 24) small follicles that may be detected initially by ultrasonography at a diameter of 1 to 2 mm (Jaiswal et al., 2004). Growth rates are similar among follicles of the wave for about 4 days, after which a single follicle is selected to continue growth (dominant follicle) while the remainder (subordinate follicles) become atretic and regress (Adams, 1999; Jaiswal et al., 2004). Cattle exhibit a 2- or 3-wave pattern interovulatory interval which refers to emergence of 2 or 3 follicular waves during an interovulatory interval, of which the last follicular wave give rise to an ovulatory follicle (Figure 5.1).

Greater than 95% of bovine estrous cycles are composed of either 2 or 3 follicular waves (reviewed in Adams, 1999). Some authors report a preponderance (>80%) of the 2-wave pattern (Ginther et al., 1989a; Rajamahendran and Taylor, 1990; Ahmad et al., 1997; Bleach et al., 2004; Burns et al., 2005) while others report a preponderance (>80%) of the 3-wave pattern (Sirois and Fortune, 1988; Noseir, 2003; Celik et al., 2005), and still others have reported a more even distribution of 2- and 3-wave patterns (Savio et al., 1990; Evans et al., 1994; Price and Carriere, 2004). The temporal characteristics of 2- and 3-wave IOI have been examined (Noseir, 2003; Ginther et al., 1989d; Adams, 1994), but factors predictive of a specific pattern have not been identified, and the repeatability of a given wave pattern within individuals is not known. Although the subject has not been studied extensively, there appears to be no breed-, or age-specific predilection for a given wave pattern in *Bos taurus* (Driancourt, 2001). An increase in the proportion of 3-wave patterns has, however, been associated with a low plane of nutrition (Murphy et al., 1991; Rhodes et al., 1995) and heat stress (Badinga et al., 1993; Wolfenson et al., 1995). In Bos indicus, no seasonal effect on wave pattern was detected (Zeitoun et al., 1996), but the pattern was influenced by parity. The majority of Nelore heifers (65%) exhibited a 3-wave pattern, whereas the majority of cows (83%) exhibited a 2-wave pattern (Figueiredo et al., 1997). Others have reported that up to 27% of estrous cycles in *Bos indicus* cows consist of 4 waves of follicular development, compared to only 7% in *Bos indicus* heifers (reviewed in Bo et al., 2003).

Pregnancy rates in cattle with 2- versus 3-wave patterns were compared (Ahmad et al., 1997; Townson et al., 2002; Bleach et al., 2004) based on the notion that the preovulatory follicle in the 2-wave pattern grows for a longer period (Ginther et al., 1989a) and may contain a relatively aged oocyte. However, results were contradictory; no difference in pregnancy rate in 2- versus 3-wave pattern was detected in some studies (Ahmad et al., 1997; Bleach et al., 2004), whereas a lower pregnancy rate following ovulation at the end of the 2-wave pattern was reported in another study (Townson et al., 2002).

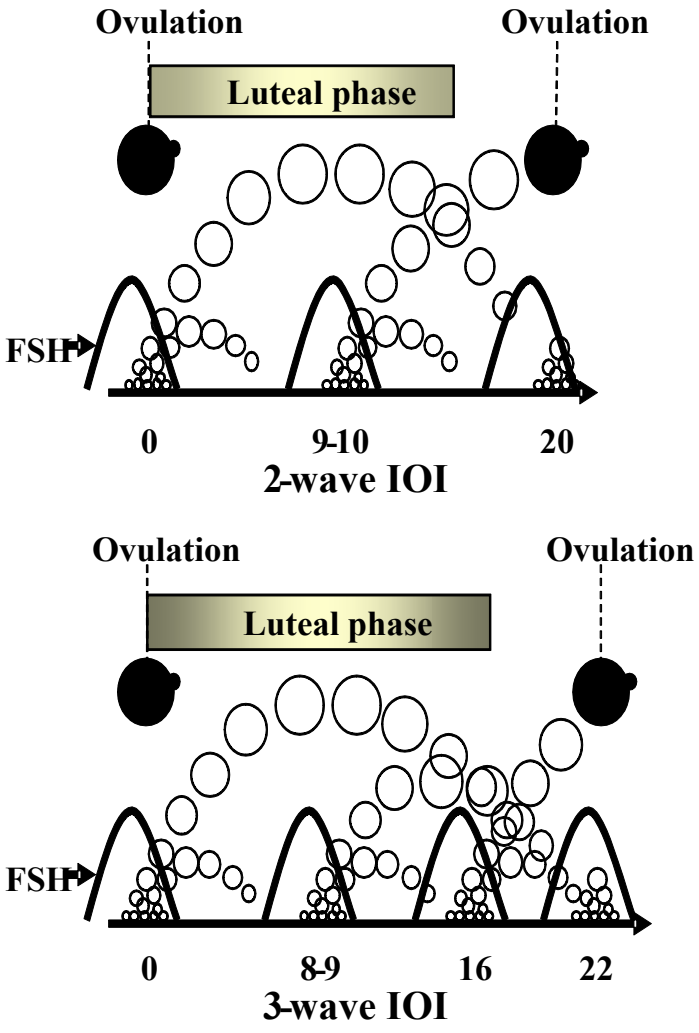

Figure 5.1 Schematic representation of 2- and 3-wave pattern interovulatory interval (IOI) in cattle. Wave emergence is preceded by a surge in the circulating concentrations of FSH. Ovulation takes place after the end of the luteal phase.

The objectives of the present study were to determine the repeatability of the 2- and 3-wave patterns within individuals, and to identify follicular and luteal characteristics predictive of the 2- versus 3-wave pattern of follicular development during the interovulatory interval (IOI) in cattle. Predictive factors associated with a 2- versus 3-wave pattern will provide insight into mechanisms controlling the pattern, and will have important implications on breeding management and the development of effective protocols for ovarian synchronization, superstimulation, and fixed-time artificial insemination. It was hypothesized that the wave-pattern (i.e., 2- or 3-wave) is repeatable within individuals. Based on the indirect findings from previous studies, it was also hypothesized that the dominant follicle of Wave 1 regulates the wave pattern.

5.3 Materials and Methods

5.3.1 Data set

Ovarian follicular and luteal data were collected by ultrasonographic monitoring of 91 complete interovulatory intervals (IOI) of 31 Hereford-cross heifers. The heifers were between 14 and 28 months of age and were maintained at the University of Saskatchewan Goodale Research Farm (52^{o} North and 106^{o} West). Ultrasonographic examinations were conducted during all four seasons of the year and uniform husbandry conditions were maintained among study periods. During each observational period, the ovaries were examined daily by transrectal ultrasonography using a 5-MHz (Aloka SSD-500; Tokyo, Japan) or 7.5-MHz (Aloka SSD-900; Tokyo, Japan) linear-array transducer until at least 2 successive ovulations (at least 1 IOI) were detected. Individual follicles and luteal structures were identified and recorded using the previous day's record of the topographic location and diameter of follicles and corpora lutea (Knopf et al., 1989; Jaiswal et al., 2004).

A subset of 75 IOI from 15 heifers that were monitored for multiple (2 to 6) successive IOI was used for assessing repeatability. Of these 15 heifers, 13 were monitored during different seasons of the year, providing data from 56 IOI specifically to assess seasonality of the wave

pattern. The respective observational periods began on the 21st day of March, June, September, and December (Vernal and Autumnal equinoxes, and Summer and Winter solstices).

5.3.2 Statistical analyses

Follicular data were centralized to the day of ovulation (Day 0). The IOI (n = 91) were classified according to the number of waves that emerged between successive ovulations. Wave emergence was detected from the follicular records by retrospective identification of a dominant follicle at an initial diameter of 4 to 5 mm (Ginther et al., 1989a). The largest follicle of the wave was defined as the dominant follicle and remaining follicles from the same wave were defined as the subordinates (Ginther et al., 1989a; Knopf et al., 1989). The growing phase of the dominant follicle was defined by the first day of its detection at 4 to 5 mm until it ceased its progressive increase in diameter. The regressing phase was marked by the first day on which the dominant follicle began a progressive decrease in diameter until it could no longer be individually identified at a diameter of ≥4 mm. The static phase comprised the period between the last day of the growing phase and the first day of the regressing phase (Ginther et al., 1989a). For the purposes of statistical analyses, the duration of dominance of the dominant follicle of Wave 1 was taken as the period of its growing and static phases together.

The follicular wave pattern was considered to be non-alternating if in consecutive IOI, a 2-wave pattern was followed by a 2-wave pattern, or a 3-wave pattern was followed by a 3-wave pattern. The proportions of alternating versus non-alternating patterns during consecutive IOI and during different seasons of the year were compared by Chi-square analyses. The subset of 75 IOI from 15 heifers permitted 60 comparisons of alternating and non-alternating patterns among heifers. Data on 56 IOI from 13 heifers were used to examine the effect of season (winter, spring, summer and fall) on repeatability of the wave-pattern and permitted 43 comparisons of alternating and non-alternating patterns among heifers.

Student's t-tests were used to make comparisons between 2- versus 3-wave patterns in i) the day of wave emergence, ii) maximum diameter of the dominant follicle, iii) duration of growing and static phases of the dominant follicle, iv) onset of regression of the dominant follicle; v) the day when the dominant follicle of Wave 2 became larger than that of Wave 1, vi) maximum diameter of corpus luteum (CL), vii) the day of onset of CL regression, and viii) the collective

number of follicles ≥4 mm detected on the day of wave emergence for each wave of the IOI (i.e., the sum of the number of follicles detected on 2 days for 2-wave patterns and 3 days for 3-wave patterns).

The diameter profile of the dominant follicle of Wave 1 was compared among 2- and 3-wave patterns during the growing phase (Days 0 to 7; Day 0 = ovulation), static phase (Days 8 to 12), and regressing phase (Days 13 to 19). The growth rate of the dominant follicle of Wave 2 and the diameter profile of CL were also compared between 2- and 3-wave patterns. The effect of Day and group was determined by analysis of variance for repeated measures using the mixed procedure of the Statistical Analysis System (SAS version 9 for Microsoft Windows; SAS Institute Inc., Cary, NC; Littell et al., 2000). Five covariance structures (compound symmetry; autoregressive order 1; unstructured; unstructured 1; and Huynh-Feldt) were fitted to the data and the best model was selected based on the smallest Akaike information criteria values. The duration of dominance (days) of the dominant follicle of Wave 1 was compared among 2- and 3-wave IOI and also with 2- and 3-wave IOI of varying durations.

Correlations between i) the duration of dominance of the dominant follicle of Wave 1 and the day when the dominant follicle of Wave 2 became larger, ii) the duration of dominance of the dominant follicle of Wave 1 and the day of onset of luteal regression, iii) the duration of dominance of the dominant follicle of Wave 1 and the number of waves per IOI, iv) the duration of dominance of the dominant follicle of Wave 1 and the duration of IOI, v) the day of onset of luteal regression and the number of waves per IOI, and vi) the day of onset of luteal regression and the duration of IOI, were determined using the correlation procedure (proc corr) in SAS.

5.4 Results

The 2-wave pattern was detected in 62/91 IOI (68%) and the 3-wave pattern was detected in 29/91 IOI (32%). The duration (mean ± SEM) of 2- and 3-wave IOI was 19.8 ± 0.2 and 22.5 ± 0.3 days, respectively ($P < 0.01$). The majority of IOI ≤21 days were of the 2-wave pattern (56/64, 88%), whereas only 8/64 (12%) were of the 3-wave pattern (Figure 5.2). In contrast, the majority of IOI ≥22 days were of the 3-wave pattern (21/27, 78%), whereas only 6/27 (22%) were of the 2-wave pattern. Five of the 15 heifers (33%) examined through 5 or 6 consecutive IOI consistently exhibited a 2-wave pattern, whereas none exhibited the 3-wave pattern

exclusively throughout all IOI (Figure 5.3). The proportion of non-alternating patterns was more than 2-fold greater than the proportion of alternating patterns (42/60 [70%] versus 18/60 [30%]; P < 0.01). Similarly, the proportion of non-alternating patterns was more than 2-fold greater than alternating patterns through consecutive seasons (32/43 [74%] versus 11/43 [26%]; P < 0.01). The proportion of 2- versus 3-wave patterns was not affected by the season of year (P = 0.61; Figure 5.4) and was similar (P = 0.18) during the pasture-based (Spring and Summer) and non-pasture-based (Fall and Winter) seasons.

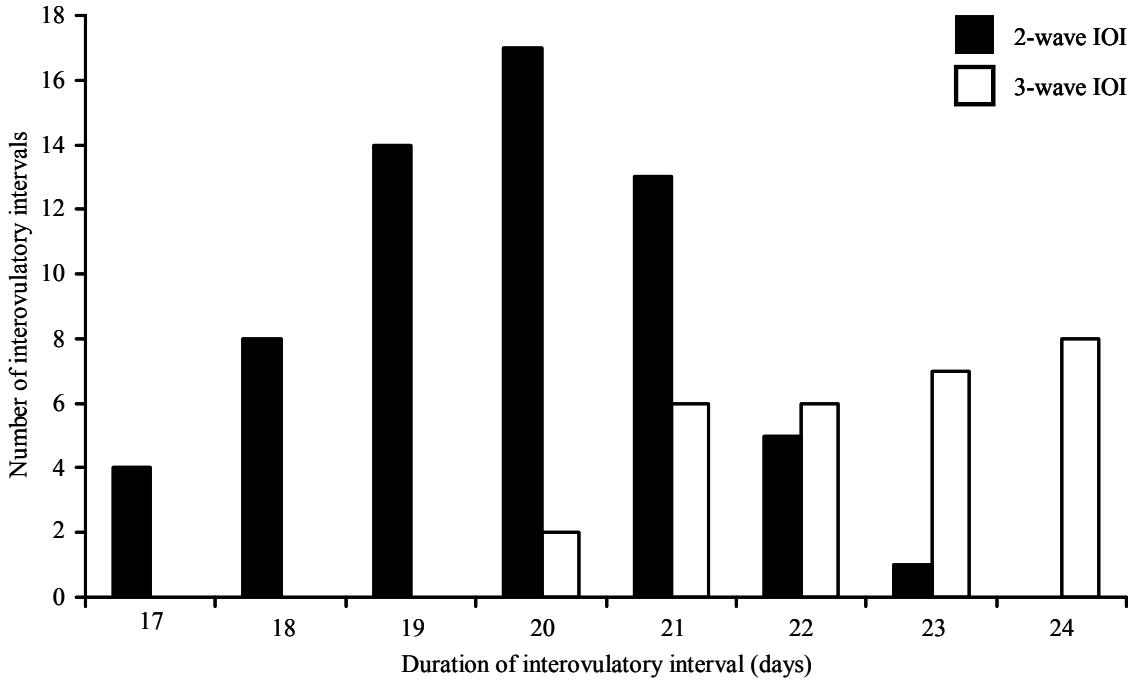

Figure 5.2 Frequency distribution of the duration of 2-wave (n = 62) and 3-wave (n = 29) interovulatory intervals (IOI) in cattle.

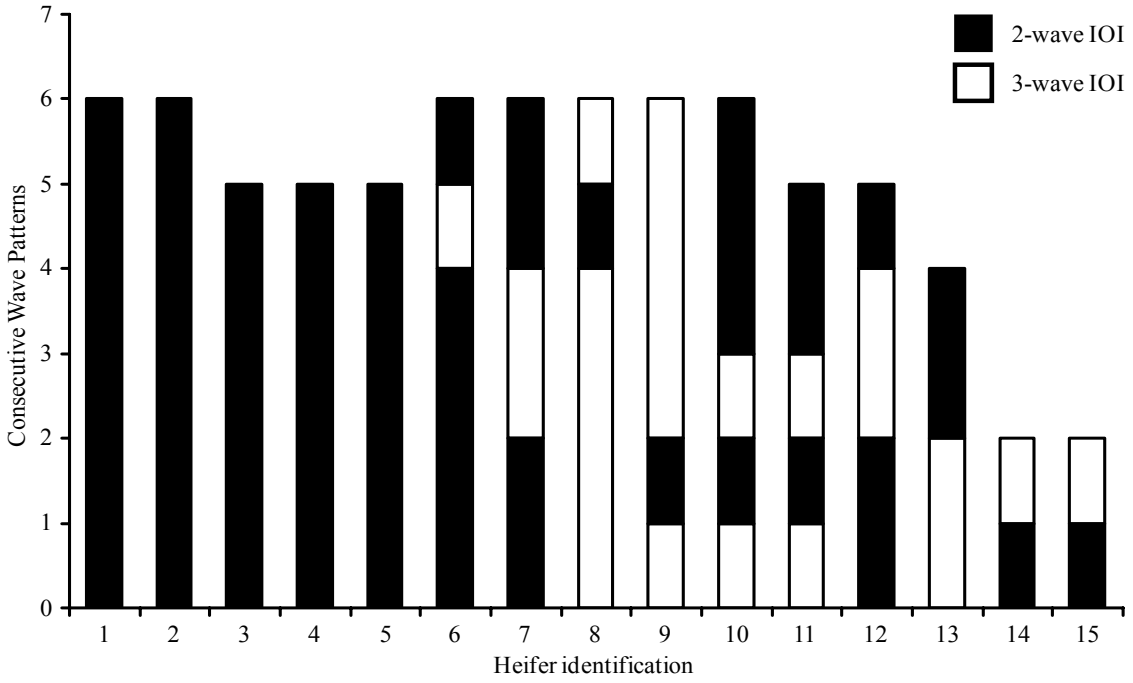

Figure 5.3 Distribution of 2- and 3-wave interovulatory intervals (IOI) in heifers (n = 15 heifers) examined during consecutive IOI (n = 75).

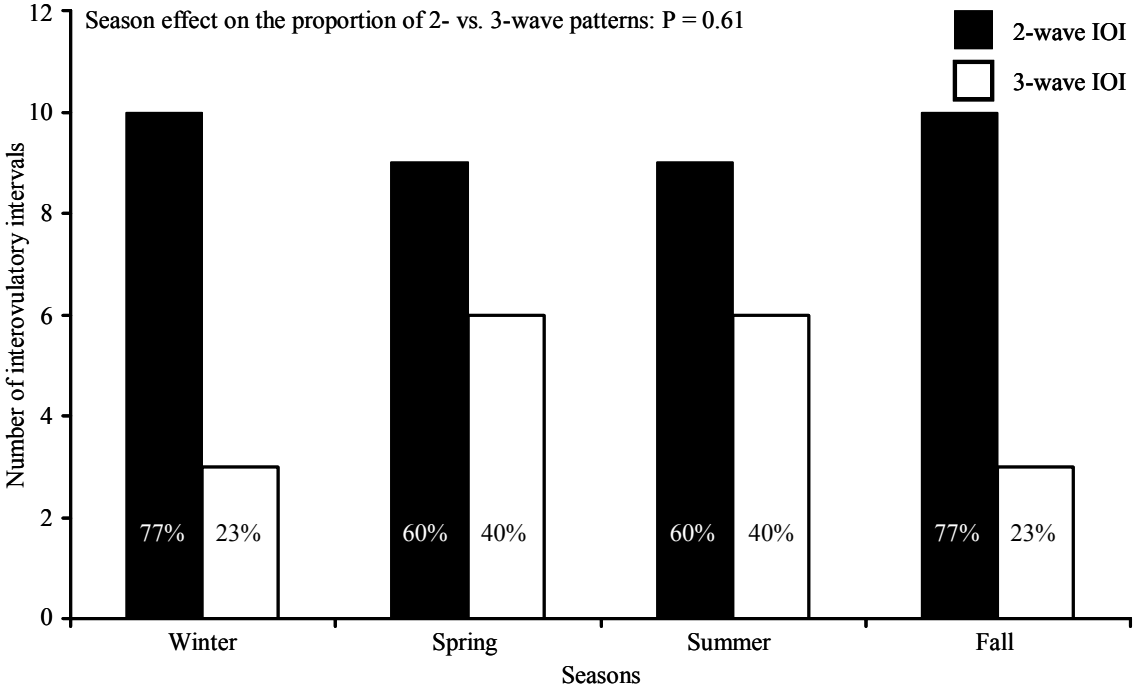

Figure 5.4 Proportions of 2- versus 3-wave interovulatory intervals (IOI) in cattle during Winter, Spring, Summer and Fall seasons.

The growing and static phases of the dominant follicle of Wave 1 were longer in 2- versus 3-wave patterns ($P \leq 0.01$; Table 5.1). The onset of the regressing phase of the dominant follicle of Wave 1 and the day when the dominant follicle of Wave 2 reached maximum diameter were delayed in the 2-wave pattern compared to the 3-wave pattern ($P < 0.01$). The onset of regression of the corpus luteum was earlier in the 2- versus 3-wave pattern ($P < 0.01$). The sum of the number of follicles ≥4 mm detected on the day of wave emergence for each wave of the IOI (2 waves in 2-wave IOI and 3 in 3-wave IOI) was greater in 3-wave versus 2-wave patterns (48.4 ± 3.2 versus 34.9 ± 2.0, respectively; $P < 0.01$).

The diameter profile of the CL was similar between 2-wave versus 3-wave patterns until the onset of regression, which began earlier in 2-wave patterns ($P = 0.01$; Table 5.1, Figure 5.5A). The duration of dominance of the dominant follicle of Wave 1 was longer in 2- versus 3-wave patterns ($P < 0.01$; Table 5.1, Figure 5.5B and 5.6). The growth rate of the dominant follicle of Wave 2 was similar between 2- and 3- wave patterns (Figure 5.7) until 5 days after wave emergence, when the growth rate decreased more rapidly in the 3-wave pattern than in the 2-wave pattern.

Table 5.1 Follicular and luteal characteristics of 2- versus 3-wave interovulatory intervals (IOI; mean ± SEM) in cattle.

	2-Wave IOI (n = 62)	3-Wave IOI (n = 29)	P-value
Interovulatory interval (days)	19.8 ± 0.2	22.5 ± 0.3	0.01
Emergence of Wave 1 (Day)*	-0.4 ± 0.1	-0.2 ± 0.1	0.54
Emergence of Wave 2 (Day)*	9.1 ± 0.2	8.0 ± 0.3	0.04
Maximum diameter of Wave 1 dominant follicle (mm)	15.0 ± 0.2	14.6 ± 0.4	0.70
Growing phase of Wave 1 dominant follicle (days)	8.0 ± 0.2	7.0 ± 0.4	0.01
Static phase of Wave 1 dominant follicle (days)	5.8 ± 0.2	3.8 ± 0.4	<0.01
Start of regressing phase of Wave 1 dominant follicle (Day)*	13.8 ± 0.3	10.7 ± 0.4	<0.01
Day* when Wave 2 dominant follicle became largest	14.9 ± 0.2	12.7 ± 0.4	<0.01
Maximum diameter of CL (mm)	24.7 ± 0.4	25.1 ± 0.5	0.30
Day* of onset of regression of CL	14.1 ± 0.3	16.6 ± 0.4	<0.01
Sum of the number of follicles ≥4 mm detected on the day of wave emergence (2 versus 3 emerging waves)	34.9 ± 2.0	48.4 ± 3.2	<0.01

*Day 0 = ovulation

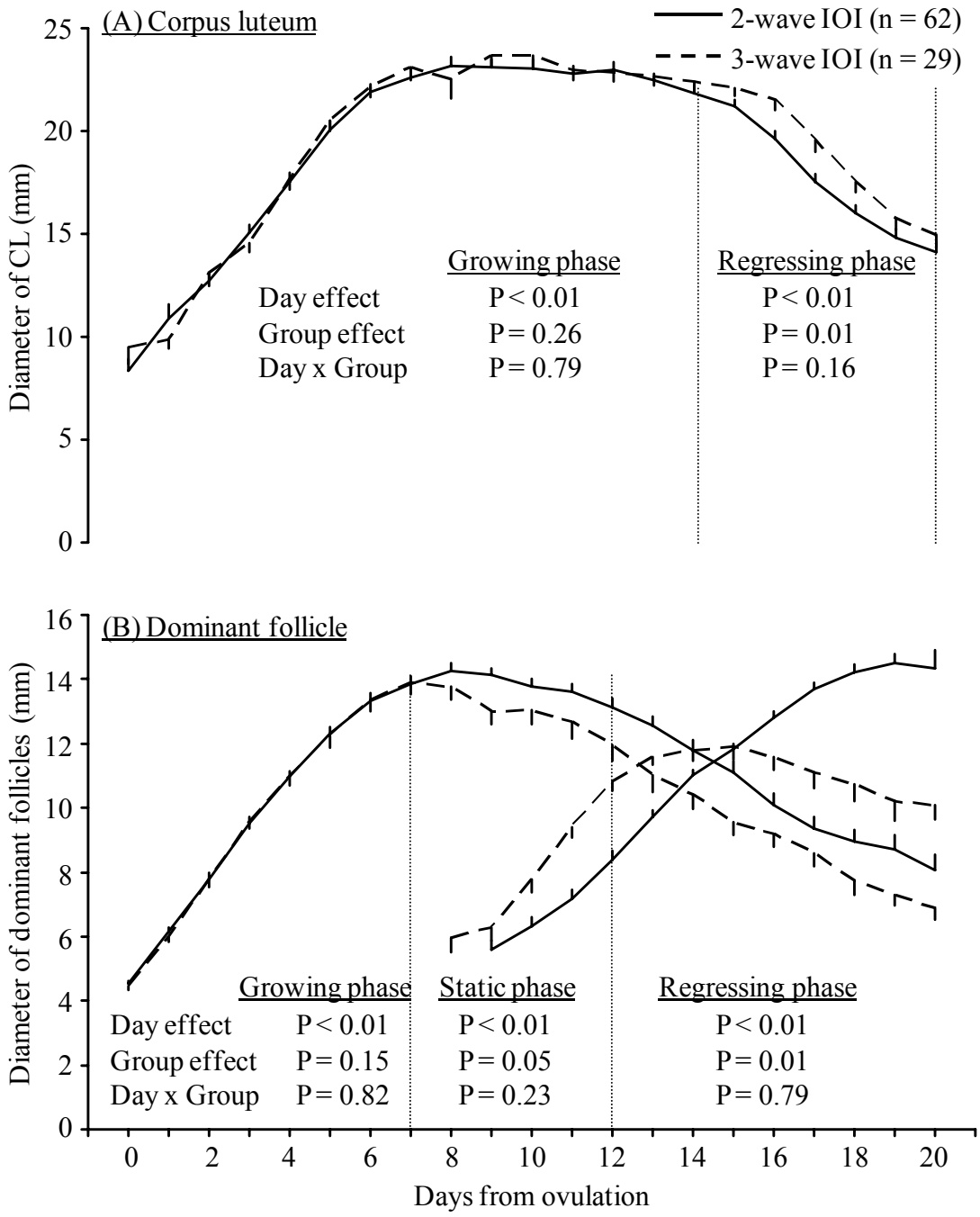

Figure 5.5 Diameter profiles of the corpus luteum (A), and dominant follicles (B) of Wave 1 and 2 during 2- versus 3-wave interovulatory intervals (IOI) in cattle. Statistical comparisons were made for the periods of luteal function and regression (Day 0 = ovulation), and for the growing, static and the regressing phases of the dominant follicle of Wave 1.

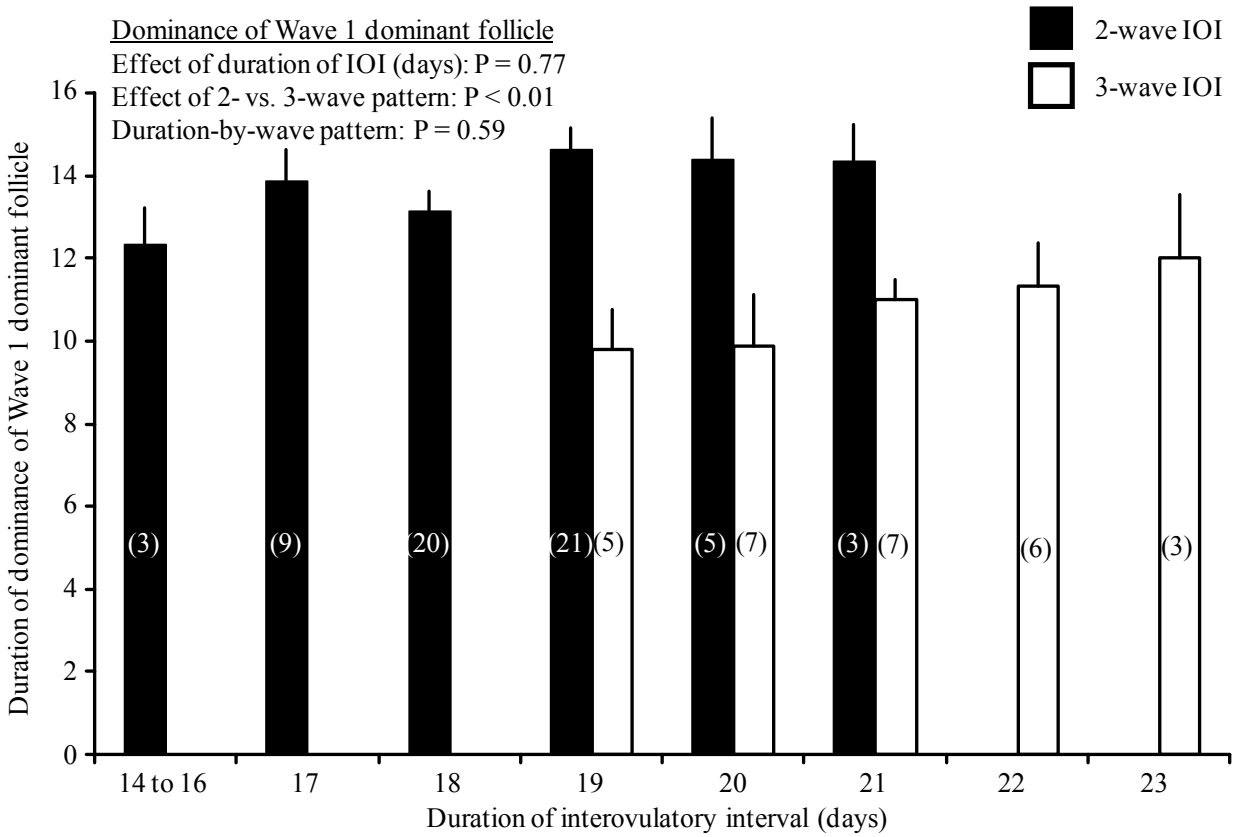

Figure 5.6 Comparison of duration of dominance (days) of the dominant follicle of Wave 1 in 2- versus 3-wave interovulatory intervals (IOI) and among 2- and 3-wave IOI of varying durations in cattle.

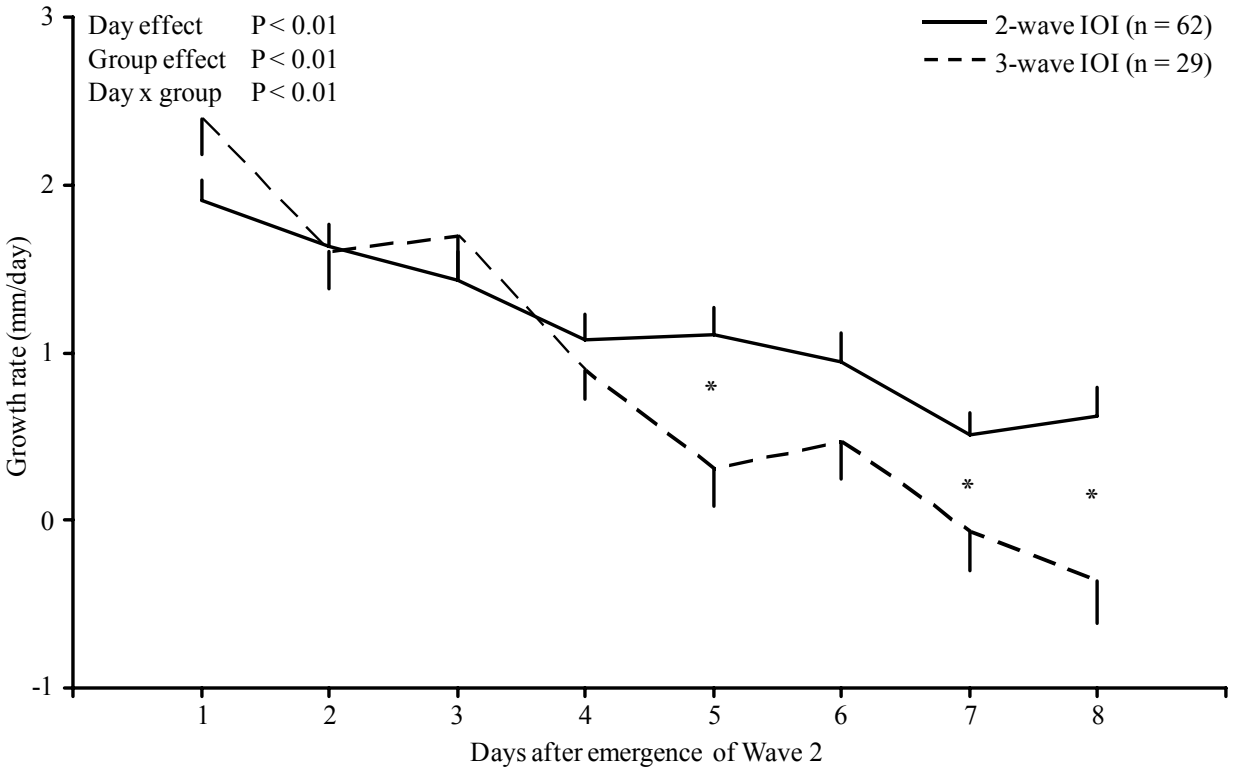

Figure 5.7 Growth rate of the dominant follicle of Wave 2 of 2- versus 3-wave interovulatory intervals (IOI) in cattle. Data were centralized to the day of emergence of Wave 2.
*Values differed between 2- and 3-wave patterns ($P < 0.05$).

The duration of the dominance of the dominant follicle of Wave 1 was inversely related to the day of onset of luteal regression, the number of waves per IOI, and the duration of the IOI ($P \leq 0.01$; Table 5.2). The day when the dominant follicle of Wave 2 became the largest follicle was directly related to the duration of dominance of the dominant follicle of Wave 1 ($P < 0.01$; Table 5.2). The day of onset of luteal regression was directly related to the number of waves per IOI, and the duration of IOI ($P \leq 0.03$; Table 5.2).

Table 5.2 Follicular and luteal correlates of 2- versus 3-wave interovulatory intervals (IOI) in cattle.

Correlate	r-value	p-value
Duration of dominance of Wave 1 versus day when Wave 2 dominant follicle became largest	0.81	0.01
Duration of dominance of Wave 1 versus day of onset of CL regression	-0.87	0.01
Duration of dominance of Wave 1 versus number of waves (2 or 3) per IOI	-0.96	0.01
Duration of dominance of Wave 1 versus duration of the IOI	-0.89	0.01
Day of onset of CL regression versus number of waves per IOI (2 or 3 waves)	0.65	0.03
Day of onset of CL regression versus duration of IOI	0.89	0.01
Duration of IOI versus number of waves per IOI (2 or 3 waves)	0.61	0.05

5.5 Discussion

Results of the present study document that the duration of the interovulatory interval in heifers is predictive of the wave pattern (i.e., 2-wave or 3-wave). The hypothesis that the wave pattern (2- or 3-wave pattern) is repeatable among individuals was supported, however, this finding is in contrast to a recent study (Price and Carriere, 2004) in which the occurrence of 2- and 3-wave patterns in Holstein heifers was reported as random. The conclusion was based on observations from only two consecutive estrous cycles (Price and Carriere, 2004) as compared to 2 to 6 consecutive estrous cycles in the present study. Results of the present study are also suggestive of a regulatory role of the dominant follicle of Wave 1 on the wave-pattern. The dominant follicle of Wave 1 in the 2-wave pattern had a longer duration of dominance by three days than its 3-wave counterpart. Furthermore, the onset of regression of the dominant follicle of Wave 1 occurred later in 2- versus 3-wave patterns, and was associated with a subsequent delay in the attainment of maximum diameter by the dominant follicle of Wave 2, as well as early onset

of luteolysis. Hence, factors that influence the development of the dominant follicle of Wave 1 may in turn be responsible for regulating the IOI pattern.

The period of dominance of the dominant follicle of Wave 1 was longer in 2- versus 3-wave patterns in previous studies (Fortune et al., 1988; Savio et al., 1988; Sirois and Fortune, 1988), but regulation of the wave-pattern was attributed primarily to the length of the luteal phase (Fortune and Sirois, 1989; Ginther et al., 1989d). The notion that the dominant follicle of Wave 1 regulates the wave pattern by influencing both the emergence of the next wave and the length of the luteal phase is supported from the findings that i) an extended life-span of the dominant follicle delayed the peak in the circulating concentrations of FSH and subsequent emergence of Wave 2 (Kastelic et al., 1990b; Ko et al., 1991; Adams et al., 1992a), whereas a shorter period of functional dominance hastened the emergence of the next follicular wave (Adams et al., 1992a; 1993b), ii) elimination of visible follicles from the ovaries on Day 10 of the cycle (Fogwell et al., 1985) or suppression of the dominant follicle (Salfen et al., 1999) increased the lifespan of the CL, whereas large estrogenic follicles (dominant follicles) exert a negative effect on the life-span of the CL (Godoy et al., 1985), iii) a short period of dominance of the dominant follicle of Wave 1 due to poor nutrition (Murphy et al., 1991; Rhodes et al., 1995; Mackey et al., 2000) or heat stress (Badinga et al., 1993; Wolfenson et al., 1995) resulted in early emergence of the dominant follicle of Wave 2 (Badinga et al., 1993; Wolfenson et al., 1995; Driancourt, 2001) and an increased proportion of 3-wave patterns (Murphy et al., 1991; Badinga et al., 1993; Rhodes et al., 1995; Wolfenson et al., 1995).

The role of the dominant follicle in the regulation of 2- and 3-wave patterns is evident from studies on follicular dynamics in prepubertal and pregnant heifers. The progressive increase in the interwave interval during transition from the prepubertal to the postpubertal phases in heifers was associated with a progressive increase in the diameter of the dominant follicle (reviewed in Adams et al., 1994). Conversely, the progressive decrease in the interwave interval in pregnant heifers was associated with a progressive decrease in the diameter of the dominant follicle with the advancement of pregnancy (Ginther et al., 1996). In addition, the prevalence of 4-wave patterns in *Bos indicus* compared to *Bos taurus* breeds (16% versus 0%) may be consequent to the smaller size and shorter period of dominance of the dominant follicle of Wave 1 in *Bos indicus* (reviewed in Bo et al., 2003)..

In contrast to the concept of a regulatory role of the dominant follicle of Wave 1, previous studies have implicated other factors that influence the 2- versus 3-wave pattern including i) lower circulating concentrations of FSH at the time of emergence of Wave 1 in 3-wave versus 2-wave IOI (Parker et al., 2003), and ii) the inability of the dominant follicle of Wave 2 in 3-wave IOI to achieve a diameter >10 mm and circulating estradiol concentrations of >5.0 pg/ml (Noseir, 2003). The latter is in contrast to the present and previous studies (Ginther et al., 1989a) wherein the diameter of the dominant follicle of Wave 2 in 3-wave patterns attained a mean diameter of 13 mm, similar to the size of an ovulatory follicle in 3-wave patterns (Ginther et al., 1989a). The high correlation between the period of follicular dominance of Wave 1 and the number of follicular waves, compared to the correlation between the CL life-span and the number of follicular waves supports the hypothesis of a regulatory role of the dominant follicle of Wave 1 rather than the intrinsic life-span of the CL (Fortune and Sirois, 1989).

Information is lacking about the endocrine differences underlying 2- versus 3-wave patterns. *In vitro* studies have shown that LH-induced synthesis and release of progesterone from the luteal cells is reduced in the presence of higher concentrations of estradiol (Akbar et al., 1972; Williams and Marsh, 1978). *In vivo*, exogenous estradiol enhances the negative effect of progesterone on LH secretion (Goodman et al., 1981). Dominant follicles are the primary source of the circulating concentrations of estradiol (Ireland and Roche, 1983). Perhaps extended dominance of Wave 1 in 2-wave patterns (Fortune et al., 1988; Savio et al., 1988; Sirois and Fortune, 1988) is associated with extended estrogen production (Singh et al., 1998), resulting in greater suppression of luteal progesterone production and early luteal regression in 2-wave patterns. In 3-wave patterns, the stimulatory effect of LH on progesterone synthesis and release may be more profound due to the shorter duration of dominance (shorter period of estradiol production) of the dominant follicle of Wave 1 (Fortune et al., 1988; Savio et al., 1988; Sirois and Fortune, 1988).

The difference in LH pulse frequency at emergence and during the growing phase of the dominant follicle of Wave 1 in 2- versus 3-wave patterns may provide an explanation for differences in follicular dominance associated with the occurrence of 2- versus 3-wave patterns observed in the present study. As well, temporal differences between 2- versus 3-wave patterns in the appearance of estradiol-induced oxytocin receptors in the endometrium (Goff, 2004) will permit test of the hypothesis that Wave 1 estradiol production regulates the timing of

prostaglandin-induced luteolysis. In this regard, results of the present study may be used in the design of future prospective studies by enabling prediction and pre-assignment of cattle with 2- versus 3-wave patterns.

Reproductive senescence is associated with the depletion of the follicular reserve that occurs around 15 to 20 years of age in cattle (Erickson, 1966a). In the present study, greater attrition of follicles was observed in 3-wave pattern. No differences have been detected in the number of follicles that emerged during successive waves, either within (Sirois and Fortune, 1988; Parker et al., 2003) or between 2- and 3-wave patterns (Sirois and Fortune, 1988; Boni et al., 1997; Singh et al., 2004; Burns et al., 2005). Hence, due to the emergence of an additional wave in 3-wave patterns, the sum of the number of follicles ≥4 mm detected on the day of wave emergence for each wave was greater in 3-wave versus 2-wave IOI. The findings provide rationale for the hypothesis that depletion of the follicular reserve and the onset of reproductive senescence occurs earlier in individuals exhibiting predominantly 3- versus 2-wave patterns.

In conclusion, the duration of the IOI was predictive of the wave pattern, and the pattern (i.e., 2- wave or 3-wave IOI) was repeatable within individuals throughout the year. The dominant follicle of Wave 1 of the 2-wave pattern had a longer period of dominance than its 3-wave counterpart; hence, factors that influence the dominant follicle of Wave 1 may be responsible for regulating the IOI pattern. There is a greater follicular attrition in 3- versus 2-wave interovulatory intervals.

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Chapter 6

DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES UNDER DIFFERENT PROGESTATIONAL ENVIRONMENTS

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6.1 Abstract

The objective of the present study was to determine the effect of progesterone (and hence, LH pulsatility) during the final stages of oocyte maturation on the competence of the oocyte to develop into an embryo. A short-term (4 days) high-progesterone, similar to the early phase of high-progesterone during ovulatory wave of 2- (6 days) and 3-wave (3 days) cycles was compared with short-term (4 days) and long-term (9 days) low-progesterone environments. Wave emergence was synchronized by follicle ablation and all heifers (n=10/group) received a once-used CIDR. Heifers in the low- and prolonged-low-progesterone groups were treated with 500 μg cloprostenol (PGF) at Day -1 (Day 0=wave emergence) while the high-progesterone group was treated with PGF on Day 3. Starting at Day 0, all heifers were treated with 200 mg FSH divided bid over 4 days. The CIDR were removed from low- and high-progesterone groups on Day 3, and from the prolonged-low-progesterone group on Day 8. Heifers were treated with LH and inseminated at 24 and 48 h, respectively after CIDR removal and were slaughtered 3 days after artificial insemination. The progesterone was high in the high-progesterone group ($P<0.01$), and the pulse frequency of LH was elevated in low- and prolonged-low-progesterone groups ($P<0.01$). Multiple follicles developed under the prolonged-low progesterone environment, but failed to ovulate. The oocyte/embryo recovery rate relative to the number of ovulations was higher in the high- versus low-progesterone group (68% versus 50%, $P<0.01$), but high progesterone was associated with a lower proportion of fertilized oocytes/embryos (73% versus 94%; $P<0.01$). It was concluded that in superstimulated heifers, the exposure of oocytes to a short-term low-progesterone environment increases their fertilizing capacity. However, post-fertilization, the developmental competence of ova that were exposed to short-term high- or low-progesterone environments was similar.

6.2 Introduction

Results of recent studies in cattle support the hypothesis that the competence of the oocyte to become fertilized and develop into an embryo is associated with the developmental status of the follicle that contains it (Salamone *et al*., 1999; Vassena *et al*., 2003). Endocrine events during the preovulatory period ultimately result in the resumption of meiosis and final stages of maturation of the oocyte of the extant dominant follicle (Moor *et al*., 1981). High circulating concentrations of estradiol in cattle has been shown to disrupt the gap junctions between the oocyte and cumulus cells (Mihm *et al*., 1994a; Revah and Butler, 1996; Austin *et al*., 1999), whereas, high circulating concentrations of LH in rats has been shown to disrupt the gap junctions between the cumulus and mural granulosa cells (Wert and Larsen, 1990). Disruption of gap junctions between the oocyte at its granulosa cell investment results in a loss of cell-to-cell communication, including transport of meiosis-inhibiting substance (Larsen *et al*., 1986; Motlik *et al*., 1986; Wert and Larsen, 1990).

The effects of differing circulating concentrations of progesterone on the development of the dominant follicle in cattle have been attributed to changes in LH secretion. Progesterone suppressed the growing phase of the dominant follicle in a dose-dependent manner (Adams *et al*., 1992a), and was associated with suppression of LH pulse-frequency (Ireland and Roche, 1982; Roberson *et al*., 1989; Stock and Fortune, 1993). Luteal-phase concentrations of progesterone resulted in a reduction in LH pulse frequency (Rahe *et al*., 1980), suppression of the dominant follicle, and a shorter interwave interval (Adams *et al*., 1992a; Lucy *et al*., 1992). Conversely, maintenance of subluteal-phase concentrations of progesterone resulted in elevated LH pulse-frequency (van Cleef *et al*., 1992; Kojima *et al*., 2003) and the development of a persistent, over-sized, estrogen-active dominant follicle (Sirois and Fortune, 1990; Adams *et al*., 1992a; Savio *et al*., 1993a; Stock and Fortune, 1993; Revah and Butler, 1996; Kojima *et al*., 2003) while still inhibiting the pre-ovulatory LH surge (Hobson and Hansel, 1972).

Persistent oversized dominant follicles, as a result of subluteal phase progesterone concentrations (e.g., prolonged progestagen treatment for estrus synchronization), have been associated with poor fertility (Hansel *et al*., 1961; Hill *et al*., 1971; Henricks *et al*., 1973; Roche, 1974; Beal *et al*., 1988; Jochle, 1993; Savio *et al*., 1993a; Stock and Fortune, 1993). The uterine environment (Wehrman *et al*., 1997) and the circulating concentrations of progesterone (Stock

and Fortune, 1993) were not affected following the ovulation of a persistent follicle. Lower fertility following the ovulation of a persistent follicle may be due to the development of oocyte under prolonged-high estrogenic environment (Sirois and Fortune, 1990; Savio *et al*., 1993b; Stock and Fortune, 1993; Wehrman *et al*., 1993; Mihm *et al*., 1994a) consequent to the higher pulse frequency of LH (Roberson *et al*., 1989; Savio *et al*., 1993b). Prolonged exposure to high pulse frequency of LH causes maturation of an oocyte long before it is ovulated ("early maturation") and accounts for poor fertility (Revah and Butler, 1996) due to the formation of abnormal (Mihm *et al*., 1994b; Revah and Butler, 1996) or retarded embryo that fails to reach the 16-cell stage (Ahmad *et al*., 1995).

The effects of circulating concentrations of progesterone (ergo, LH) have also been used to explain differences in the development of the ovulatory follicle of 2- versus 3-wave interovulatory intervals (IOI). In 2- versus 3-wave IOI, the ovulatory follicle grows for a longer period (9 days versus 6 days; Ginther *et al*., 1989; Jaiswal *et al*., unpublished observations in chapter 2) and attains a greater diameter (16 versus 14 mm, Ginther *et al*., 1989; 15 versus 14, Jaiswal *et al*., unpublished observations). Although the preovulatory follicle of 2- and 3-wave IOI is exposed to a similar decline in circulating progesterone during the final 3 days of maturation (Ginther *et al*., 1989), the preovulatory follicle in 2-wave IOI develops under a high-progesterone environment for a longer period (6 versus 3 days; Ginther *et al*., 1989). The oocyte that is released at ovulation is 3 days older relative to the day of wave emergence in 2-wave versus 3-wave IOI. Hence, it has been postulated that the ovulatory follicles of 2-wave IOI release aged oocytes that are less fertile than their 3-wave counterparts (Ahmad *et al*., 1997; Townson *et al*., 2002; Bleach *et al*., 2004; Celik *et al*., 2005).

The objective of the present study was to determine the effect of progesterone (and hence, LH pulsatility) during the final stages of oocyte maturation on the competence of the oocyte to develop into an embryo. The experiment was designed to induce an early growing phase of high progesterone (similar to a 2-wave cycle), low progesterone (similar to a 3-wave cycle), or prolonged-low progesterone (similar to persistent oversized dominant follicles). It was hypothesized that the long-term low but not the short-term low- or high- progesterone environments affect the developmental competence of fertilized oocytes.

6.3 Materials and Methods

6.3.1 Animals & Treatments

The experiment was done in June and August, and the cattle were maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52^{o}N and 106^{o}W). Hereford crossbred heifers (n = 49), 20 to 24 months of age and weighing from 480 to 680 kg, were treated with a single luteolytic dose of cloprostenol (500 μg Estrumate, Schering-Plough Animal Health, Pointe-Claire, Quebec, Canada) intramuscularly. The ovaries were examined daily by transrectal ultrasonography using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). The first 30 heifers that ovulated were assigned randomly to one of three groups (n = 10 heifers per group) designed to induce high-, low-, or prolonged-low-circulating concentrations of progesterone (Figure 6.1).

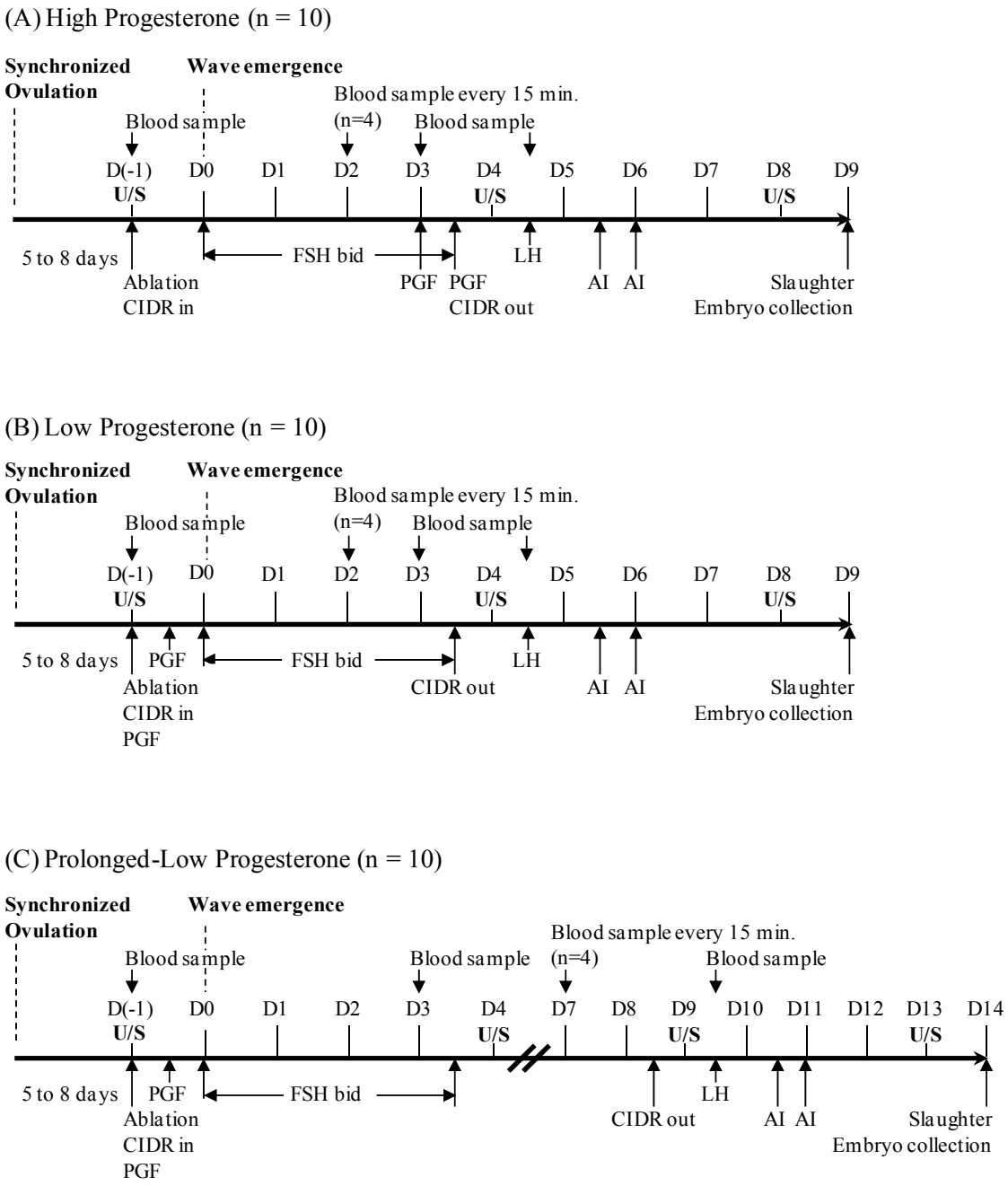

Figure 6.1 Experimental protocols used for creating *in vivo* high- (A), low- (B), and prolonged-low- (C) progesterone environments. Five to 8 days after ovulation, all follicles ≥5 mm were ablated to induce new wave emergence. In the presence of a functional corpus luteum, a once used CIDR device was inserted in vagina to create a high-progesterone environment (A), whereas, in the absence of corpus luteum (luteolysis), a once used CIDR devices were inserted in vagina for a variable period to create a low- (B) or prolonged-low-progesterone (C)

environments. Heifers in all groups were treated with FSH to obtain more ova/embryos for statistical comparison. Day 0 = expected day of wave emergence and the first of FSH treatment; AI: artificial insemination; CIDR: controlled internal drug release device; FSH: follicle stimulating hormone; LH: luteinizing hormone; PGF: prostaglandin F2α; U/S: ultrasonography.

To minimize and synchronize the interval to new wave emergence among heifers, transvaginal ultrasound-guided ablation of all follicles ≥5 mm was done between 5 and 8 days after ovulation (Bergfelt *et al*., 1994). Heifers in the low- and prolonged-low-progesterone groups were treated with two luteolytic doses of cloprostenol 12 h apart on the day of follicular ablation, whereas heifers in the high-progesterone group were not given prostaglandin so their CL remained functional. A previously used (i.e., partially spent) intravaginal progesterone-releasing device (1.9 g progesterone; CIDR-B, Bioniche Animal Health, Belleville, Ontario, Canada) was inserted immediately after follicular ablation to maintain sub-luteal phase plasma concentrations of progesterone in the low-progesterone groups (van Cleef *et al*., 1992) and to supplement endogenous progesterone in the high-progesterone group. Beginning 24 h post-ablation (i.e., expected time of new follicular wave emergence, Day 0; Bergfelt *et al*., 1994), heifers were given 25 mg of pFSH intramuscularly every 12 hours for 4 days (total dose of 200 mg NIH-FSH-P1 Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), which is half the standard superstimulatory dose for cattle (Gonzalez et al., 1990). On Day 3, heifers in the high-progesterone group were given 500 µg cloprostenol in the morning and in the evening, and the intravaginal progesterone device was removed from heifers in the low- and high-progesterone groups in the evening. The intravaginal progesterone device was removed from heifers in the prolonged-low-progesterone group on the evening of Day 8. All heifers were given a single intramuscular dose of pLH (25 mg Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) 24 h after CIDR removal to induce ovulation. Heifers were exposed to breeding bulls 12 h after LH treatment and artificially inseminated 24 and 36 h after LH treatment by one inseminator using frozen semen of the same ejaculate of a single bull. The heifers were sent to slaughter at 108 h post-LH treatment to recover ova/embryos from the excised reproductive tracts. Follicular development and ovulation were monitored by transrectal ultrasonography on Day -1 (day of follicle ablation), Day 4, and on the day before heifers were sent to slaughter. In

addition, the ovaries of heifers in the prolonged-low-progesterone group were examined by ultrasonography on Day 9.

6.3.2 Embryo collection

Reproductive tracts were transported at ambient temperature to the laboratory in polythene plastic bags within 3 hours of slaughter. The number of CL in each ovary was recorded as an estimate of the number of ovulations. The oviducts were sectioned about 1 cm caudal to the utero-tubal junction, to include a small part of the tip of the uterine horn, and were dissected from the mesosalphinx to straighten them. Each oviduct was flushed with 20 ml of phosphate buffered saline (supplemented with 0.4% BSA) by inserting a blunt 18 gauge needle into the infundibulum and collecting the flush into petridishes (100 x 15 mm; Becton Dickinson, Franklin Lakes, NJ) at the utero-tubal junction. The remaining part of the uterine horn was dissected from the mesometrium and the base of each horn (near the bifurcation) was ligated with large tissue forceps. A stab-incision was made near the base of the ligated uterine horn to permit insertion of the tip of a 60 ml catheter-tipped syringe and infusion of 75 ml of flushing medium. The flush was collected into petridishes at tip of the uterine horn. Medium flushed from the oviducts and horns was passed through a 70 micron filter apparatus (Emcon®, Veterinary Concepts, Spring Valley, WI) and ova/embryos were evaluated under stereo-microscope.

6.3.3 Blood sampling and radioimmuno-assays

To estimate the LH, estradiol, and progesterone concentrations, jugular blood samples were collected in heparinized tubes (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) on Day -1, Day 3, and immediately before LH treatment (i.e., Day 4 in high- and low-progesterone groups, and Day 9 in the prolonged-low-progesterone group). To compare LH pulse characteristics under different progestational environments, blood samples were collected at 15-min intervals for 8 h (n = 4 heifers per group) starting at 36 h before CIDR removal (i.e., on Day 2 in the high- and low-progesterone groups, and on Day 7 in the prolonged-low-progesterone group). Samples (5 ml) were collected via an intravenous catheter (vinyl tubing; 1.0 mm inner

diameter × 1.5 mm outer diameter; Product code SV70; Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia) that was inserted into the jugular vein on the day before serial sampling, so as to minimize the effect of handling stress on plasma gonadotropin concentrations. The catheter was filled with heparinized saline between blood collections (1000 U.S.P. units of sodium heparin per liter of saline; Hepalean, Organon Teknika Inc., Toronto, Ontario, Canada). Blood samples were centrifuged for 15 min at 1500 x g within 60 min of collection, and the plasma was stored at -20^{o}C.

Plasma concentrations of LH were measured using a double antibody radioimmunoassay, and were expressed as NIDDK-bLH4 units (Evans *et al*., 1994; Honaramooz *et al*., 1998). The range of the standard curve was 0.06 to 8 ng/ml. The intra-assay coefficients of variation were 7% (low reference sera; mean 0.43 ng/ml) and 2% (high reference sera; mean, 0.99 ng/ml). The LH-pulse frequency, pulse amplitude, means, and basal concentrations were calculated as described previously (Honaramooz *et al*., 1998).

Plasma concentrations of progesterone were determined (Kastelic *et al*., 1999) using a solid-phase radioimmunoassay (catalogue # TKPG5; Lot # TKPG21443; Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The range of the standard curve was 0.1 to 40 ng/ml. The intra-assay coefficients of variation were 6% (low reference sera; mean 1.5 ng/ml), 8% (medium reference sera; mean 2.6 ng/ml), and 3% (high reference sera; mean 15.0 ng/ml).

Plasma concentrations of estradiol-17β were estimated using a double-antibody radioimmunoassay kit (Catalog # KE2D5, Diagnostics Products Corporation, Los Angeles, CA; Joseph *et al*., 1992). The range of the standard curve was 2 to 500 pg/ml. The intra-assay coefficients of variation were 2% and 4% for samples with means of 28.3 and 94.9 pg/ml, respectively.

6.3.4 Data analyses

Statistical analyses were performed using the Statistical Analysis System software package (SAS learning edition 2.0; SAS Institute Inc., Cary, NC). The effect of treatment (high-, low-, and prolonged-low-progesterone) was compared for LH profile (mean and basal concentrations, pulse frequency and amplitude) by analysis of variance using the General Linear Model procedure (proc GLM). The effect of treatment and day on hormone concentrations and the

number of follicles in different diameter categories were determined by analysis of variance for repeated measures using the mixed procedure (Littell *et al*., 2000). If main effects or their interaction were statistically significant ($P < 0.05$), multiple comparisons were made using Tukey's post-hoc test. The values are expressed in mean ± SEM unless otherwise stated. Recovery rates of ova/embryos, ratio of ova to embryos, and proportion of different stage embryos were compared between groups by chi-square analyses. The mean number of different stage embryos was compared between the short-term low- and high-progesterone groups by Student's t-test.

The experimental protocol was approved by the University of Saskatchewan Protocol Review Committee under the umbrella of the University committee on Animal Care and Supply, and was performed in accordance with the guidelines of the Canadian Council on Animal Care.

6.4 Results

In accordance with the induced progesterone milieu, the plasma concentrations of progesterone on Day 3 and Day 4 (high- and low-progesterone groups) or Day 9 (prolonged-low-progesterone) were higher in the high-progesterone group compared to the low- and prolonged-low-progesterone groups ($P < 0.01$; Figure 6.2). Within groups, there was a decline ($P < 0.01$) in the circulating progesterone concentrations within 24 h after CIDR removal, i.e., between Day 3 and Day 4 (high- and low-progesterone groups) or Day 8 and Day 9 (prolonged-low progesterone group).

The influence of progesterone treatments on LH pulse characteristics are summarized in Table 6.1. Plasma LH concentration on Day 3 were similar among groups ($P = 0.37$; Figure 6.3). In all groups, the circulating concentrations of LH on the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group) were higher ($P < 0.01$) than on Day 3.

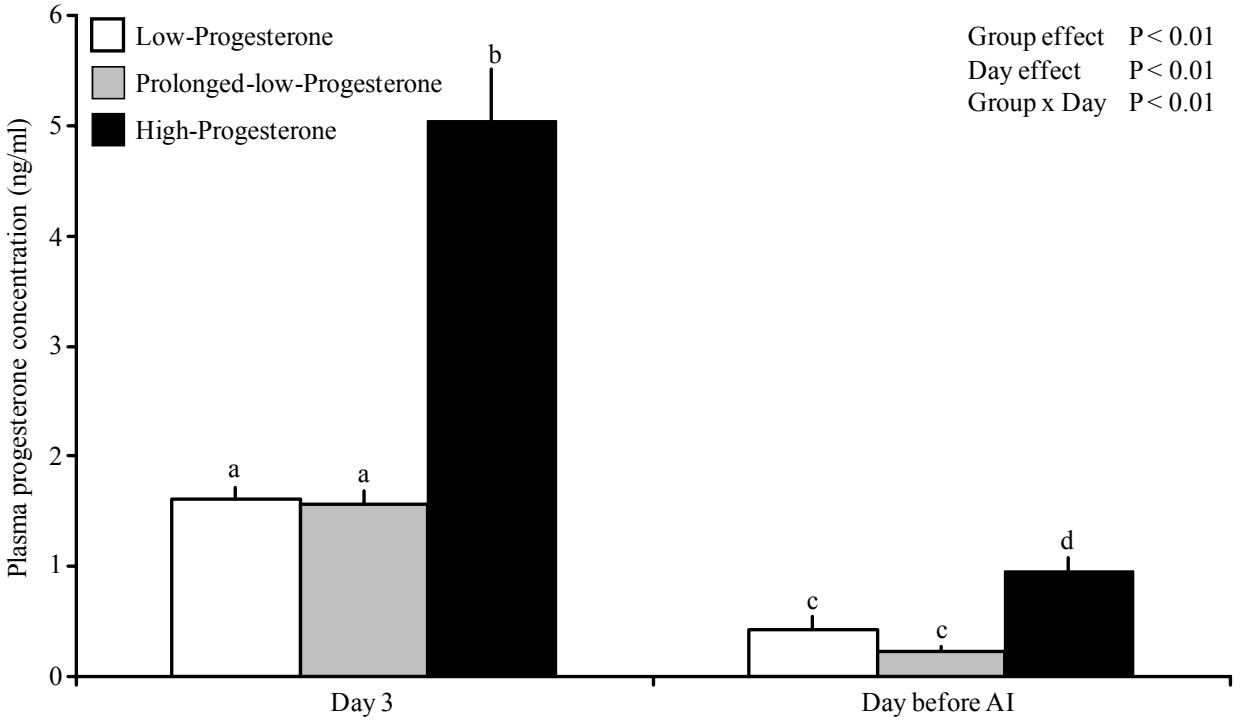

Figure 6.2 Circulating plasma concentrations of progesterone (mean ± SEM) on Day 3 (Day 0 = 1st FSH treatment) and on the day before artificial insemination in heifers treated to induce low-, prolonged-low-, and high-progesterone environments. [abcd] Values with no common superscripts were different ($P < 0.05$).

Table 6.1 Plasma LH pulsatility 36 h before CIDR removal in heifers treated to induce high-, low-, and prolonged-low-progesterone environments (mean ±SEM)

	High	Low	Prolonged-low
Mean	0.12 ± 0.0 [a]	0.14 ± 0.0 [a]	0.12 ± 0.0 [a]
Basal	0.10 ± 0.0[a]	0.11 ± 0.0[a]	0.10 ± 0.0[a]
Pulse amplitude	0.34 ± 0.1[a]	0.20 ± 0.1[a]	0.17 ± 0.0[a]
Pulse frequency/8 hr	1.75 ± 0.5[a]	6.00 ± 0.0[b]	5.25 ± 0.9[b]

[ab] Within rows, values with no common superscript are different ($P < 0.05$)

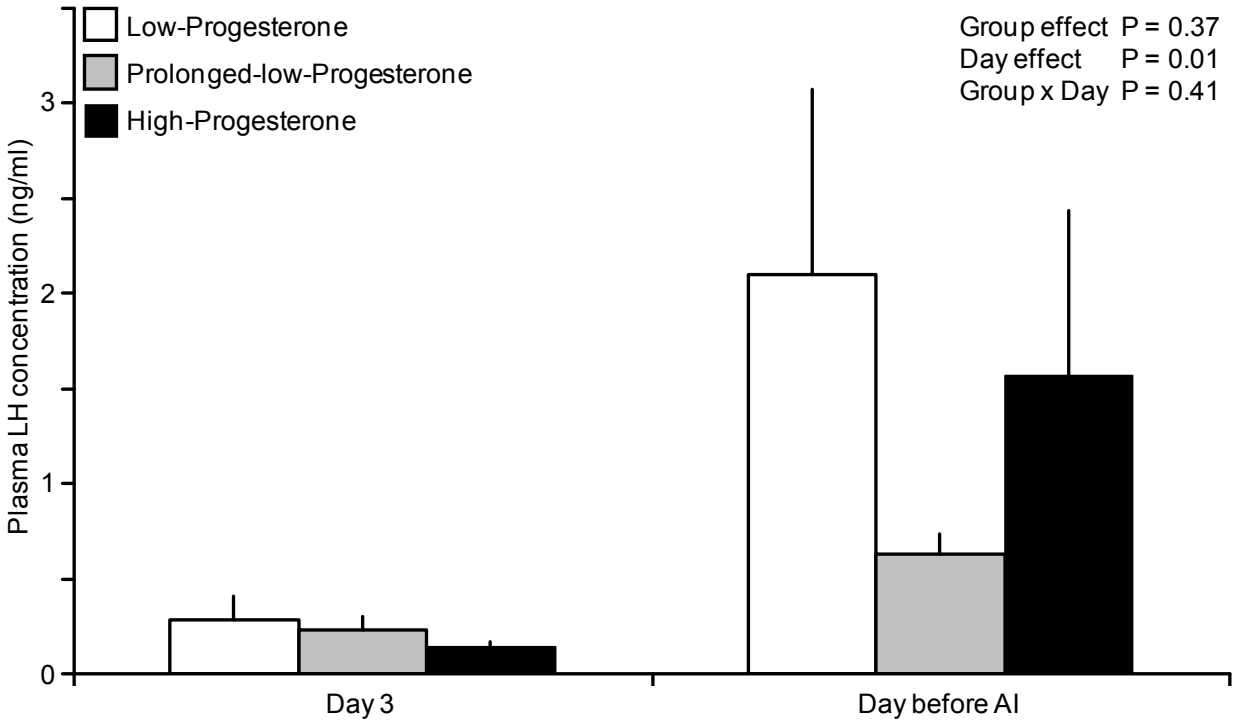

Figure 6.3 Circulating plasma concentrations of LH (mean ± SEM) on Day 3 (Day 0 = 1st FSH treatment) and on the day before artificial insemination in heifers treated to induce low-, prolonged-low-, and high-progesterone environments.

The circulating concentrations of estradiol-17β differed between groups ($P < 0.01$; Figure 6.4). Within the high- and low-progesterone groups, there was an increase ($P < 0.01$) in the

circulating concentrations of estradiol ($P < 0.01$) from Day 3 to the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group); however, the concentrations remained unchanged ($P = 0.90$) in prolonged-low-progesterone group.

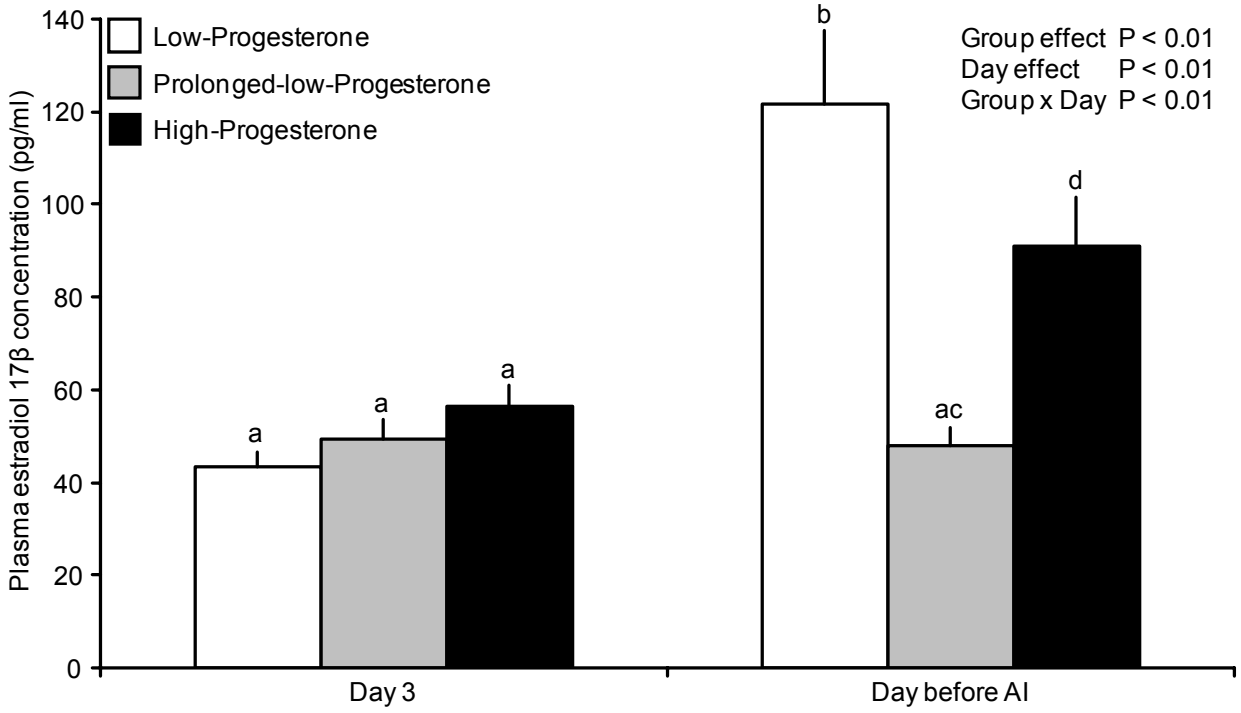

Figure 6.4 Circulating plasma concentrations of estradiol-17β (mean ± SEM) on Day 3 (Day 0 = 1st FSH treatment) and on the day before artificial insemination in heifers treated to induce low-, prolonged-low-, and high-progesterone environments. [abcd] Values with no common superscripts were different ($P < 0.05$)

Comparison of follicle numbers in different size categories on the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group) is shown in Figure 6.5. The prolonged-low-progesterone group had the greatest number of follicles ≥12 mm ($P = 0.02$) and tended ($P = 0.07$) to have the greatest number of follicles 9 to 11 mm in diameter. There was no difference ($P \geq 0.39$) among groups in the total number of follicles or in the number of follicles <9 mm in diameter.

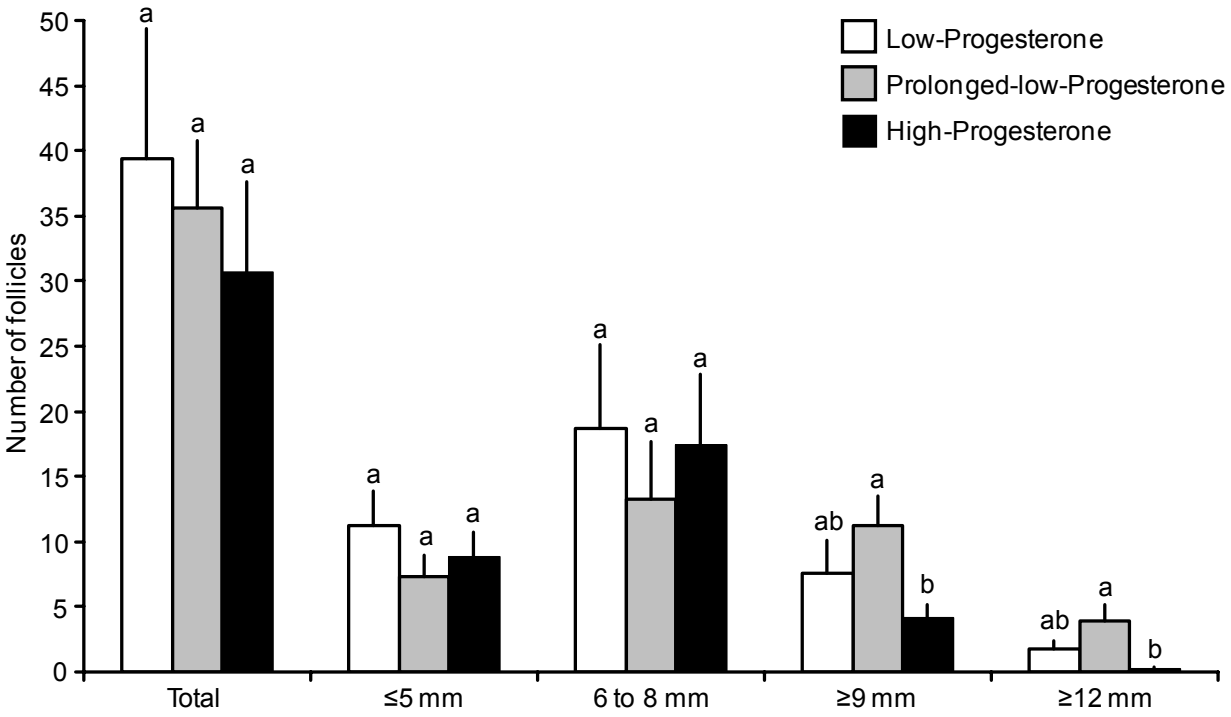

Figure 6.5 Number of follicles in different size categories 12 h before an ovulatory dose of LH was given in heifers treated to induce low-, prolonged-low-, and high-progesterone environments. [a,b] Within follicle categories, values with no common superscripts were different ($P < 0.05$).

No CL was detected and no oocytes were recovered from heifers in the prolonged-low-progesterone group; hence, data from this group were excluded from analyses related to ova/embryo recovery. There was no difference ($P = 0.83$) in the mean number of CL detected at the time of slaughter between the high-progesterone and low-progesterone groups (Table 6.2). The recovery of ova/embryo per number of CL was higher ($P < 0.01$) in the high-progesterone group (139/204; 68%) than the low-progesterone group (111/223; 50%), but the proportion of fertilized ova per total ova recovered was higher ($P < 0.01$) in the latter (94% versus 73%). With respect to the total embryos recovered, the proportion of different stage embryos did not differ ($P > 0.10$) among groups. The proportion of ova/embryos recovered from the oviduct was lower in the low-progesterone than the high-progesterone group ($P < 0.01$; Table 6.2).

Table 6.2 Comparison of response to the superstimulatory treatment and rate of ova/embryo transport in the genital tract of heifers under low (n = 10) versus high-progesterone (n = 10) environments

End-points	Low-progesterone	High-progesterone
Number of CL detected at slaughter (mean ± SEM)	22.3 ± 4.1[a]	20.4 ± 4.7[a]
Oocytes/embryos recovered per total ovulations	111/223 (50%)[a]	139/204 (68%)[b]
Fertilized oocytes/embryos per total recovered	104/111 (94%)[a]	102/139 (73%)[b]
2-cell embryos	0/104 (0%)[a]	2/102 (2%)[a]
mean per heifer ± SEM	0[a]	0.2 ± 0.1[a]
4-cell embryos	8/104 (8%)[a]	13/102 (13%)[a]
mean per heifer ± SEM	0.8 ± 0.5[a]	1.3 ±0.6[a]
8-cell embryos	55/104 (53%)[a]	58/102 (57%)[a]
mean per heifer ± SEM	5.5 ±1.1[a]	5.8 ± 2.3[a]
16-cell embryos	41/104 (39%)[a]	29/102 (28%)[a]
mean per heifer ± SEM	4.1 ± 1.4[a]	2.9 ± 1.9[a]
Ova/embryos recovered from oviduct	17/111 (15%)[a]	51/139 (37%)[b]

[ab]Values in a row with no common superscripts are different ($P < 0.05$)

6.5 Discussion

The present study was designed to compare the *in vivo* developmental competence of oocytes developing under a short-term high- (i.e., early growing phase of preovulatory follicle of 2-wave

IOI) versus short-term low-progesterone (i.e., early growing phase of 3-wave cycle) environment and also under a long-term low-progesterone environment (e.g., persistent dominant follicle). A superstimulation model was used in the experimental protocol to increase the number of ovulations and ova/embryos available for statistical comparison, with the limitation that the duration of the growing phase of the high- and low-progesterone treatment groups must be similar. The treatment protocol was effective in inducing the intended hormonal milieu in accordance with the previous studies (Ireland and Roche, 1982; Roberson *et al*., 1989; Sirois and Fortune, 1990; Savio *et al*., 1993b; Stock and Fortune, 1993; Custer *et al*., 1994; Kinder *et al*., 1996; Binelli *et al*., 1999; Vinoles *et al*., 1999); however, the experimental design did not permit to compare the difference in the length of growing phases between high- and low-progesterone groups as would be expected in 2-wave versus 3-wave IOI.

The fertilization capacity of oocytes that were exposed to the short-term low-progesterone environment was increased, but the developmental competence post-fertilization was not different from oocytes that were exposed to a short-term high-progesterone environment. Multiple follicles developed under the prolonged-low progesterone environment, but failed to ovulate. The study also revealed that the recovery rate of ova/embryos (number of ova/embryos recovered out of total number of corpora lutea detected) was high in the high-progesterone group as compared to the low-progesterone group. The ova/embryos that developed under a low-progesterone milieu were transported to the uterine horns faster than those that developed under a high-progesterone milieu.

Estradiol facilitates the transport of the fertilized or unfertilized ovum towards the uterus by induction of normal peristaltic activity (Ingersoll, 1962). In contrast, progesterone suppresses the frequency and amplitude of oviductal contractions (Maia and Coutinho, 1970). The low ova/embryo recovery rate in the low-progesterone group may be attributed to the loss of ova/embryos consequent to their more rapid rate of transport from the oviducts to the uterine horns under the influence of high estradiol as evident from the greater recovery of ova/embryos from the uterine horns. The day of embryo collection in the present study was based on a finding in superovulated cows in which the majority of oocytes/embryos were collected from the oviducts 3 days after second insemination (Holy *et al*., 1992). The rate of oocyte/embryo transport (Holy *et al*., 1992) is comparable with the high-progesterone group in which majority were recovered from the oviducts.

The high pulse frequency of LH consequent (ergo low-progesterone) stimulates estradiol production (Desaulniers *et al*., 1995) by making aromatizable substrate available for the production of estradiol by the granulosa cells of developing follicles (Lucy *et al*., 1992). The higher fertilization rate of oocytes in the low-progesterone group may be attributed to the functional development of follicles as evident from the high circulating concentrations of estradiol (Badinga *et al*., 1992; Price *et al*., 1995; Stewart *et al*., 1996). Additionally, the high estradiol and low-progesterone environment in cows around the time of estrus prompts the secretion of oviductal proteins which enables the oviductal epithelial cell proliferation (Slayden and Brenner, 1994) and thus makes the uterine environment conducive for the efficient transport of egg (Fuentealba *et al*., 1988), sperm capacitation (Anderson and Killian, 1994), sperm/egg binding (Staros and Killian, 1998), and fertilization process (Binelli *et al*., 1999). The LH receptors are expressed in the oviducts and the oviductal proteins are secreted in a dose-dependent manner in response to treatment with hCG (Sun *et al*., 1997). Thus the effect of high-estradiol-low-progesterone environment may be mediated through the resulting high concentrations of LH. However, it needs to be determined whether the high fertilization rate in low-progesterone environment was due to the effect of oviductal environment on both sperm and ova or due to the competence of the oocyte. In spite of the difference in the fertilization rate of ova among high- and low-progesterone group heifers, the recovery of similar proportions of different stage embryos in these groups indicates that the differential progesterone milieu before ovulation affects the fertilizing capacity of oocytes but not their developmental competence after fertilization is achieved.

The complete anovulation in all 10 heifers that were exposed to prolonged-low-progesterone may be due to the induction of atresia in follicles as evident from the lower circulating concentrations of estradiol (Lucy *et al*., 1992; Savio *et al*., 1993b). The heifers were superstimulated to obtain large number of oocytes as a study material. However, multiple follicles developed during superstimulatory treatment compete for available LH (Adams *et al*., 1993b) which is evident by the low circulating concentrations of estradiol (Revah and Butler, 1996) and smaller sized follicles (Adams *et al*., 1993b; Revah and Butler, 1996). The small follicles requires higher than normal dosage of LH for ovulation (Sartori *et al*., 2001). The possibility of the luteinization of follicles may be ruled out because had there been a luteinization, there would have been an increase in the circulating concentrations of progesterone.

In conclusion, oocyte/embryo collection rate relative to the number of ovulations was greater in the high progesterone group, and was attributed to greater efficiency in recovery from the oviducts compared to the uterine horn. In superstimlulated heifers, the exposure of oocytes to a short-term low-progesterone environment *in vivo* increased the fertilizing capacity, but the developmental competence post-fertilization was not different from oocytes that were exposed to a short-term high-progesterone environment. Multiple follicles developed under the prolonged-low progesterone environment, but failed to ovulate.

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Chapter 7

GENERAL DISCUSSION

The results of studies presented in this thesis revealed that i) the attenuation of the FSH surge following vaccination against GnRH does not inhibit the wave-like emergence of follicles 1 to 3 mm in diameter (Chapter 2); ii) in anti-GnRH vaccinated heifers, the growth rate of follicles 1 to 3 mm in response to exogenous FSH was similar to controls (Chapter 3); and iii) antral follicles <1 mm develop in a wave-like fashion (Chapter 4); iv) within individuals, the pattern (i.e., 2- wave or 3-wave interovulatory interval) is repeatable throughout the year, and the factors that influence the development of the first wave dominant follicle may be responsible for regulating the pattern (Chapter 5); v) in FSH-treated heifers, oocytes that develop under a high-progesterone milieu are less capable of being fertilized, whereas follicles that develop under a prolonged-low progesterone milieu fail to ovulate (Chapter 6);.

7.1 Fundamental pattern of development of antral follicles

In cattle, follicles are classified as primordial, primary, secondary or tertiary based on morphology (Lussier et al., 1987; Braw-Tal and Yossefi, 1997, Lundy et al., 1999). The tertiary follicles are also called vesicular follicles, however, the term “antral follicle”, meaning a follicle with a cavity, is commonly used. The antrum is fully formed when a follicle reaches about 0.29 mm in size. The antral follicle then develops to a preovulatory size of 16 mm and is termed a “Graafian follicle”. The developmental pattern of antral follicles ≥1 mm (Pierson and Ginther, 1988; Savio et al., 1988; Sirois and Fortune, 1988; Jaiswal et al., 2004) has been characterized as “wave-like”, which refers to a synchronous growth of a group of follicles, termed “wave emergence” (reviewed in Adams, 1999) in response to a surge in the circulating concentrations of FSH (Adams et al., 1992b; Gong et al., 1995).

In spite of the fact that granulosa cells in the ovary are the only target site for FSH action (Simoni et al., 1997) and that FSH receptors are expressed in follicles immediately after their activation from a primordial pool (Camp et al., 1991; Xu et al., 1995; Bao et al., 1997), the role of FSH in the regulation of preantral and small antral follicles has been a subject of debate. It has

been argued that FSH receptors (FSHr) in small follicles may not have coupled to the adenylate cyclase second messenger system and hence, may be non-functional until the follicle reaches a secondary stage of development in the bovine (Wandji et al., 1992) and other species (Sokka and Huhtaniemi, 1990; O'Shaughnessy et al., 1997; Oktay et al., 1997). The inferences were made mainly due to lack of noticeable effect of FSH on small follicles which may have been due to minimal requirement for FSH during early stages of follicular development (Govan and Black, 1975) coupled with the extremely slow rate of growth of small follicles (Scaramuzzi et al., 1980). However, a recent study detected functional FSHr in the precursor cells of the granulosa cells in the hamster ovary (Roy and Albee, 2005). The functionality of the FSHr was tested by measuring cAMP production following *in vitro* culture of the fetal ovaries with FSH. The study also demonstrated that the suppression of fetal concentrations of circulating FSH using anti-FSH antibody resulted in a decreased number of primordial follicles being formed in a dose-dependent manner. The functionality of the FSHr was also demonstrated by a novel study in hamsters in which DNA synthesis in preantral and small antral follicles was increased in response to the periovulatory changes in the circulating concentrations of FSH (Roy et al., 1987).

FSH plays an important role at all stages of development. Studies in various mammalian species including cattle have demonstrated that FSH is required in i) the transition of flattened pregranulosa cells to cuboidal granulosa cells (Jones and Krohn, 1961; Edwards et al., 1977; Lintern, 1977; Arendsen, 1982; Dahl et al., 1988; Gougeon et al., 1992) which marks the formation of a primary follicle from a primordial stage follicle (Braw-Tal and Yossefi, 1997); ii) the growth of preantral follicles (Abir et al., 1997; Gulyas et al., 1977; Hirshfield, 1985; Wang and Greenwald, 1993a; 1993b; Hulshof et al., 1995; Ralph et al., 1995; 1996; Cecconi et al., 1999; Gutierrez et al., 2000); and iii) the formation of the antrum which marks the formation of the antral follicle (Roy and Treacy, 1993). In the fetus as well as in adult cows, the growth of follicles at all stages of development is associated with the circulating concentration of FSH (Tanaka et al., 2001).

The argument that small antral follicles are sensitive to but not dependent on FSH came from studies in which sheep were hypophysectomized to completely remove the source of FSH. Even after several weeks of hypophysectomy, the growth of preantral (Dufour et al., 1979) or antral follicles up to 2 mm in diameter (Driancourt et al., 1987; McNatty et al., 1990) did not stop completely. However, the decrease in the number of small preantral and antral follicles in these

studies is indicative of a functional role of FSH. It needs to be determined whether the growth of follicles was functional or was merely morphological. In the studies in which FSH secretion was suppressed by a GnRH-agonist (Gong et al., 1996) or by immunization against GnRH (Prendiville et al., 1995; 1996), the follicles grew to 3 mm size but not beyond. However, close monitoring of the data revealed that the concentration of FSH was decreased but not completely suppressed and also the data were not centralized. Furthermore, the suppression of gonadotropins using a GnRH-agonist suppresses the development of follicles but the expression of FSHr on follicles is not affected (Hampton et al., 2004). The threshold concentrations of FSH required to recruit follicles of different size categories are specific to each individual (human data, Brown, 1978), to follicle size category (ewe data, Picton and McNeilly, 1991), and to the individual follicle (ewe data, Fry and Driancourt, 1996).

There are strong similarities between antral follicles ≥1 mm and <1 mm in terms of i) morphology (Lussier et al., 1987; Braw-Tal and Yossefi, 1997); ii) expression of FSH receptors (Camp et al., 1991; Xu et al., 1995; Bao and Garverick 1997); iii) intensity of mRNA expression for FSHr in the granulosa cells of follicles up to 2 mm in size (Bao and Garverick, 1998), etc. Additionally, the periodic emergence of waves of follicles ≥1 mm in response to periodic surges in the circulating concentrations of FSH (reviewed in Adams, 1999), and the consistency in the number of follicles ≥2 mm (Boni et al., 1997; Singh et al., 2004), or ≥3 mm (Burns et al., 2005) recruited into successive waves suggest that the follicular development may get organized into waves at an early stage of development.

It was therefore hypothesized that small antral follicles develop in a wave-like fashion in association with the circulating concentrations of FSH. To test this hypothesis, the first study (Chapter 4) was conducted with a specific objective to elucidate the effect of prolonged suppression of FSH on the recruitment of follicles ≥1 mm. To suppress the circulating concentrations of FSH, heifers were immunized against GnRH with one primary and two booster doses of anti-GnRH vaccine at 28 day intervals. It was speculated that the effect of FSH suppression on the recruitment of follicles ≥1 mm would provide indirect evidence about the role of FSH on follicles <1 mm and that the finding would form a basis for the strategic planning of experiments to characterize the dynamics of follicles <1 mm.

The vaccination did not suppress the mean concentrations of FSH and was ineffective in inhibiting the periodic surges of FSH. Rather, the FSH surges were attenuated. There were

distinct peaks of 1 to 3 mm follicles in association with the rising concentrations of FSH but these follicles failed to grow beyond 4 mm after the first booster vaccination. Importantly, the study revealed an inverse correlation between the number of 1 to 3 mm follicles and the mean as well as peak concentrations of FSH. This finding has strengthened the observations drawn from previous studies on heifers (Singh et al., 2004; Burns et al., 2005; Malhi et al., 2005) in which the concentration of FSH and the number of ultrasonographically visible follicles were inversely related. The present finding provides a strong basis for the use of systemic concentrations of FSH as a marker for the selection of high-fecundity animals for achieving optimum superovulatory response.

The threshold concentrations of FSH required to recruit follicles of different size categories are specific to the individual subject (human data, Brown, 1978), the follicle size category (ewe data, Picton and McNeilly, 1991), and the individual follicle (ewe data, Fry and Driancourt, 1996). Perhaps the distinct peaks of 1 to 3 mm follicles and absence of their growth beyond 4 mm in response to attenuated FSH surges are indicative of their different threshold requirements for FSH. The small antral follicles may have been misinterpreted as gonadotropin-sensitive due to a difference in the threshold requirement for FSH by the small antral follicles compared to large antral follicles,

In the previous study (Chapter 4), it was speculated that if antral follicles <1 mm are dependent on FSH, there would be a complete absence of 1 to 3 mm follicles in response to the immunization against GnRH. Another study (Chapter 5) was conducted simultaneously to elucidate the effect of exogenous FSH on follicles developed under suppressed circulating concentrations of FSH. It was speculated that if follicles <1 mm are dependent on FSH, then they would respond to exogenous FSH and would be visible ultrasonographically as ≥1 mm follicles. However, in the previous study (Chapter 4), there was a periodic emergence of 1 to 3 mm follicles in the heifers immunized against GnRH in association with the (attenuated) FSH peaks. The study in Chapter 5 revealed that the growth rates of three largest follicles did not differ between the vaccinated and control heifers. This finding supports a previous study in heifers, in which FSHr remained functional even after suppression of circulating concentrations of FSH using chronic treatment with a GnRH-agonist (Hampton et al., 2004).

During the formation of primordial follicles, functional FSHr are expressed on the pregranulosa cells (Roy and Albee, 2005). Thereafter, most of the primordial follicles remain

quiescent for a variable time period. The FSHr remains functional even after the long-term suppression of gonadotropins (Hampton et al., 2004), hence, it is speculated that the functional machinery of FSHr may still remain intact in the primordial follicles during quiescence and that FSH might have a regulatory role in the activation of a primordial follicle and its development through preantral and antral stages. Although, the wave-like development of antral follicles <1 mm in association with surges in circulating concentrations of FSH has been demonstrated in the last study (Chapter 6), experiments are required to determine the dependence of antral follicles <1 mm on circulating FSH. The effects of complete suppression of FSH and treatment with exogenous FSH on the dynamics of antral follicles <1 mm may be determined. The finding that antral follicles <1 mm develop in a wave-like fashion opens avenue to characterize the developmental pattern of preantral follicles which comprises primary and secondary follicles and now with the advent of high-resolution ultrasound biomicroscope (Visual Sonics Inc., Canada; Figure 7.1) follicles as small as 70 μm can be monitored on a daily basis.

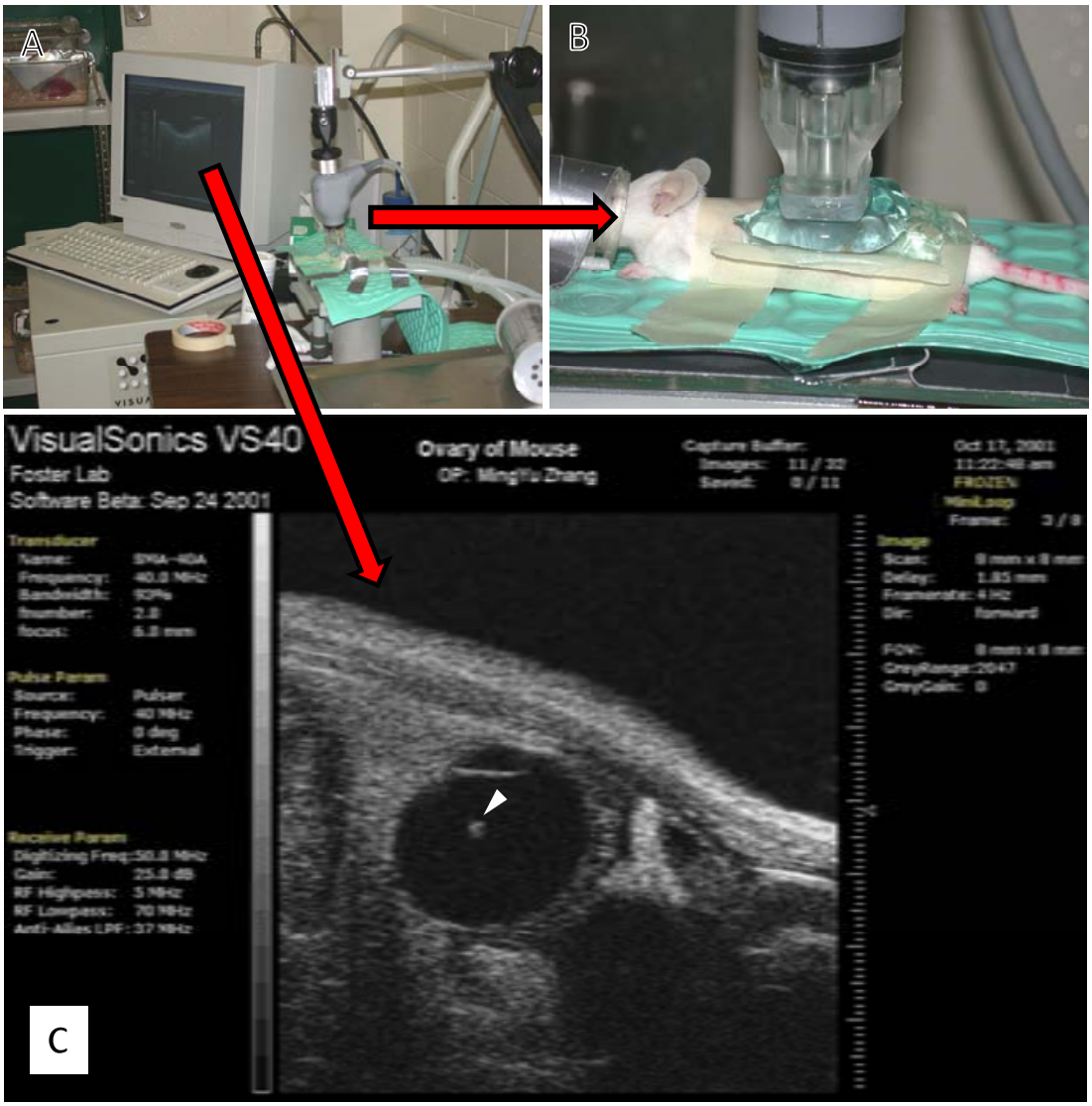


Figure 7.1 Ultrasound biomicroscope for the non-invasive monitoring of follicles as small as 70 µ. Ultrasound biomicroscope (A); mouse being scanned in sternal recumbence (B); and image of approximately 1 mm follicle (courtesy Dr. Jaswant Singh) using ultrasound biomicroscope (C).

7.2 Regulation of 2- and 3-wave interovulatory intervals

Cattle exhibit 2 or 3 waves of follicular activity during an interovulatory interval (IOI). Although the single-wave (Evans et al., 1994) and 4-wave IOI (Rhodes et al., 1995, Zeitoun et al., 1996) have been reported, the majority (>95%) of IOI are composed of either 2 or 3 follicular waves (reviewed in Adams, 1999). A preponderance (>80%) of 2-wave IOI (Ginther et al., 1989;

Rajamahendran and Taylor, 1990; Ahmad et al., 1997; Bleach et al., 2004; Burns et al., 2005), 3-wave IOI (Sirois and Fortune, 1988; Noseir, 2003; Celik et al., 2005), or an uniform distribution of 2- and 3-wave IOI (Savio et al., 1990; Evans et al., 1994; Price et al., 2004) have been reported. There appears to be no clear seasonal- (Bos indicus breed; Zeitoun et al., 1996), breed- or age-specific (Bos Taurus breed; Driancourt, 2001) predilection for a given wave pattern.

The findings from our study (Chapter 2) are indicative of repeatability of the pattern within individuals and also of a regulatory role of the dominant follicle of Wave 1 on the wave-pattern. The late emergence of Wave 2 and early onset of luteolysis in 2-wave patterns may be consequent to the longer dominance of the dominant follicle of Wave 1 which was predicted by the delayed onset of its regression.

The dominant follicles are the major source of estradiol in the circulation (Ireland et al., 1979; Schappa et al., 1982). Estradiol regulates the synthesis and release of uterine prostaglandin (Bartol et al., 1981), a major luteolytic agent (Lauderdale, 1972; Liehr et al., 1972; Rowson et al., 1972; Fairclough et al., 1981) and thereby plays a leading role in the process of luteolysis (Kimball and Hansel, 1974). The luteolytic role of estradiol was also confirmed by a midcycle treatment of heifers with exogenous estrogen which resulted in the reduced weight of luteal tissue (Brunner et al., 1969) and premature luteolysis (Wiltbank et al., 1961). Hence, large follicles, being the major source of estrogen in the circulation (Ireland et al., 1979; Schappa et al., 1982) are implicated in the spontaneous regression of the corpus luteum (Godoy et al., 1985).

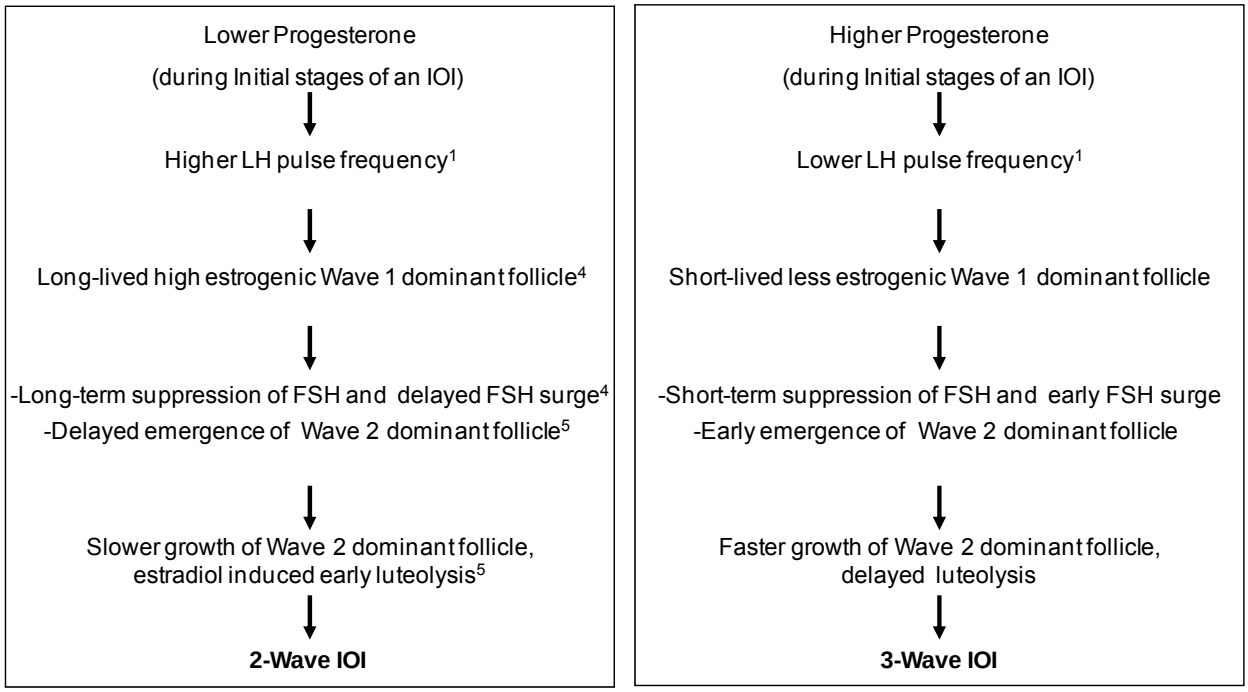
The growth of the dominant follicle, the duration of its dominance and the estrogen content of the dominant follicle depend on the pulse frequency of LH (Sirois and Fortune, 1990; Stock and Fortune, 1993; Ahmad et al., 1995; Revah and Butler, 1996; Kojima et al., 2003), which is inversely related to the circulating concentrations of progesterone (Ireland and Roche, 1982). Studies in which the circulating concentrations of progesterone were manipulated using exogenous progesterone indicated that the high pulse frequency of LH consequent to the lower circulating concentrations of progesterone (Savio et al., 1993; Sirois and Fortune, 1990) extended the dominance and increased the size of the dominant follicle (Adams et al., 1992a; Savio et al., 1993; Sirois and Fortune, 1990). However, higher circulating concentrations of progesterone reduce the life span of the dominant follicle (Adams et al., 1992a), which may be due to induction of atresia (Lucy et al., 1992) consequent to a decrease in the pulse frequency of LH (Rahe et al., 1980; Ireland and Roche, 1982).

Although previous studies (Sirois and Fortune, 1988; Fortune et al., 1988; Savio et al., 1988) indicated a longer dominance of the dominant follicle of Wave 1 in 2- versus 3-wave IOI, it was speculated that the wave pattern is regulated by the length of the luteal phase (Fortune and Sirois, 1989; Ginther et al., 1989d; Taylor and Rajamahendran, 1991). The notion in the present study that the dominant follicle of Wave 1 regulates the wave pattern is supported from the findings that i) the extended period of dominance of a dominant follicle delays the peak in FSH and subsequent emergence of Wave 2 (Kastelic et al., 1990; Ko et al., 1991; Adams et al., 1992a), whereas a shorter period of dominance hastens the emergence of the next follicular wave (Adams et al., 1992a; 1993b; Ginther et al., 1996); ii) a shorter period of dominance extends the luteal phase which in turn allows extra time for the emergence of a third wave (Driancourt, 2001); and iii) elimination of the visible follicles from the ovaries on Day 10 of the cycle increases the lifespan of the corpus luteum (Fogwell et al., 1985). Though information on the secretion of estradiol by dominant follicles of Wave 1 of 2- versus 3-wave patterns is lacking, it is hypothesized that the dominant follicle of Wave 1 of a 2-wave pattern is more estrogenic compared to its 3-wave pattern counterpart and that it remains estrogenic for a longer duration.

It is hypothesized that the 2-wave pattern is a default pattern, and an alteration in the dominance of the dominant follicle of Wave 1 induces the occurrence of a 3-wave pattern. In the present study, 5 heifers exhibited a consistent 2-wave pattern, but none of the heifers exhibited a consistent 3-wave pattern. The heifers whose average body condition score falls within the normal range of 5 to 7, exhibit 2-wave patterns (Ahmad et al., 1997). However, conditions such as acute nutritional restriction (Murphy et al., 1991; Grimard et al., 1995; Rhodes et al., 1995; Mackey et al., 2000), and heat stress (Badinga et al., 1993; Wolfenson et al., 1995; Guzeloglu et al., 2001) result in disruption of the growth of the dominant follicle (Murphy et al., 1991; Grimard et al., 1995; Rhodes et al., 1995) and the preponderance of a 3-wave pattern (Murphy et al., 1991; Badinga et al., 1993; Grimard et al., 1995; Rhodes et al., 1995; Wolfenson et al., 1995; Mackey et al., 2000; Guzeloglu et al., 2001). In underfed beef cows, the retarded size of the dominant follicle reduced the inter-wave interval, and the preponderance of a 3-wave pattern (Grimard et al., 1995) may therefore be due to the low frequency of LH.

Based on our findings and the available literature, a model is proposed (Figure 7.2) to explain the regulation of 2- and 3-wave patterns. According to this model, if the pulse frequency of LH is high during the initial phase of an IOI, the dominant follicle attains a longer duration of

dominance, becomes more estrogenic and induces an early cascade for luteolysis. However, if the LH pulse frequency is low during the initial phase of an IOI, the dominant follicle does not attain long-term dominance which then extends the lifespan of the CL and results in the induction of an additional follicular wave as in a 3-wave pattern, i.e., a "tug of war" like situation happens at the beginning of the cycle.



[1]Ireland & Roche (1982), Journal of Reproduction and Fertility, 64: 295-302; [2]Badinga et al. (1993), Theriogenology, 39: 797-810; [3]Murphy et al.(1991), Journal of Reproduction and Fertility, 92: 333-338; [4]Adams et al. (1992a), Journal of Reproduction and Fertility., 95: 627-640; [5]Godoy et al.(1985), Journal of Animal Science., 60: 519-527.

Figure 7.2 Model depicting the regulation of 2- versus 3-wave pattern

LH is luteotropic in nature (Kaltenbach et al., 1968; Denamur et al., 1973; Hansel et al., 1973) and hence supports the luteal life-span. It provides substrate to the dominant follicle for the production of estradiol (Lucy et al., 1992) and thereby maintains the follicular dominance (Badinga et al., 1992). Hence, if the pulse frequency of LH is higher during the initial phase of an

IOI, it would impart longer dominance to the dominant follicle and would augment the development of the luteal tissue. However, since estradiol has an antiluteotrophic effect during the initial days of an IOI (Lemon, 1975) and luteolytic effects during diestrus (Pratt et al., 1991), the dominant follicle may be selectively benefited with the higher pulse frequency of LH and acquires a luteolytic role. It is speculated that there is a low pulse frequency of LH during the initial phase of a 3-wave IOI which lead to an early rise in circulating concentration of progesterone which then further suppresses the pulse frequency of LH. As a result, the dominant follicle of the first wave does not acquire longer dominance and a 3-wave pattern is induced. The late occurrence of mid-cycle progesterone peak in a 3-wave pattern (Townson et al., 2002) may also be consequent to the low pulse frequency of LH during the initial phase of an IOI. Since progesterone inhibits the development of a luteolytic signal (Lamming and Mann, 1995) through its endometrial receptors (Meyer et al., 1988), the delayed luteolysis in 3-wave patterns (Ginther et al., 1989) may be consequent to the (speculated) higher concentrations of progesterone during the initial phase of an IOI. It is postulated that the pulse frequency of LH during initial phases of an IOI regulates a wave pattern.

Reproductive senescence is associated with the depletion of the follicular reserve that occurs around 15 to 20 years of age in cattle (Erickson, 1966). In the present (Chapter 2) as well as previous studies (Jaiswal et al., 2004), a higher attrition of follicles was observed in the 3-wave IOI. Considering the total pool of primordial follicles at birth (Erickson, 1966b; Tanaka et al., 2001) and the average number of follicles recruited at wave emergence (Ginther et al., 1996), a mathematical model is proposed (Appendix 3). According to this model, cows with 3-wave IOI exhibit 210.9 extra waves of follicles during 20 years of life, which is equivalent to the activation of approximately 9068.7 extra follicles or 5.76 years of reproductive life compared to cows exhibiting predominantly 2-wave IOI. This implies that the primordial follicle pool may be depleted earlier in cows exhibiting predominantly 3-wave IOI, triggering early onset of reproductive senescence. It is hypothesized that the depletion of follicular reserve and the onset of reproductive senescence occurs earlier in individuals exhibiting predominantly 3-wave versus 2-wave IOI. In women, the onset of menopause begins half-way through life and poses a risk of acquiring many debilitating diseases like osteoporosis, Alzheimer's disease, etc. A recent study (Baerwald et al., 2003) in women has reported a wave-like developmental pattern of follicles and

prevalence of 2- and 3-wave pattern IOI. Perhaps a similar association between the prevalence of 3-wave pattern IOI and the early onset of menopause in women may be investigated.

7.3 Development of the preovulatory follicle and developmental competence of the oocyte

The dominant follicle of each wave has the capability to ovulate (Savio et al., 1990), however, it is the dominant follicle of the last wave which ovulates (Bergfelt et al., 1991; Lucy et al., 1992), i.e., the wave that produces a dominant follicle during the progesterone decline consequent to the luteolysis is ovulatory. In the presence of luteal phase progesterone, the dominant follicle undergoes atresia and a new follicular wave emerges (Savio et al., 1993a).

Preovulatory follicles differ because of their exposure to different concentrations of circulating progesterone. The circulating concentration of progesterone and the duration of exposure to progesterone have an effect on fertility. For example, in estrus synchronization treatments wherein a long-term source of low-dose progesterone (e.g., Melengestrol Acetate (MGA) in feed, Norgestomate ear implants, Progesterone Releasing Intravaginal Device-PRID, etc.) is used (Hansel et al., 1961; Hill et al., 1971; Henricks et al., 1973; Roche, 1974; Butcher and Pope, 1979; Beal et al., 1988; Brink and Kiracofe, 1988; Macmillan et al., 1991; Jochle, 1993), the preovulatory follicle is exposed to sub-luteal concentrations of progesterone for a longer duration., The preovulatory follicle attains a larger size and releases an aged oocyte which is less capable of being fertilized due to the prolonged exposure to sub-luteal concentrations of progesterone (Sanchez et al., 1993; Savio et al., 1993a; Stock and Fortune, 1993; Wehrman et al., 1993; Mihm et al., 1994a; Ahmad et al., 1995; Kinder et al., 1996). The aged oocyte forms abnormal (Breuel et al., 1993; Mihm et al., 1994b) or retarded embryos that result in early embryonic death (Ahmad et al., 1995). The oocyte from the preovulatory follicle in the 2-wave pattern is larger and older than those of 3-wave patterns (Ginther et al., 1989d; Bleach et al., 2004). Based on the rationale that prolonged growth of the preovulatory follicle compromises fertility, it has been argued by many (Ahmad et al., 1997; Townson et al., 2001) that the oocytes from the ovulatory follicle of 2-wave patterns are less competent than their 3-wave pattern counterparts.

The *in vivo* developmental competence of oocytes that were grown under high-, low- and persistent-low progesterone environments were compared (Chapter 3). The embryos were

collected from excised tubular genitalia of the different treatment groups and their development was compared. The study revealed that the oocytes that developed under a high-progesterone milieu were less capable of being fertilized. However, once fertilized, their developmental competence was similar to the oocytes developed under the low-progesterone milieu.

Progesterone plays an important role in the maturation and fertilization of oocytes. It has an inverse relationship with the pulse frequency of LH (Ireland and Roche, 1982) and LH has a positive correlation with the estradiol production by the developing follicle (Roberson et al., 1989; Savio et al., 1993b; Desaulniers et al., 1995). Studies in cattle have shown that estradiol disrupts the gap junctions between cumulus cells and the oocyte (Mihm et al., 1994a; Revah and Butler, 1996; Austin et al., 1999). In rats, LH disrupts the gap junction between the mural and cumulus cells (Wert and Larsen, 1990). The disruption of gap junctions blocks the flow of meiosis-inhibiting factor from the granulosa cells to the oocyte. As a result, meiosis is resumed and the oocyte starts maturing (Wert and Larsen, 1990). It is speculated that maturation of oocytes in the high-progesterone group might have been affected due to the low concentrations of estradiol in this group and consequently, fertility was compromised. The reduced concentration of estradiol is indicative of degenerative changes in follicles (Badinga et al., 1992; Price et al., 1995; Stewart et al., 1996) and is associated with poor fertility (Perry et al., 2005). Additionally, the use of superovulatory treatment to obtain large numbers of oocytes as study material might have contributed to the low fertility since multiple follicles that develop during superstimulatory treatment compete for available LH (Adams et al., 1993b), secrete less estradiol (Revah and Butler, 1996), and tend to remain small (Adams et al., 1993b; Revah and Butler, 1996).

The high estradiol and low-progesterone environment in cows during proestrus and estrus regulates the secretion of proteins in the oviduct in a specific pattern which enables the proliferation of oviductal epithelial cells (Slayden and Brenner, 1994). The luminal environment of the oviduct becomes conducive to the efficient transport of the egg (Fuentealba et al., 1988) and to processes like sperm capacitation (Anderson and Killian, 1994), sperm/egg binding (Staros and Killian, 1998) and fertilization (Binelli et al., 1999). Estradiol facilitates the movement of spermatozoa towards the ovary by induction of antiperistaltic activity. It also facilitates transport of the ovum before and after fertilization, towards the uterus by induction of normal peristaltic activity (Ingersoll, 1962). In contrast, progesterone suppresses the frequency and amplitude of tubal contractions (Maia and Coutinho, 1970). Furthermore, an *in vitro* study in cattle (Sun et al.,

1997) demonstrated that LH binds to receptors in the oviduct and stimulates the secretion of proteins in a dose dependent manner. Hence, the higher pulse frequency of LH in the low-progesterone group might also have contributed to the induction of a uterine-tube environment suitable for normal fertilization. However, it needs to be determined whether the high fertilization rate in the low-progesterone group was due to the oviduct environment and its suitability to sperm and/or ova or whether it was due to competent oocytes.

In spite of the difference in the fertilization rate of ova among low- and high-progesterone group heifers, the recovery of similar proportions of different stage embryos in these groups indicates that the progesterone environment before ovulation affects the fertilizing capacity of oocytes but not the developmental competence of ova which are already fertilized. In 2-wave patterns, the preovulatory follicle is exposed to a high progesterone environment for 7 days compared to 3 days in 3-wave patterns and the overall duration of its development is therefore longer (Ginther et al., 1989d). However, the duration of growth of the preovulatory follicle preceding natural estrus does not affect fertility (Zimbelman et al., 1970; Beal et al., 1988; Chenault et al., 1990). The follicle attains competence for ovulation at a diameter of approximately 10 mm (Sartori et al., 2001), however, the oocyte within it attains developmental competence when the follicle is as small as 3 mm (Sirard and Blondin, 1996). The phase of the cycle including early luteal, late luteal or follicular does not affect the quality of the cumulus-oocyte complex (Wit et al., 2000). In contrast, the manipulation of the growth period of the preovulatory follicle affects the developmental competence of the oocyte. In cattle, the developmental competence of the oocyte is compromised when it is induced to ovulate from smaller follicles (≤11 mm). Follicle size, however, does not affect fertility if ovulation is spontaneous (Perry et al., 2005).

Fertility is compromised in synchronization protocols using melengestrol acetate for both < 10 days (Beal et al., 1988; Chenault et al., 1990) or >10 days (DeBois and Bierschwal, 1970; Zimbelman et al., 1970). Furthermore, the persistent follicle that develops during long-term progestogen treatment is usually larger (22 mm; Vasconcelos et al., 2001) than the preovulatory follicle in a 2-wave pattern (17 mm; Ginther et al., 1989d). The persistent follicle attains a larger size due to the effect of the higher pulse frequency of LH and produces higher concentrations of estradiol, which is detrimental to fertility (Sirois and Fortune, 1990; Savio et al., 1993b; Stock and Fortune, 1993; Wehrman et al., 1993; Mihm et al., 1994). Since the effect of the persistent

follicle on the uterine environment (Wehrman et al., 1997) and on the development of the CL has been ruled out (Stock and Fortune, 1993), the lower fertility following ovulation of a persistent follicle may be subsequent to the degenerative changes in the oocyte and not to the aging of the oocyte. It is speculated that the degenerative changes in the oocyte may be associated with poor nutrient supply as a result of severed gap junctions between oocyte and cumulus cells in response to the prolonged exposure to higher pulse frequency of LH and higher concentrations of estradiol..Although the preovulatory follicle in the 2-wave pattern develops under a high-progesterone environment for 7 days, compared to 3 days in a 3-wave pattern, the exposure to declining concentrations of progesterone is similar (i.e., 3 days; Ginther et al., 1989d). Additionally, the estradiol concentrations 6 days prior to ovulation are statistically similar in the 2- and 3-wave patterns (Ahmad et al., 1997; Savio et al., 1993a). Hence, during the final days of maturation, the preovulatory follicles in both 2- and 3-wave patterns develop under similar hormonal environments. Except for one study (Townson et al., 2002), none of the studies detected a difference in the pregnancy rate following ovulation of preovulatory follicles of 2- versus 3-wave patterns (Ahmad et al., 1997; Bleach et al., 2004; Celik et al., 2005).

A proper progesterone environment after breeding is necessary for the establishment of successful pregnancy and for improving the pregnancy rate (Mann et al., 1999; Silva et al., 2002). Although the hormonal environment during the development of a preovulatory follicle does not affect the capacity of the CL to produce progesterone (Stock and Fortune, 1993), the morphological development of the preovulatory follicle during 2- and 3-wave patterns may have significance for the induction of a suitable uterine environment following breeding. The 2-wave equivalent follicular activity following ovulation prompts higher estradiol secretion and affects the pregnancy rate (Ahmad et al., 1997), which may be due to the formation of a CL with reduced capacity to produce progesterone. The formation of the CL depends on the development of the preovulatory follicle (Robinson et al., 2005). In cows, large preovulatory follicles give rise to corpora lutea which secretes higher amounts of progesterone (Robinson et al., 2005). In contrast, the GnRH-induced ovulation of smaller follicles results in a slower rate of increase in the circulating concentration of progesterone after artificial insemination (Perry et al., 2005). Small follicles secrete less estradiol (Vasconcelos et al., 2001). As a result, the expression of LH receptors on granulosa cells is decreased (Kessel et al., 1985; Wang and Greenwald, 1993) and their luteinization is affected (McNatty et al., 1979). Consequently, the luteinized granulosa cells

produce less progesterone (McNatty et al., 1979; Vasconcelos et al., 2001). The relationship between the size of the preovulatory follicle and the size of the resulting CL is also evident from the present ($r = 0.38$; $P < 0.01$) and previous studies (Vasconcelos et al., 2001). However, it needs to be determined whether the progesterone secretion is related to the size of the CL or the mass of the luteal tissue.

Chapter 8

GENERAL CONCLUSIONS

8.1 Developmental dynamics of antral follicles <1 mm (Chapters 2 to 4)

i) Follicles developed under the low basal FSH concentrations remained smaller in diameter (≤3 mm), but were as responsive to exogenous FSH as the follicles that were developed under the physiological concentrations of FSH.

ii) Attenuation of physiological surge of FSH obliterated wave pattern of follicles ≥4 mm, but follicles ≤3 mm continued to be recruited in a wave-like fashion.

iii) An inverse relationship exists between the number of follicles recruited into a follicular wave and the peak concentrations of FSH.

iv) The dynamic change in the number of different size categories of antral follicles <1 mm on different days after ovulation indicates a wave-like pattern of development.

The findings infer that antral follicles of all size categories, i.e., <1 mm and ≥1 mm develop in a wave-like fashion in response to circulating concentrations of FSH (Figure 8.1).

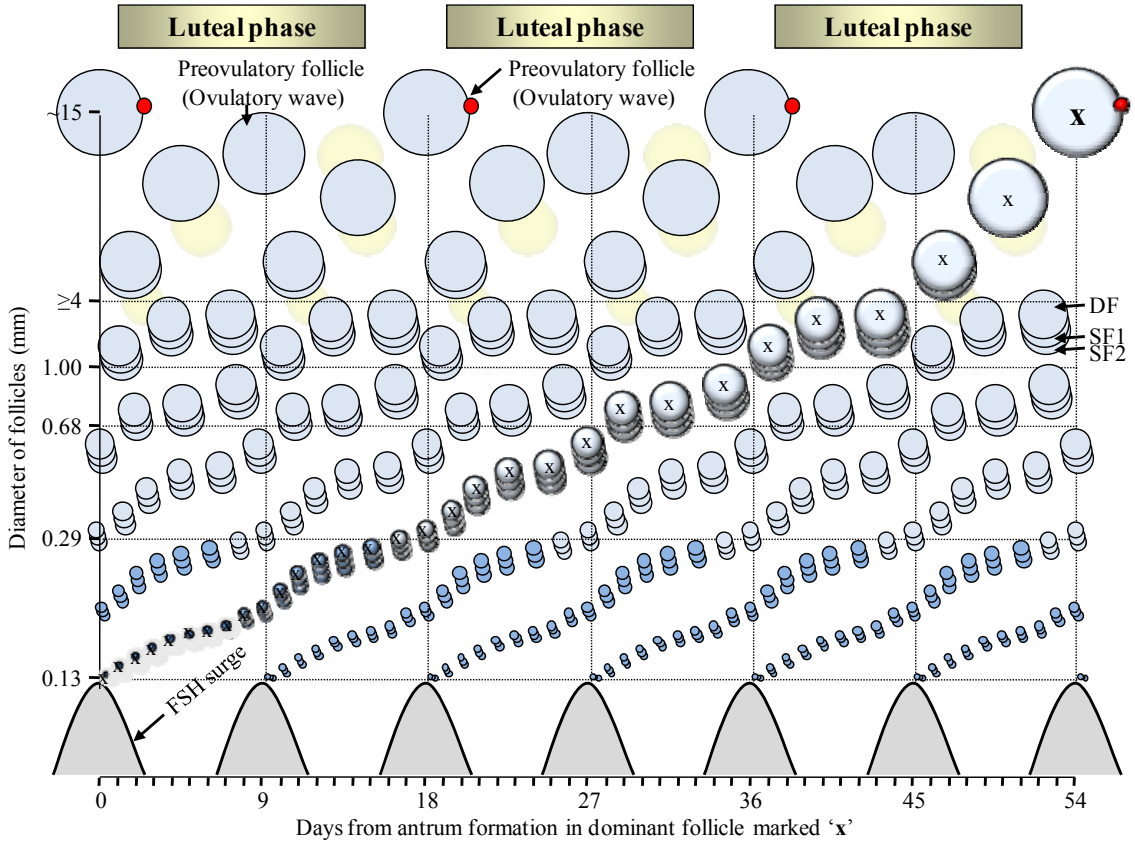


Figure 8.1 Schematic representation of the wave-like developmental pattern of antral follicles of all size categories, i.e., 0.13 mm to <1 mm (histologically characterized) and ≥1 mm (ultrasonographically characterized). The wave-like developmental pattern of antral follicles <1 mm (chapters 2,3,4) and ≥1 mm (Pierson and Ginther, 1984; Pierson and Ginther, 1987a; Jaiswal et al., 2004) was characterized. The duration of growth of follicles in different size categories was based on a previous study (Lussier et al., 1987).

8.2 Repeatability of 2- versus 3- wave pattern interovulatory intervals (Chapter 5)

i) Duration of an interovulatory interval (IOI) is predictive of a wave pattern.

ii) Longer dominance of Wave 1 dominant follicle correlates with a 2-wave pattern.

iii) Wave pattern is repeatable within individuals throughout the year.

iv) Greater attrition of follicles in 3-wave pattern than 2-wave pattern due to the emergence an extra wave.

The findings infer that a wave pattern (2- or 3-wave IOI) is repeatable within individuals. The wave pattern is regulated by the dominant follicle of Wave 1 of an IOI. There might be an early depletion of follicular reserve and consequently an early onset of reproductive senescence in individuals that predominantly exhibit a 3-wave pattern.

8.3 Effect of progesterone exposure on the developmental competence of oocytes (Chapter 6)

i) Short-term high progesterone (similar to 2-wave pattern) increases the oocyte/embryo recovery rate but affects the fertilization rate.
ii) No effect of short-term high (similar to 2-wave pattern) or short-term low (similar to 3-wave pattern) on the embryonic developmental competence of oocyte.

The study infers that the difference in the duration of development of a preovulatory follicle in 2- versus 3-wave pattern does not affect the developmental competence of oocyte.

Chapter 9

FUTURE DIRECTIONS

Studies reported in this thesis have led to new hypotheses regarding the developmental dynamics of follicles and can be pursued to expand our knowledge about the physiology of folliculogenesis and to optimize the reproductive efficiency of cattle.

New Hypotheses:

i) Preantral follicles develop in a wave-like fashion in temporal relationship with FSH.

ii) Number of follicles recruited into an ultrasonographically visible follicular wave is inversely correlated with the mean as well as peak concentrations of circulating FSH.

iii) The dominant follicle of Wave 1 of a 2-wave pattern secretes higher concentration of estradiol for a longer duration than that of a 3-wave pattern.

iv) Higher pulse frequency of LH vis-à-vis lower circulating concentrations of plasma progesterone during the initial phase of an interovulatory interval inducts a 2-wave pattern.

v) The 2-wave pattern is a default pattern of an interovulatory interval and conditions (e.g., heat stress, poor nutrition, etc.) that affects the dominance of a dominant follicle of Wave 1 inducts the occurrence of a 3-wave pattern.

vi) The follicular reserve depletes earlier in individuals that predominantly exhibit a 3-wave pattern than those that exhibit a 2-wave pattern.

Chapter 10

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APPENDICES

Appendix 1 Classification and developmental characteristics of bovine follicles

Follicle stage	Follicle type	FSHr & LHr	Granulosa cell layers*	Granulosa cells per section *	Follicle (mm)	Oocyte (μm)	Zona pellucida	Theca interna
Primordial	1		1	<10 flattened	<0.04	30	Absent	Absent
Transitory	1a		Entered growth phase and is surrounded by a mixture of flattened and cuboidal cells					
Primary	2	FSHr forms (Granulosa)	1-1.5	10-40** cuboidal	0.04-0.08	31	Begins to form	Begins to form
Secondary	3 Small preantral		2-3	41-100	0.08-0.13	50		
	4 Large preantral	LHr forms (Theca)	4-6	101-250***	0.13-0.25	69	Partially formed	Partially formed
Tertiary (vesicular)	5 Small antral		>6	>250	0.25-0.5	93	Fully formed	
	Large antral/ preovulatory	LHr forms (Granulosa)		40 x 10^6	16-18	132		

(Lussier et al., 1987; Xu et al., 1995; Braw-Tal and Yossefi, 1997; Lundy et al., 1999)

*Largest cross-section of the follicle: section that contains nucleolus of an oocyte

* Oocyte growth commences when there are at least 40 granulosa cells in the largest cross-section (fourth generation of follicle cells)

*** Antrum formation begins when there are at least 250 granulosa cells in the largest cross-section.

Appendix 2 Developmental rates of bovine follicles

Follicle size range (mm)	Granulosa cell generation	Time spent in each size class		Atresia %	Mitotic activity
		Hours	Days		
0.13-0.28	6	365.5	15.1	1.6	
0.29-0.67	8	284.9	11.9	6.6	
0.68-1.52	10	99.5	4.15	40.5	Maximum
1.53-3.67	11	83.2	3.47	30	
3.68-8.57	12	186.2	7.76	67.4	
>8.57	13			60	

(Lussier et al., 1987; Fortune, 1994)

Appendix 3 Mathematical interpretation of the association between 3-wave IOI pattern and the possibility of early onset of reproductive senescence in cows

Assumption: 2-wave and 3-wave cows begin with the same number of follicles and follicular depletion complete at 20 years in 2-wave cows

Basis for mathematical calculation		2-wave cows	3-wave cows
Age at puberty		1 year	1 year
Total follicles @ birth	Erickson, 1966b	68000	68000
	Tanaka et al., 2001	16200	16200
IOI (reviewed in Adams, 1999)		20 days	23 days
IOI/year		18.3	15.9
Waves/year		36.6	47.7
Extra waves/year			11.1
Extra waves after puberty in 20 years (life span of a cow)[1]			210.9
Equivalent reduction in years of reproductive life compared to 2-wave cows[2]			5.76
Average number of follicles ≥4 mm detected at wave emergence (Ginther et al., 1996)		24	24
Approximate total follicles activate to grow/wave (based on average ≥4 mm follicle/wave and % atresia from and preceding <4 mm size category; atresia at 1.53-3.67 adjusted to 15%		43	43
Follicles activated/year [4]		1573.8	2051.1
Follicles activated to 10 years of age[5]		14164.2	18459.9
Follicles activated to 19 years of age[6]		29902.2	38970.9
Extra follicles activated			9068.7

[1]11.1 extra waves per year x 19 years = 210.9

[2]210.9 waves per year ÷ 36.6 waves per year of 2-wave cows = 5.56 years

[3]If 63.7% = proportion of follicles <4 mm that are atretic (refer appendix 2), then estimate that 36.3% of a given wave survive to ≥4 mm; assuming 24 follicles ≥4 mm/wave and y = number of follicles undergone atresia then 24/y = 36.3/63.7 and y=24 x 63.7/36.3 = 43 total follicles started to grow per wave

[4]2-wave IOI: 43 follicles x 36.6 waves per year = 1573.8 follicles
3-wave IOI: 43 follicles x 47.7 waves per year = 2051.1 follicles

[5]2-wave IOI: 1573.8 follicles per year x 9 years = 14164.2 follicles
3-wave IOI: 2051.1 follicles per year x 9 years = 18459.9 follicles

[6]2-wave IOI: 1573.8 follicles per year x 19 years = 29902.2 follicles
3-wave IOI: 2051.1 follicles per year x 19 years = 38970.9 follicles

Appendix 4 Procedure for counting follicles (<1 mm) and determining ovarian volume from images obtained using Trestle Med Micro imaging system of ovarian tissue sections

Post-processing JPG images with Adobe Photoshop CS2

Purpose of this processing is to: 1) 'almost' align the vertical axis of ovarian slices; 2) crop to the minimum dimensions so as to exclude most of the background and; 3) place the ovary at 'almost' same location in the series.

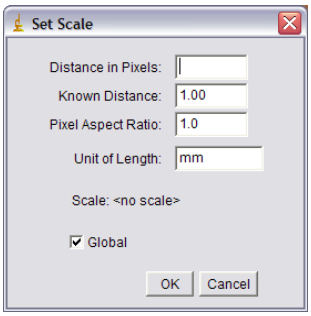
- Open image (*File-Open….*select all images of a series and click *Open*). Click through the images to go to the largest-sized ovary. Use this image for initial setup.
- Making long-axis (or one edge) of the ovary vertical (*Image-Rotate Canvas-Arbitrary…*- Enter estimated angle and direction of rotation-click *OK*). Undo the step (*Edit-Undo Rotate Canvas*) if the long-axis is not yet vertical. Repeat the procedure till you are satisfied. "Almost vertical" long-axis will be OK (it need not be perfectly vertical).
- Cropping to Fixed Dimension (*Click Marque tool* on the *Tools Bar*, i.e., the rectangle tool with dotted line at top left corner of the tool bar). *Options Menu Bar* will appear under the Regular Menus. In the *Styles*, click *Fixed Size*. Enter arbitrary *Width* and *Height* pixel dimensions. *Click* on the image to examine if the *Selection Box* (the box with moving ants) is large enough to include complete ovary. If not, enter new *Width* and *Height* pixel size and try again. Note the pixel dimensions on a piece of paper (just in case you if you have to close Photoshop before finishing!). Place your Cursor inside the box (it will change from cross-hair to arrowhead-with-rectangle) and hold left mouse button to move the box around. Bring the ovary in the centre of the selection box. Click *Image-Crop*.
- Save the image (*File-Save As…;* Set the Quality to 10 or more).
- Rotate the images 90 degree clockwise or anticlockwise (*Image-Rotate Canvas-90 CW or 90CCW*) to make the images Landscape mode (if needed). Some Images may be needed to *Flip* to match orientation.
- Process all images of the series using identical settings for the *Width* and *Height* size (very important!).

Processing Images with Image J

Download WCIF version of Image J for Windows (optimized for Confocal Microscopy) from **http://www.uhnresearch.ca/facilities/wcif/** Install the program, keep default directory locations and upgrade to recent version after installation (there is a *Upgrade Image J* Shortcut in the Shortcuts folder). Set the memory (*Edit-Options…-Memory…*) to 70% of your System Memory (maximum of 1.7Gb)

Setup Scale (Perform once per session – go to last step of this section if you already know the pixel : mm ratio)

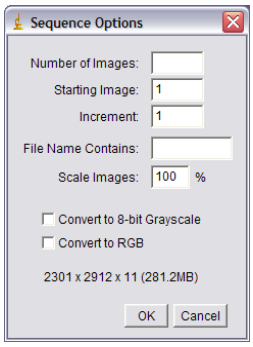
- *File-Open…* to open the (scanned) Ruler Image (need to be done only once till you change the magnification or DPI setting for your scanning, if you know the pixel : mm ratios, then directly go to last step in this section)
- Use *Line Tool* from *Tools Menu* (below the *Menu Tool Bar*) to select vertical (shorter) part of scale from the image
- *Analyze - Measure*
- Repeat procedure using Horizontal (longer) Scale (perpendicular to the first one) to verify that X and Y directions use the same pixel aspect ratio (should be 1)
- *Analyze – Set Scale* and input the values in *Distance in Pixels* (last measured value will already be there in the box), *Known Distance* (preferably in mm), *Pixel Aspect Ratio* (should be 1), *Units Length* and click *Global*.



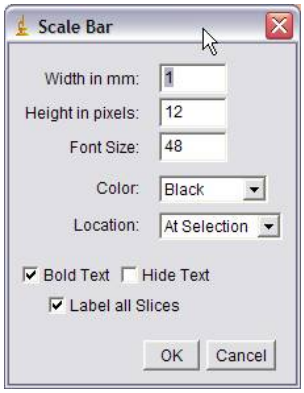
- Close the image after entering the values

Import and Align Stack

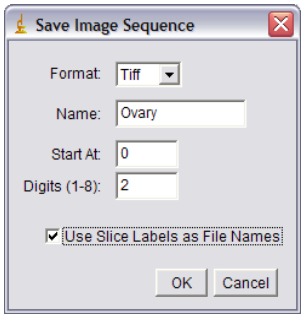
- *File – Import – Image Sequence…* to open the image stack (images from one set should be placed in one directory)



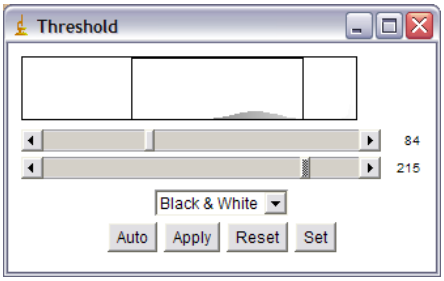
- *Plugins-Stack.Shuffling-Align Slices* (careful!! Don't use the *Align Slice*) and select *Rigid Body* in Dialog Box (This step takes long time to run)
- On the first image of the stack, select a rectangular area of the background using *Rectangle Selection Tool*. Slide through the stack to make sure that none of the remaining images have any part of the ovary touching this rectangle
- *Analyze – Tools – Scale Bar…* and fill in the *Dialog Box* with appropriate information. Select '*At Selection*' in *Location* Dialog and click *Label all Slices.* This action will imprint a Scale Bar on all images of the stack

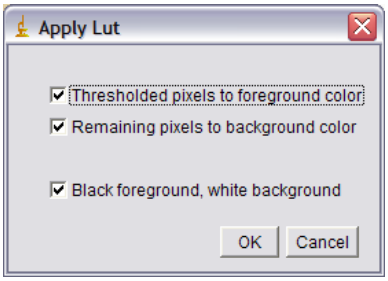


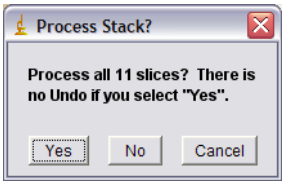
- *Edit – Selection – Select None* (or press *Ctrl+Shift+A*) to unselect the rectangle
- *File – Save As – Image Sequence…* and save as TIFF format with *Use Slice Labels as File Names* clicked. Click *OK* and click *Save* in the next Dialog Box (don't change the file name!)



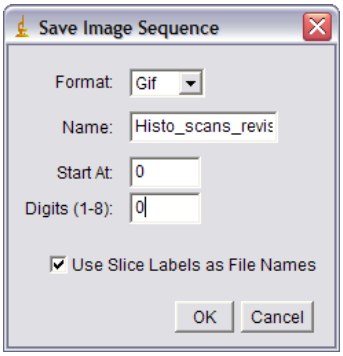
- *Image – Type – 8-bit* (this will change the stack to grayscale image)
- *Image – Adjust – Threshold* ….(or *Ctrl+Shift+T*) will open a slider box, slide controls to obtain darkest density for tissue and white density for background and follicle antrum. Click *Apply*, select all three boxes in *Apply Lut* Dialog Box and click *OK*. Click *Yes* on *Process Stack?* Dialog Box. Image stack will turn black and white (bitmap).







- *File – Save As – Image Sequence…* and save as GIF format with *Use Slice Labels as File Names* clicked. Click *OK* and click *Save* in the next Dialog Box (don't change the file name!)

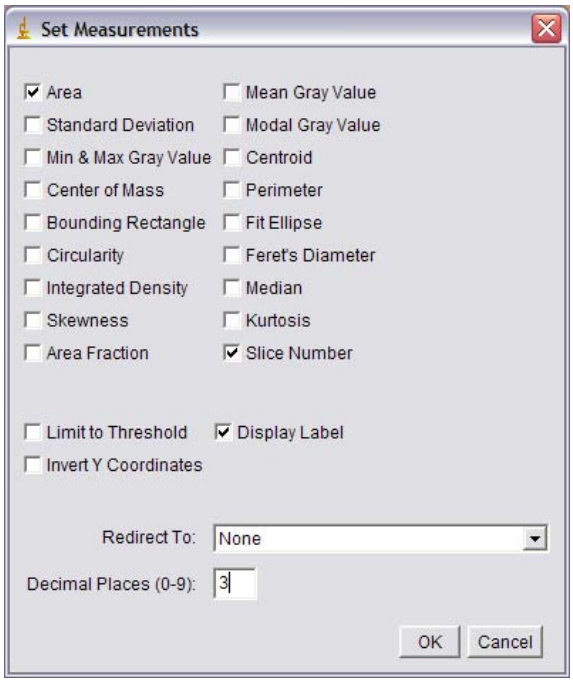


Measuring Volume of the Ovarian Stack

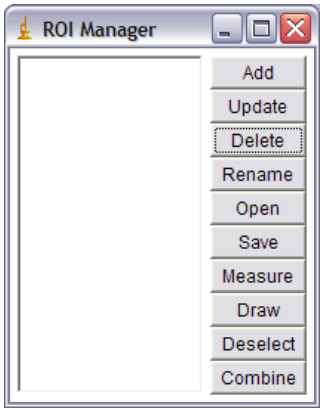
The purpose of this procedure is to calculate the volume of the ovarian tissue being analyzed. We need to exclude the volume occupied by the follicles greater than the 1mm in size. This step

will decrease the variability in ovarian volume during follicular wave development. CLs were already enucleated before processing the ovaries.

- *Image – Lookup Tables – Red* (white area will turn into red)
- *Analyze – Set Measurements.*…and click *Area, Slice Number* and *Display Label.* Set *Decimal Places* to 3

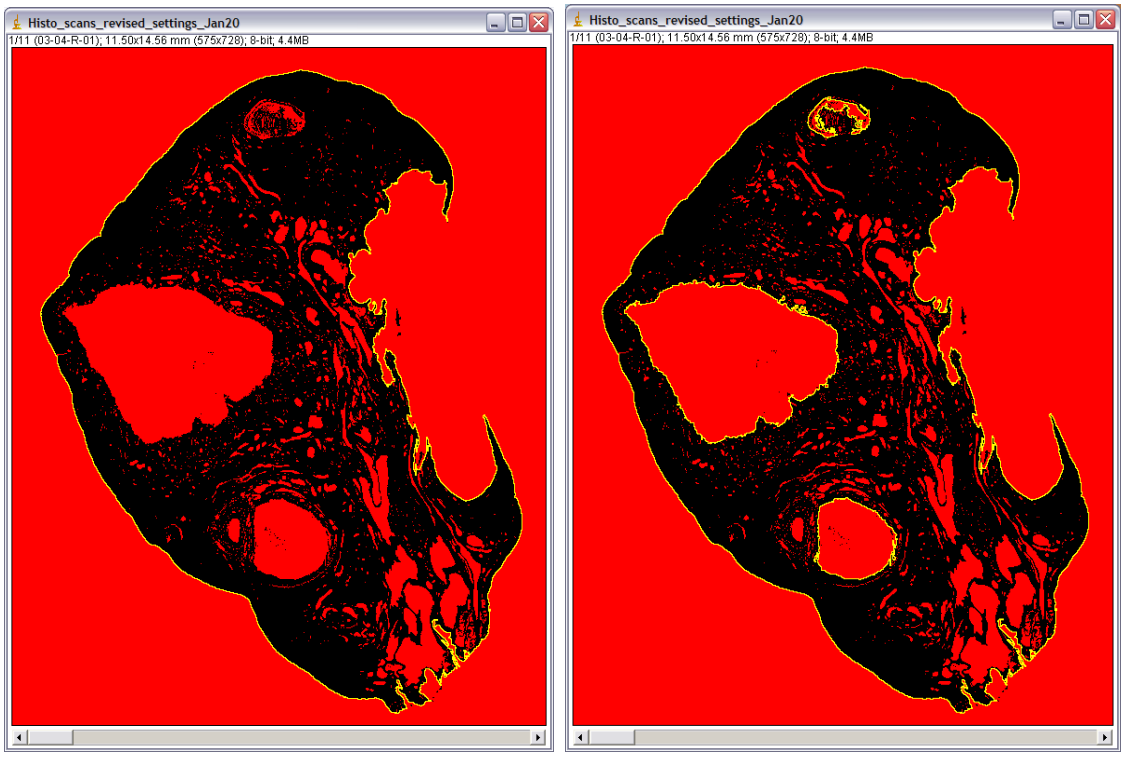


- *Analyze – Tools – ROI Manager* will open the following Window. Press *Delete* to clear the contents (if it shows some previous data). Keep this Window open

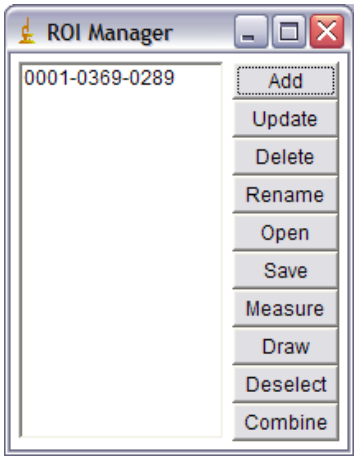


- Click the *Wand Tool* in *Tools Men*u
- Purpose of this step is select ovarian tissue excluding the area of follicles >1mm. Go to first image in the stack and click on the ovarian tissue (black part) close to the edge near right-

upper corner. A yellow line will appear outlining the ovary. Hold the *Alt* key to unselect antra (by clicking close to right-upper edge inside the antrum) of follicles larger than 1mm (a yellow selection line will appear outlining the follicle). Following example shows selection of ovarian tissue followed by exclusion of follicle antra.

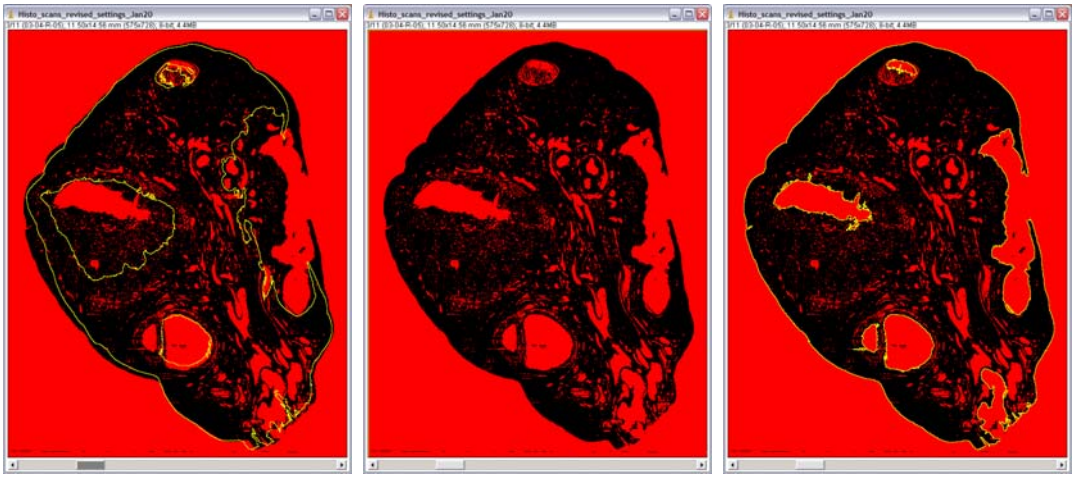


- Click Add in the ROI Manager Window

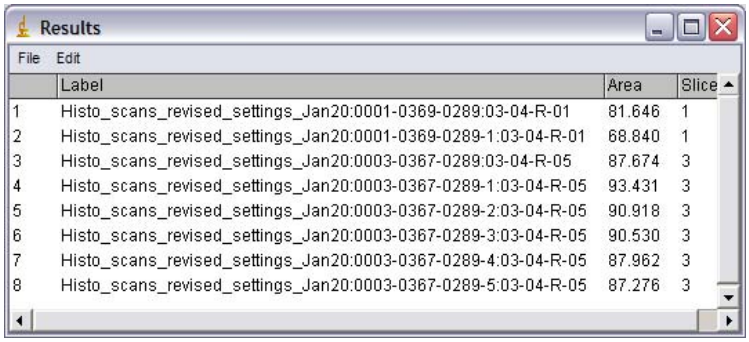


- Go to next image in the stack. It will show the yellow selection lines from previous image. Click outside the ovary to unselect (yellow lines should disappear). Repeat above two steps

to select ovarian area without antra of larger follicles and Add in the ROI Manager. Keep repeating till you reach last image



- Click Measure in the ROI Manager Window. A new Dialog Box will appear with area (in square mm) of each slice. Use slice numbers to make sure that all slices were included (in the following example measurement 3 to 8 were made on the same slice and slice 2, 4 to 11 were not used)



	Label	Area	Slice
1	Histo_scans_revised_settings_Jan20:0001-0369-0289:03-04-R-01	81.646	1
2	Histo_scans_revised_settings_Jan20:0001-0369-0289-1:03-04-R-01	68.840	1
3	Histo_scans_revised_settings_Jan20:0003-0367-0289:03-04-R-05	87.674	3
4	Histo_scans_revised_settings_Jan20:0003-0367-0289-1:03-04-R-05	93.431	3
5	Histo_scans_revised_settings_Jan20:0003-0367-0289-2:03-04-R-05	90.918	3
6	Histo_scans_revised_settings_Jan20:0003-0367-0289-3:03-04-R-05	90.530	3
7	Histo_scans_revised_settings_Jan20:0003-0367-0289-4:03-04-R-05	87.962	3
8	Histo_scans_revised_settings_Jan20:0003-0367-0289-5:03-04-R-05	87.276	3

• Go to *File – Save As* of *Results* Window and Save as ****.xls file (preferably using same name as stack in the same directory where images are located). You may choose to include results from all three stacks of a given ovary and save it as a single Excel file

Open the ****.xls file in Microsoft Excel as Tab Delimited file. Add area from all slices of a stack (or ovary) and multiply with the distance between adjacent slices (in mm) to obtain the analyzed volume for each stack (or ovary). In our case, distance between adjacent slices is 50micrometer or 0.05mm). For example, volume analyzed from above example will be 34.414 cubic mm {(81.646+68.84+87.674+93.431+90.918+90.53+87.962+87.276) x 0.05).