DEVELOPMENTAL PATTERN OF THE SMALL (1-3 mm) ANTRAL FOLLICLES IN CATTLE

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

in the Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

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ABSTRACT

Much has been improved in the basic and applied aspect of female reproduction after understanding of the wave-like developmental pattern of follicles ≥4 mm. However, it is speculated that the understanding of the developmental pattern of small follicles <4 mm may bring about efficient management of ovarian functions for essential reproductive interventions. Present studies were therefore, carried out to characterize the developmental pattern of 1-3 mm follicles in cattle using ultrasonography and to validate a method to histologically characterize the developmental pattern of follicles <1 mm using non-serial data. Transrectal ultrasonography was used once daily (n=18 Hereford-cross heifers) to examine changes in the diameter of follicles ≥1 mm for one interovulatory interval (IOI), as well as every 6 h (n = 9 Hereford-cross cows) from 5 to 13 days after ovulation to encompass emergence of Wave 2. A periodic shift in the peak number ($P < 0.05$) of 1-3 mm and ≥4 mm follicles and a significant inverse relationship ($P < 0.05$) between them suggested a wave-like developmental pattern. The number of 1-3 mm follicles detected in anovulatory waves did not differ ($P = 0.53$) between 2- versus 3-wave IOIs. A difference ($P < 0.05$) was noticed between anovulatory and ovulatory waves in 3-wave IOIs but not ($P = 0.63$) in 2-wave IOIs. The future dominant follicle was identified at 1 mm and was found to emerge 6-12 h earlier than the largest subordinate follicle ($P < 0.01$). Emergence of the future dominant ($r = 0.71$) and 1st subordinate ($r = 0.78$) follicles was temporally associated ($P < 0.05$) with a rise in circulating concentrations of FSH.

The developmental pattern of follicles not detectable by ultrasonography (<1 mm) may be assessed histologically by examining the ovaries from different animals on

different days (i.e., non-serial method) using follicle diameter or number profiles. A data set (n = 56 heifers) of follicles ≥4 mm was tabulated in a serial (same set of heifers each day; n = 7/day, N = 7) and non-serial (different set of heifers each day; n = 7/day, N = 56) manner for number and diameter profiles around emergence of the first follicular wave. Profiling of serial and non-serial data (serial and non-serial methods) revealed a change in the number of follicles (4-5 mm, 6-8 mm, ≥9 mm) over days ($P < 0.01$), but the effect of method and the day-by-method interaction were not different ($P > 0.28$). Similarly, the diameter of the dominant and first 2 subordinate follicles changed over days ($P < 0.01$), but the effect of method and the day-by-method interaction were not different ($P > 0.06$), indicating that non-serial data provide wave-like profile of follicles.

In conclusion, the hypothesis that follicles 1-3 mm develop in a wave-like manner was supported, and a new non-serial method was validated for the study of follicle dynamics using non-serial (e.g. histologic) data.

DEDICATED TO LATE PROFESSOR DR. HC PANT

ACKNOWLEDGEMENTS

Results of any research project are the outcome of teamwork. I would like to take this opportunity to thank everyone for his or her support. My parents, wife, children, brothers, sisters and their family members, all of them deserve my deepest gratitude for their selfless support to me. First of all I would like to express my deepest respect to Professor H.C. Pant (Consultant, National Dairy Development Board, India) who encouraged me and remained instrumental in sending me to this part of the world. I remained fortunate to have had good supervisors throughout my academic career and I take this opportunity to thank everyone of them: Dr. Ms. Mangla Kanchwar (B.Sc.supervisor), Dr. V.M. Mehta (M.Sc. supervisor), Dr. A.K. Misra (supervisor during my over 8 years of work in Embryo transfer & Frozen semen technology), Drs. Himanshu and Falguni Bavishi (with whom I worked on human IVF for 1 year) and Dr. Gregg Adams (supervisor for this M.Sc.). Dr. Adams is a great supervisor to work with. Whenever, I met him, I always got encouragement and thereby got lot of energy to accomplish my task. He is very kind, generous, enthusiastic, has just-do-it attitude, and most importantly has a transparent personality. I extend my sincere thank you to the members of my supervisory committee, Dr. Peter Flood, Dr. Reuben Mapletoft, and Dr. Jaswant Singh. I respectfully thank you Dr. NC Rawlings, Associate Dean of Research, for your very positive, sincere and supportive attitude. My special thanks to Mr. Bill Kerr, Manager (Goodale farm) whom I found very devoted to his work and was always there whenever I needed help during day, night, holidays, week days etc.

I would like to thank the Natural Sciences and Engineering Research Council for providing the funding for this research.

Ms. Tammy Orban and Dr. Miguel Dominguez, thank you for teaching me ultrasonography and extending everlasting friendship. I feel happy to thank Raj Duggavathi, who helped me understand statistics and to analyze my data. I must also thank the members of the Department of Veterinary Biomedical Sciences; Rob M^{c}Corkell, Marcelo Ratto, who have assisted me during the course of this project. I must also thank Sarah Caldwell, Jim Gibbons, Diane Matovich, Glenna Miller, Susan Cook, Janardhan Kyathanhali, Om Parkash Parbhakar, Laxman Gomez, Andrez Palaz, Marcello Martinez, Marcos Colazo, and Edward Bagu for their direct, indirect help and moral support.

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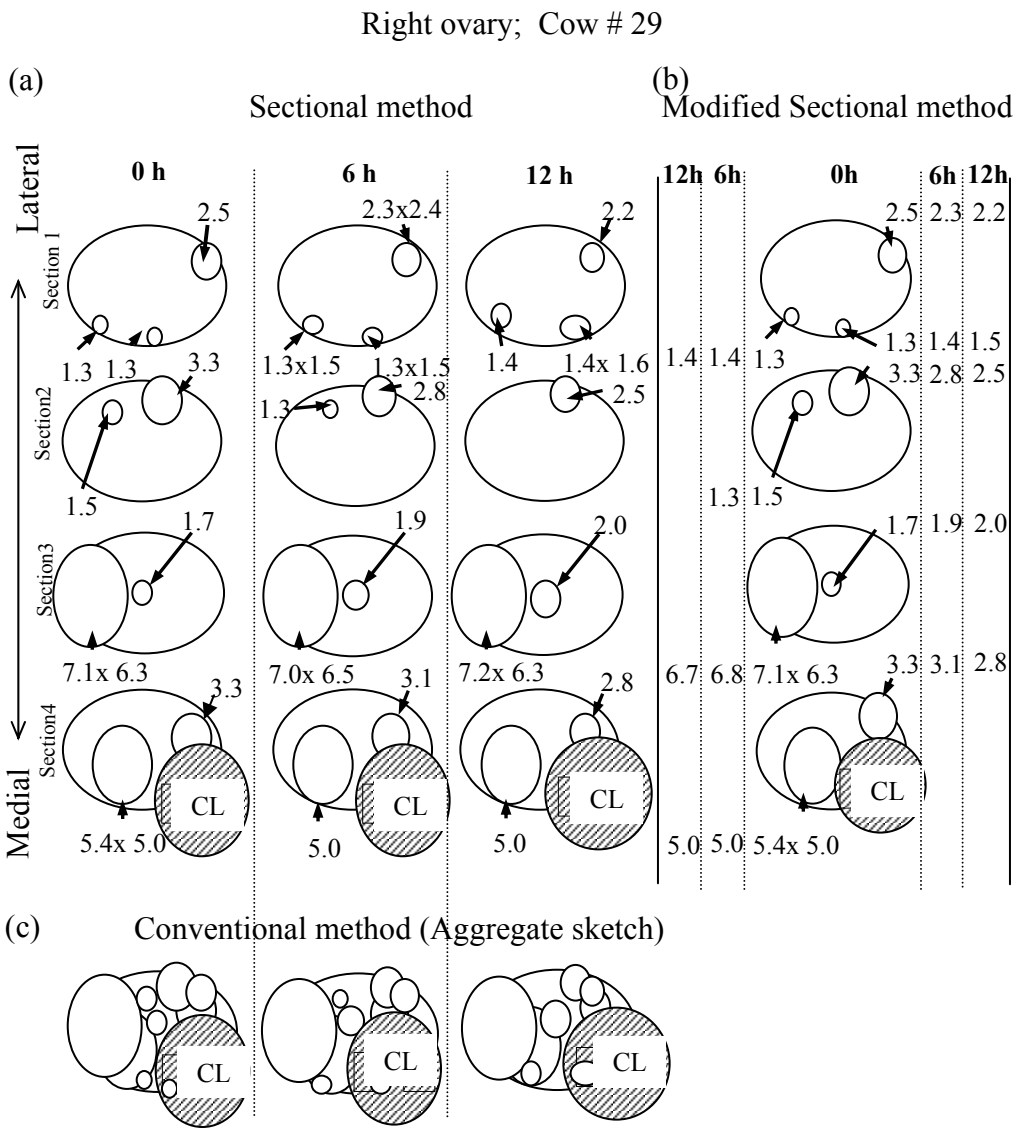

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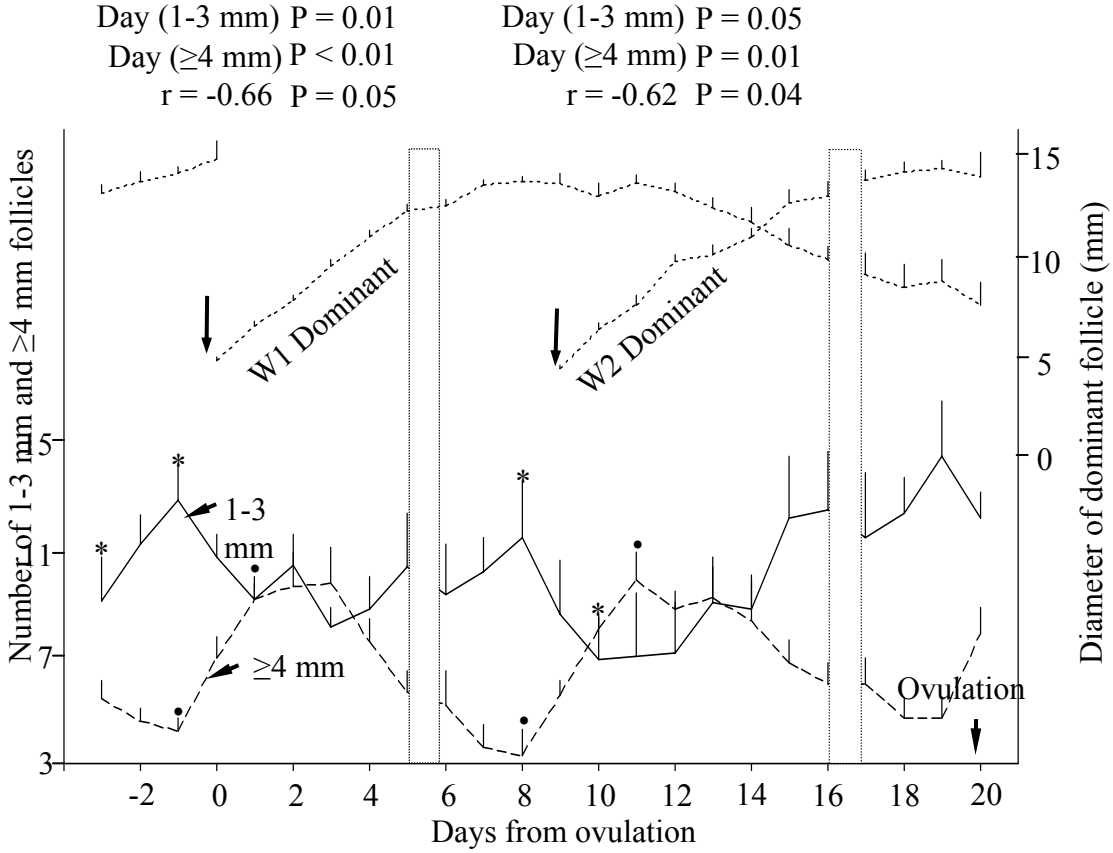

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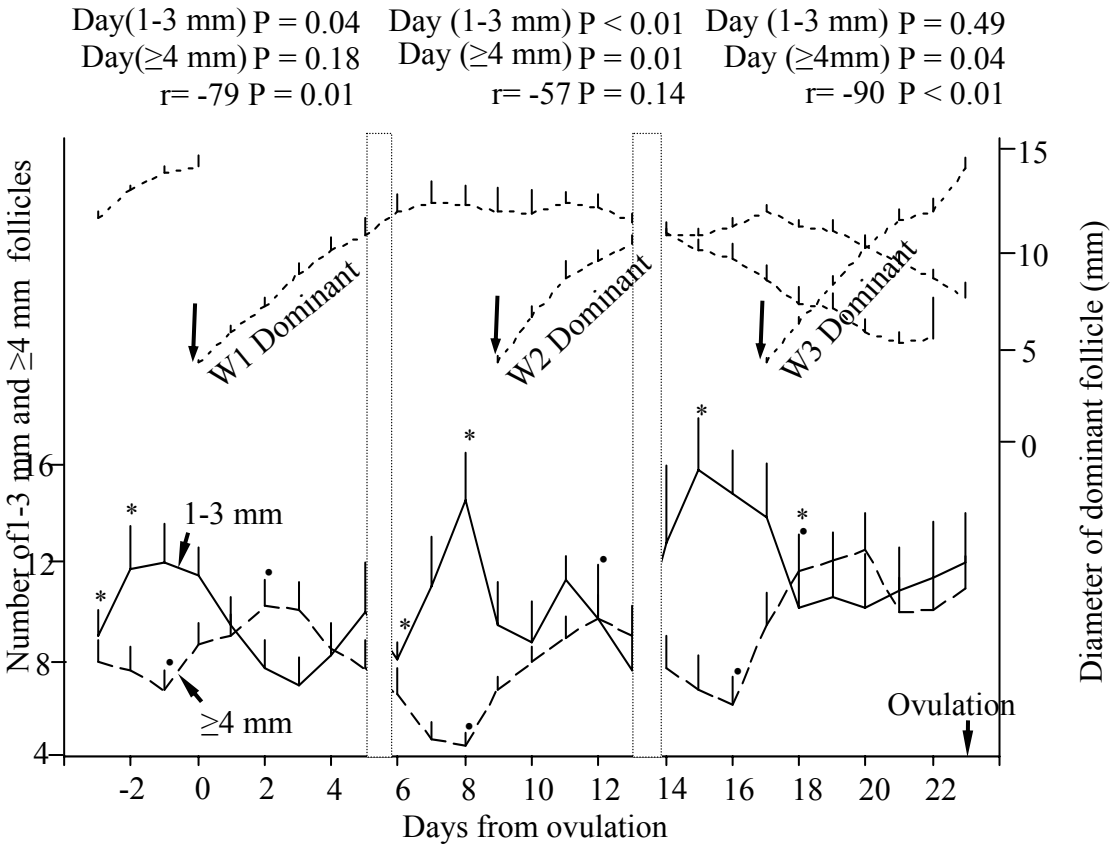

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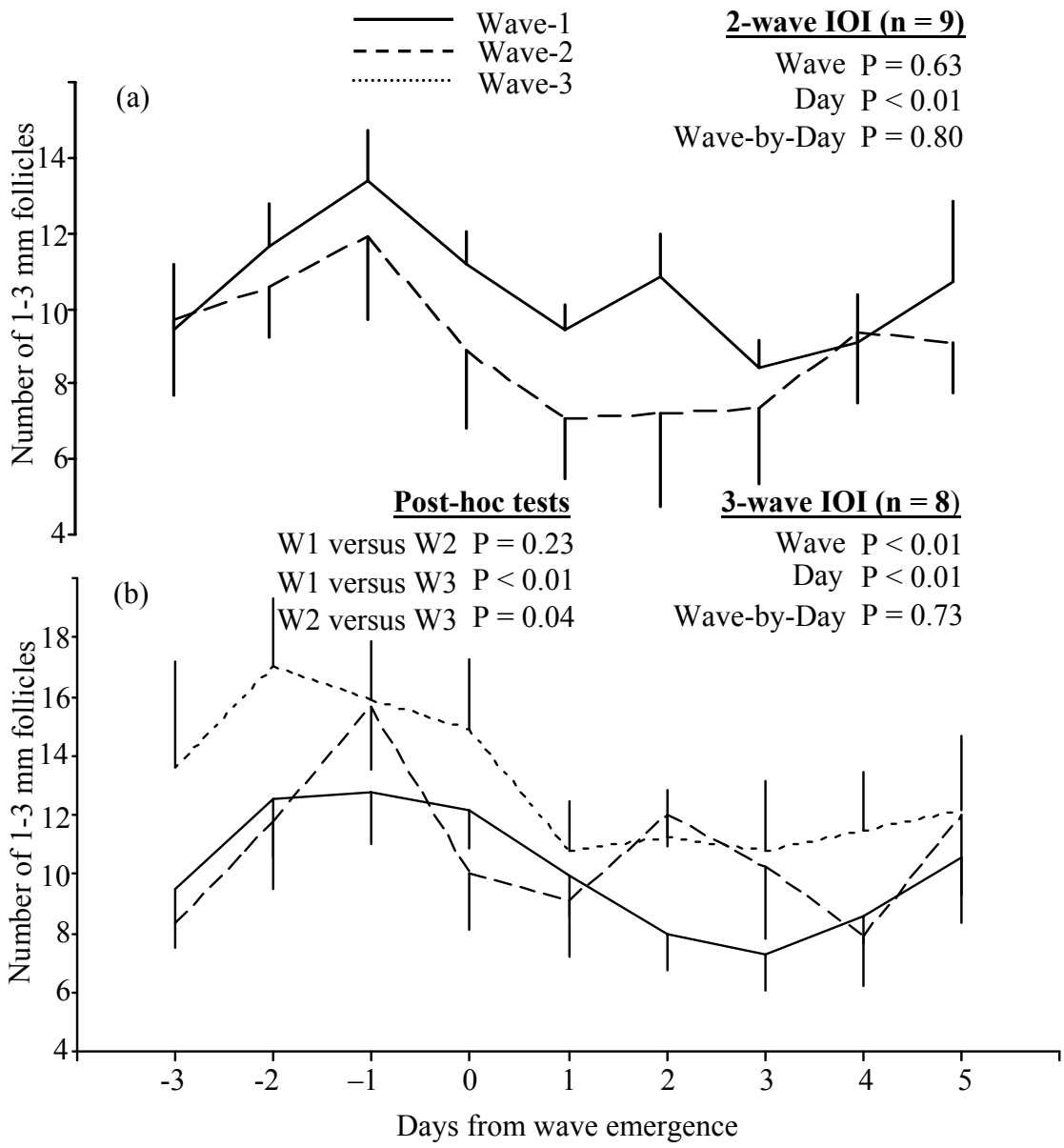

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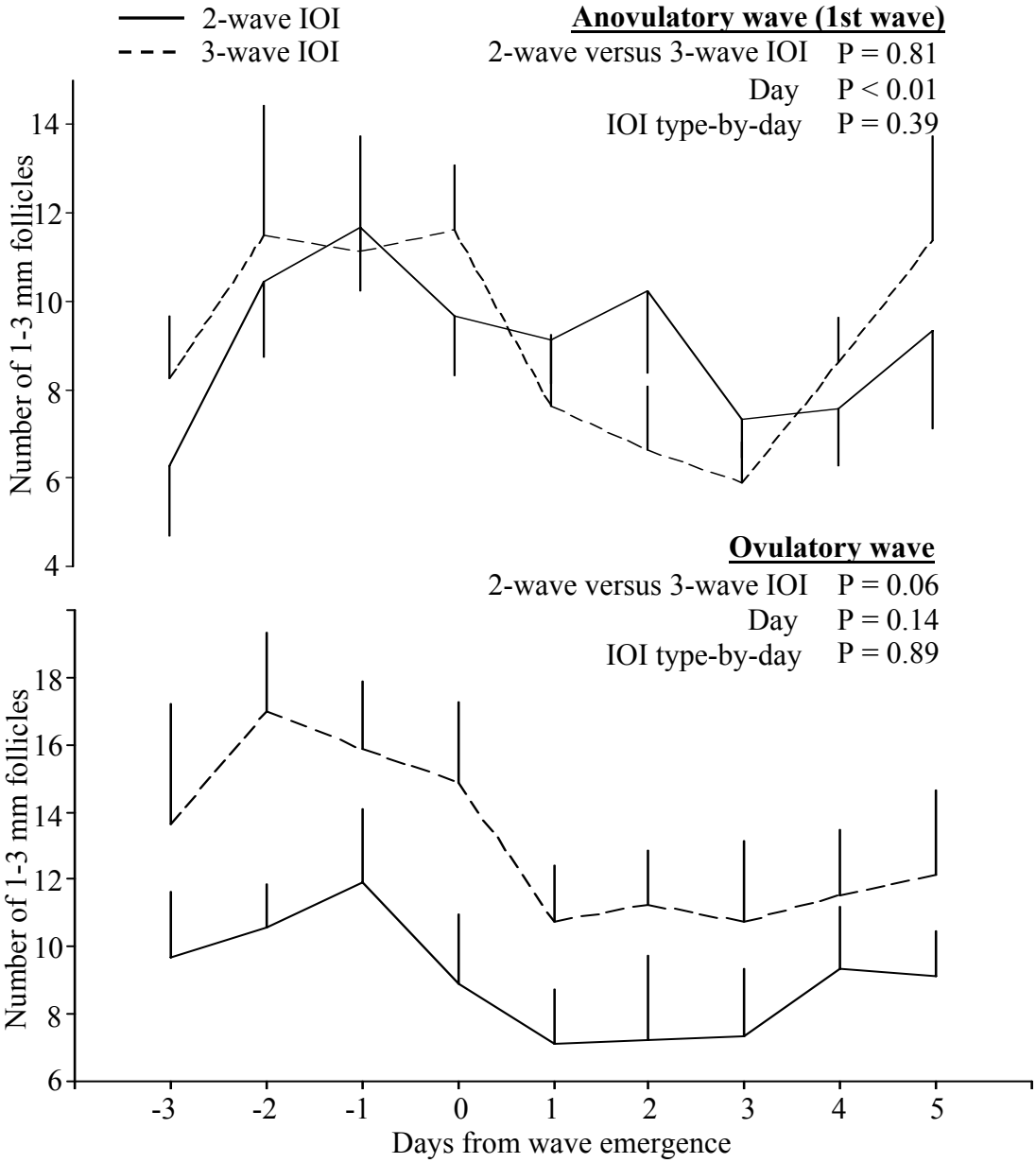

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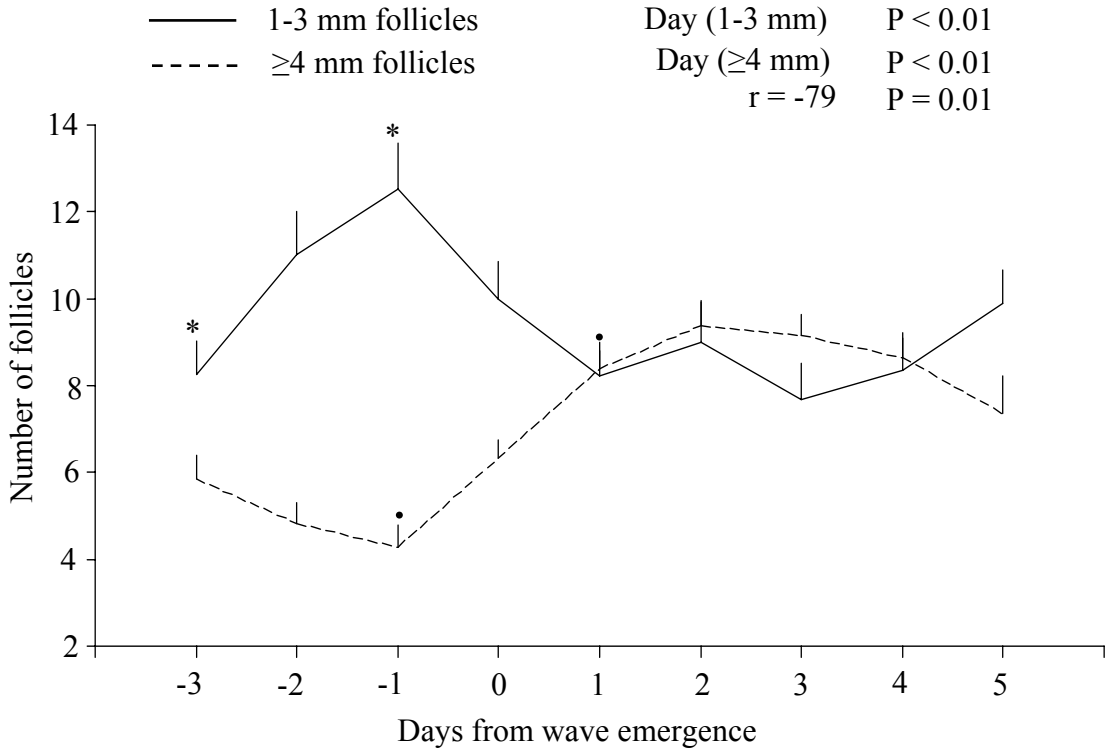

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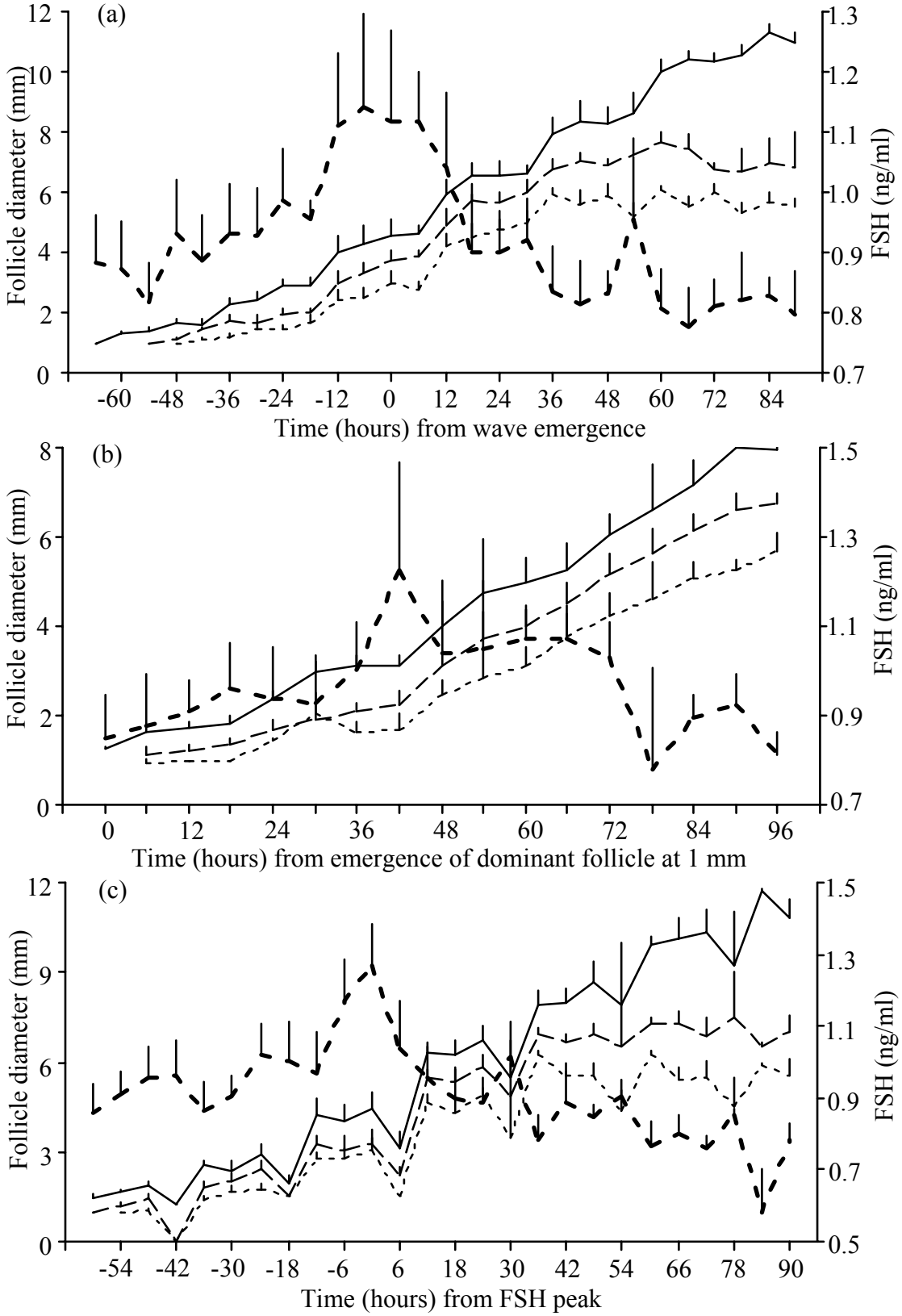
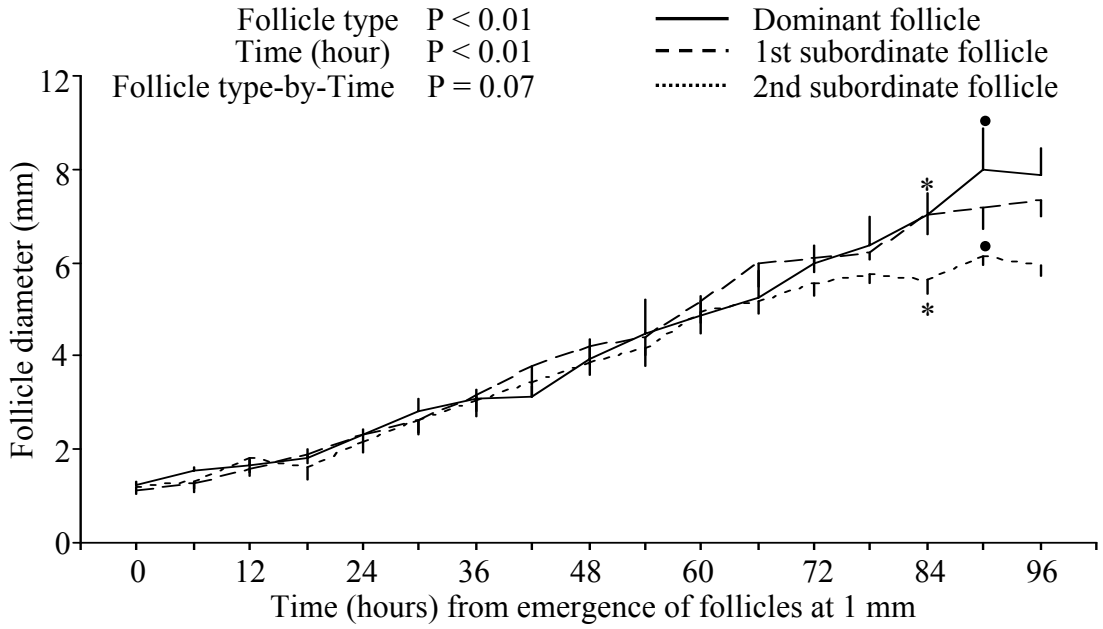
Figure 2.7


(a)
Follicle diameter (mm)
FSH (ng/ml)
Time (hours) from wave emergence
(b)
Follicle diameter (mm)
FSH (ng/ml)
Time (hours) from emergence of dominant follicle at 1 mm
(c)
Follicle diameter (mm)
FSH (ng/ml)
Time (hours) from FSH peak



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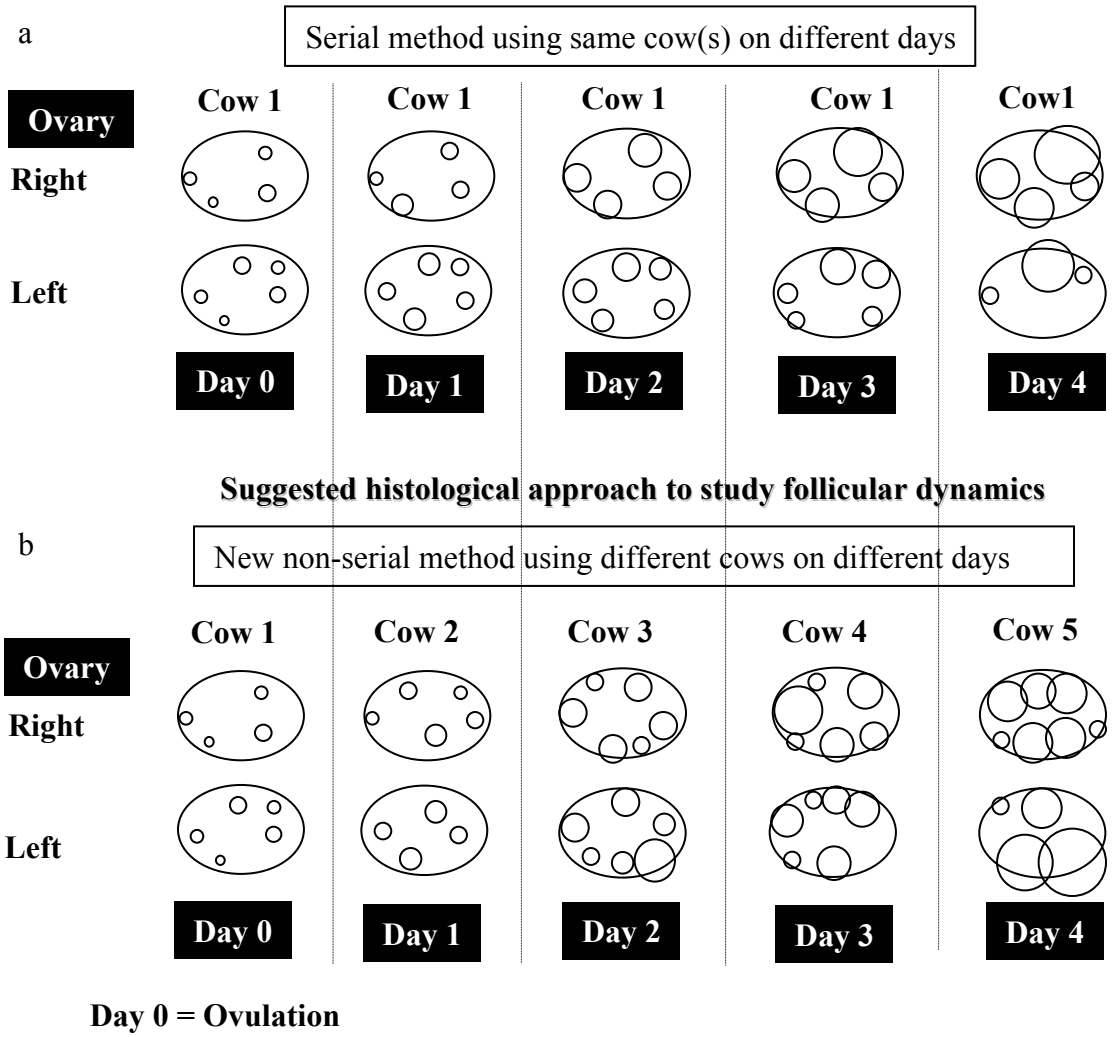

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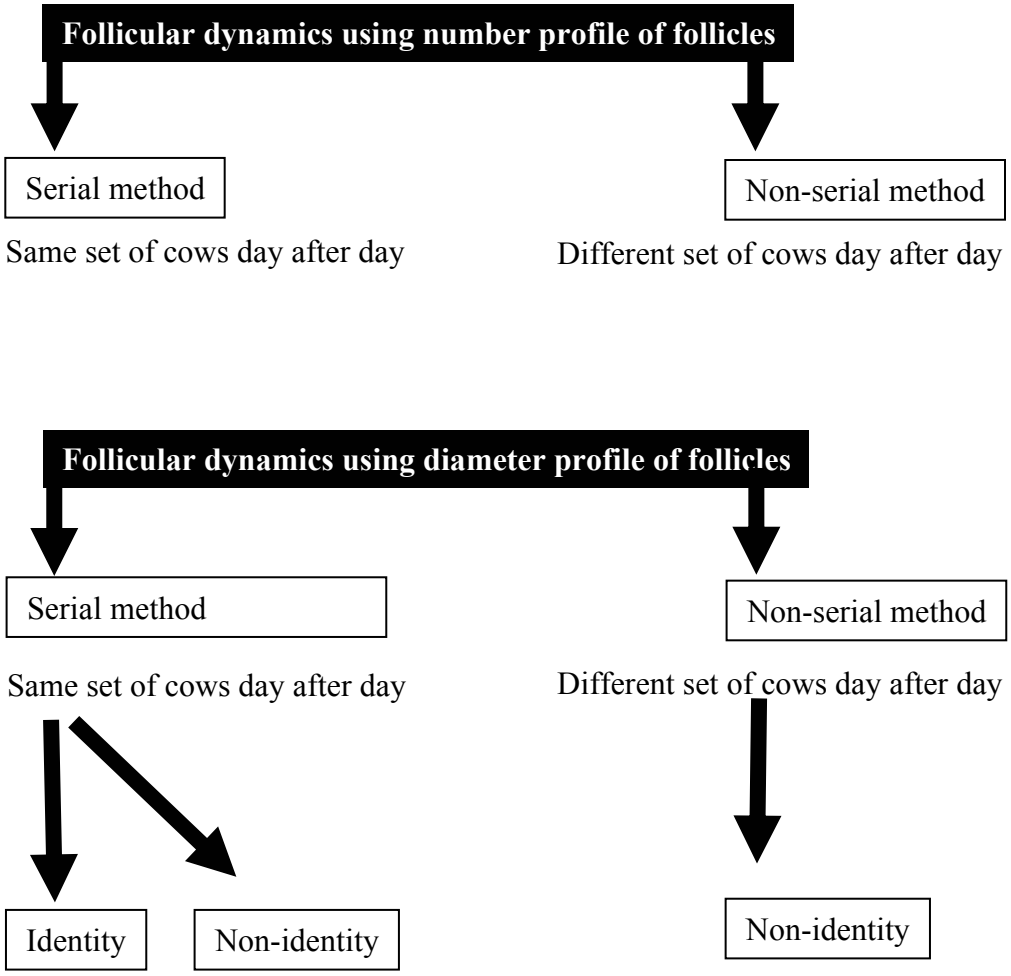

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Figure 3.3 Comparison of the number (mean ± SEM) of follicles in the (a) 4-5 mm, (b) 6-8 mm, and (c) □9 mm categories by serial (■ n=7/day; N=7) and non-serial (□ n=7/day; N=56) methods. The rise in the number of follicles in successive size categories on successive days is indicative of a wave pattern.

Figure 3.3

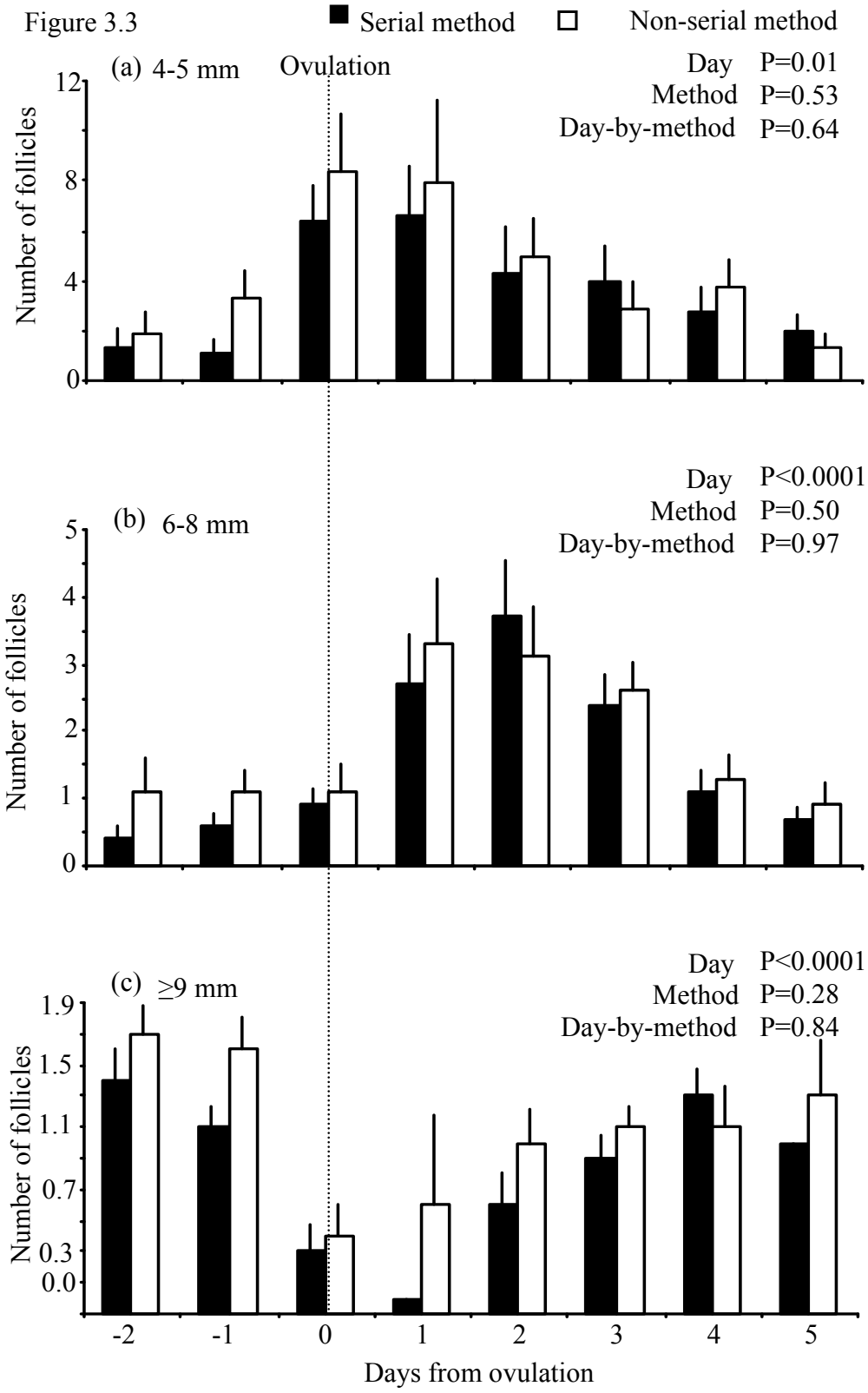
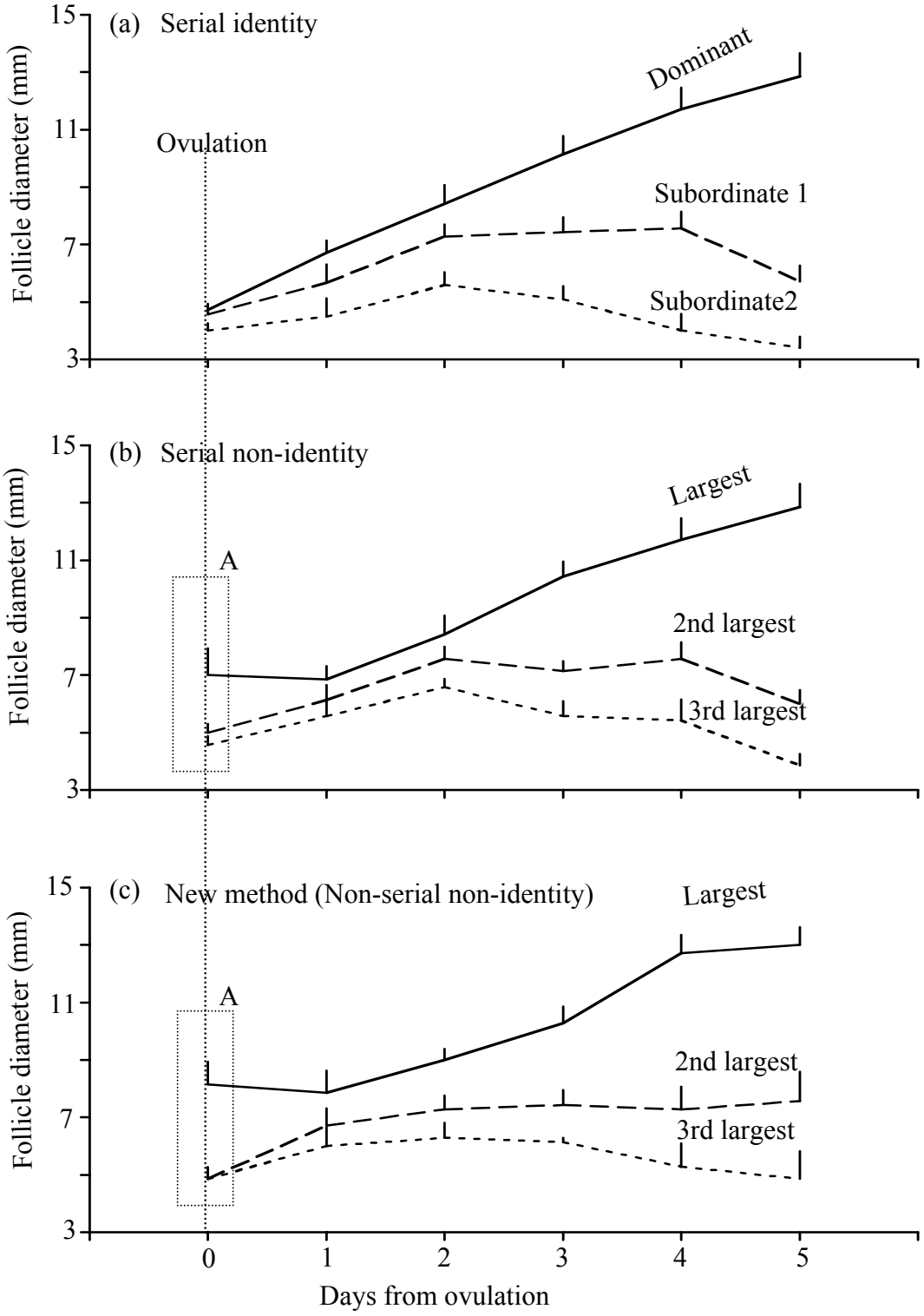

Serial method
Non-serial method
(a) 4-5 mm
Ovulation
Day P=0.01
Method P=0.53
Day-by-method P=0.64
Number of follicles
12
8
4
0
(b) 6-8 mm
Day P<0.0001
Method P=0.50
Day-by-method P=0.97
Number of follicles
5
4
3
2
1
0
(c) ≥9 mm
Day P<0.0001
Method P=0.28
Day-by-method P=0.84
Number of follicles
1.9
1.5
1.1
0.7
0.3
0.0
-2
-1
0
1
2
3
4
5
Days from ovulation

Figure 3.4 Diameter profiles (mean ± SEM) of 4 largest follicles compared by two conventional methods: (a) Serial (n=7/day; N=7) identity (b) Serial (n=7/day; N=7) non-identity; and a new method (c) Non-serial (n=7/day; N=56) non-identity. Methods were compared statistically for 3 largest follicles from Day 0 to Day 5 (Day 0=ovulation). A=area of ambiguity around ovulation, where the diameter profiles cross for successive largest follicles using non-identity methods. Largest (dominant) follicle: Day, P<0.0001; Method, P=0.06; Day x method, P=0.11. second largest (first subordinate) follicle: Day, P<0.0001; Method, P=0.39; Day x method, P=0.85. third largest (second subordinate) follicle: Day, P<0.0001; Method, P=0.42; Day x method, P=0.99.

Figure 3.4


(a) Serial identity
Dominant
Ovulation
Subordinate 1
Subordinate2
Follicle diameter (mm)
15
11
7
3
(b) Serial non-identity
Largest
A
2nd largest
3rd largest
(c) New method (Non-serial non-identity)
Largest
A
2nd largest
3rd largest
0
1
2
3
4
5
Days from ovulation

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

1. GENERAL INTRODUCTION

Artificial insemination has made possible the widespread dissemination of valuable germ-plasm from genetically superior males. Similarly, techniques such as estrus synchronization, superovulation (multiple ovulations compared to the usual single ovulation) and embryo transfer have made it possible to enhance the numbers of offspring that can be obtained from superior females. The manipulation of ovarian functions for various clinical and biotechnological purposes has been greatly improved with the understanding about the developmental pattern of later stages of follicular growth (follicle diameter ≥4 mm; Adams, 1994). However, imprecision in the degree of estrus synchrony and variability in response to superovulatory treatment remain the factors limiting the widespread implementation of advanced reproductive technologies in cattle (Adams, 1994). The limitation may be attributed to a lack of understanding of the developmental pattern of small follicles (i.e., <4 mm in diameter).

The total interval required by a bovine follicle to reach an ovulatory diameter from initial activation has been estimated to be 80-100 days (Britt, 1991). Although much has been learned by characterization of follicle dynamics during the final 10 days of development (ovulatory and anovulatory follicular waves), very little is known of the dynamics of the preceding lifespan of ovarian follicles. Recent studies have focused on factors regulating the growth of follicles as well as gene expression during different stages of follicular development. However, there is a need to understand the

fundamental pattern of development of all size categories of follicles to be able to predict or to manipulate ovarian function effectively. To address the issue of early follicular development, it is imperative to understand the embryonic development of ovarian follicles. This introduction will also include current information on large (≥4 mm) and small (<4 mm) follicles and approaches to understand small follicle development.

1.1 Embryonic development of ovarian follicles

The female gonads (ovaries) contain a large pool of follicles at various developmental stages. Follicles are blister-like sacs, which contain an oocyte or a female gamete.

1.1.1 *Formation of primordial germ cells (future oocytes) and gonadal ridges (future ovaries)*

Oocytes originate as primordial germ cells from the endoderm of the embryonic yolk sac (Byskov and Hoyer, 1994; Senger, 1997). Gonadal ridges are a pair of indifferent gonads (testis in male and ovaries in female), which develop as thickening of the coelomic epithelium on the medial aspect of the mesonephric kidneys. Thus, the early, non-differentiated gonad consists of a loose mesenchymal tissue covered by the coelomic epithelium and supported by the developing mesonephric tissue. The cell streams that connect the mesonephric tissue proper and the gonads are called the rete ovarii. The mesonephric kidney forms in the thoraco-lumber region and is the second of three consecutive nephric structures (pro-, meso-, and metanephros), that form during

the development of the urogenital system (Byskov and Hoyer, 1994; Dyce et al., 1996). The cells that originate from the coelomic epithelium have cuboidal or spherical nuclei and are classified as epithelial or somatic cells. The cells of the stratified medial aspect of the mesonephric kidney have elongated nuclei and an overall appearance of fibroblasts and are classified as mesenchymal cells (Hirshfield and DeSanti, 1995). The gonadal ridges are established by Day 32 of gestation in cattle (Erickson, 1966b).

1.1.2 *Migration of primordial germ cells to the gonadal ridge and their multiplication*

The primordial germ cells migrate by amoeboid movement from epithelium of the yolk sac via the connective tissue of the hindgut and eventually arrive at the gonadal ridges (Senger, 1997; Smitz and Cortvrindt, 2002) by Day 35 to 36 of gestation (Erickson, 1966b). Primordial germ cells are termed oogonia upon arrival at the gonadal ridges (Smitz and Cortvrindt, 2002). When the primordial germ cells arrive at the coelomic epithelium covering the gonadal ridge, they seem to be "trapped" by processes from the epithelial cells (Merchant and Alvarez, 1986). Soon, thereafter, primordial germ cells are present in the underlying tissue of the gonadal ridge. Due to the passage from coelomic epithelium to underlying gonadal tissue, at first the coelomic epithelium was mistakenly considered a germinal epithelium (Merchant and Alvarez, 1986).

During their migration, as well as upon arrival at the gonadal ridges, the primordial germ cells multiply rapidly by mitosis (Smitz and Cortvrindt, 2002). In cattle, the definitive ovary is established by Day 40 of gestation (Erickson, 1966b). Germ cell cords (ovigerous cords) develop within the ovary as accumulations of several germ cell clusters. Not all of the germ cells within the clusters are in the same mitotic cycle in

ruminants (Russe, 1983). The germ cell cords are masses of epithelial cells and oogonia (Russe, 1983; Hirshfield and DeSanti, 1995; Dyce et al., 1996) which are delineated by a basal lamina, clearly defining them from surrounding mesenchymal cells (Byskov and Hoyer, 1994). Hence, three cell types are recognizable in the early ovary: 1) the cells of the germ cell cords, which include epithelial cells (i.e., somatic cells, which are precursors to the future follicular or granulosa cells); 2) germ cells (future oocytes); and 3) the mesenchymal, stromal or interstitial cells (future theca interna and externa cells) present between the cords. In cattle, intense oogonial mitotic activity, characterized by a sudden increase in the number of mitotic figures per ovary (304 at Day 60 versus 13 at Day 50) begins at Day 60 (Erickson, 1966b). The number of mitotic divisions of the oogonia is limited (Russe, 1983). During the development of the ovary, the more mature oogonia become deeply embedded in the ovarian tissue due to the increase in the size of the growing gonad (Stein and Anderson, 1979). The more peripherally located primordial germ cells (stem cells) give rise to new oogonia (Russe, 1983).

The oogonia undergo either immediate meiosis (e.g., mouse, rat, hamster) or delayed meiosis (e.g., pig, sheep, dog, cow). In the female fetus, the beginning of oogonial meiosis is delayed up to 45 days with respect to testicular differentiation in male fetus and during this delay period, the oogonia become enclosed in ovigerous cords (Byskov and Hoyer, 1994). By the end of the delay period; i.e., Day 75 to 80 of gestation in cattle, meiosis of the oogonia begins (Erickson, 1966b; Byskov and Hoyer, 1994). Reports are contradictory as to whether the ovigerous cords begin to break up (Byskov and Hoyer, 1994; Dyce et al., 1996) or begin to form (Erickson, 1966b) with the initiation of oogonial meiosis. With the disruption of the ovigerous cords, the ovary divides into cortical and medullary parts (Erickson, 1966b; Smitz and Cortvrindt, 2002).

The proximity of the oogonia to the mesonephric cells of the rete ovarii is implicated in the initiation of meiosis as the first oogonia to begin meiosis are localized at the inner part of the cortex (reviewed by Byskov and Hoyer, 1994). The association between oogonia and mesonephric cells of rete ovarii forms the basis of the hypothesis that oocytes that are first entering meiosis are also the first to be released by ovulation later in life i.e., first in-first out, last in-last out (Polani and Crolla, 1991; Hirshfield, 1992). 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). The epithelial cells condense around the oogonia and enclose it in a single flattened layer delineated by a basal lamina (Russe, 1983; Hirshfield, 1989; Byskov and Hoyer, 1994; Eppig, 2001).

1.1.3 *Transformation of oogonia into primary oocytes and formation of primordial follicles*

The oogonial germ cells enlarge after the meiotic process is initiated and are then defined as primary oocytes (Byskov and Hoyer, 1994, Smitz and Cortvrindt, 2002). Germ cells passing through the transitory stages of the meiotic prophase appear to be extremely vulnerable (Beaumont and Mandl, 1962). In the fetal human ovary only about 5% of the peak numbers of germ cells reach the resting diplotene stage of first meiotic prophase (Baker, 1963). The vast majority of the surviving oocytes acquires a single

layer of flattened epithelial cells and thus forms primordial follicles (Erickson, 1966b). All remaining oocytes, which were not surrounded by epithelial cells invariably, degenerate (Wagenen and Simpson, 1973; Smitz and Cortvrindt, 2002). The first meiotic division of primary oocytes is arrested at the pachytene stage of prophase I (Erickson, 1965). During the pachytene stage of prophase I, the chromosomes decondense and are packed within a nucleus known as the germinal vesicle (Smitz and Cortvrindt, 2002). Primordial follicles in bovine ovaries constitute the lifetime reservoir of follicles (approximately 68000 at birth; Erickson, 1966b). Since the reserve of primordial follicles is not renewable, the original endowment is gradually depleted throughout the life of a cow (Erickson, 1966a; Gosden et al., 1983) until it is near zero when the cow is 15 to 20 years of age (Erickson, 1966a). The fate of more than 99% of all follicles entering the growing pool is atresia (Ireland, 1987).

1.2 Activation of primordial follicles

Some of the primordial follicles start to grow immediately (Russe, 1983) as soon as they are formed during fetal stage. Most of the primordial follicles remain in the resting pool, which is depleted during life either by entry of primordial follicles into the growth phase or by atresia (Erickson, 1966b, Gougeon, 1996). Once the growth of an individual follicle has begun, it is continuous and sequential until the follicle either ovulates or becomes atretic (Peters and Levy, 1966).

Throughout the reproductive life of an individual, the primordial follicles start to develop either at random or in response to an unknown trigger (Webb et al., 1992). Some studies indicate that the mechanism of activation of the primordial follicles is

hierarchical in nature and is controlled. In a study, a high percentage of primordial follicles dissected from the same ovarian tissue initiated growth when cultured in vitro. This led the authors to conclude that the ovarian stroma exerts inhibitory control over the initiation of primordial follicle growth in vivo (Wandji et al., 1996). Contrary to this, it was reported that the rate at which resting (non-growing) follicles are stimulated to initiate their development appears to depend, in part, on the size of the pool of primordial follicles (Krarup et al., 1969), and that other factors, including those present in the follicular fluid of antral follicles, may act to control the number of follicles initiating their growth (Peters et al., 1973). Experiments designed to test the theory of "first in first out, last in last out" (Polani and Crolla, 1991; Hirshfield, 1992) support the idea that oocytes entering meiosis first are also the first to be released by ovulation later in life. Perhaps, the hierarchy in the formation of the oocytes may be manifested in the form of follicular waves in succession.

1.3 Primordial follicle growth and developmental stages

In cattle, the initiation of follicle growth is characterized by the transformation and proliferation of flattened precursor cells to cuboidal cells, termed hereafter as follicular or granulosa cells (Eppig, 2001). Subsequent growth of the follicle is characterized by an increase in the number of granulosa cells and is accompanied by a rapid increase in the size of the oocyte and formation of the zona pellucida (Braw-Tal and Yossefi, 1997; Lundy et al., 1999; McNatty, 1999). There is an increase in the extent of the blood capillary network outside the basement membrane together with a concentric alignment and differentiation of theca interna cells. The fluid-filled spaces (pockets) appear

between the granulosa cells throughout these preantral growth phases. By the time 5 to 8 layers of granulosa cells have formed, these pockets of fluid coalesce to form an antral cavity. Thereafter, the growth to ovulation is referred to as antral follicular growth (Lundy et al., 1999).

1.4 Classification of the developmental stages

Folliculogenesis is the process whereby ovarian follicles develop from primary into secondary and eventually into antral follicles, which become eligible for ovulation (Senger, 1997). In cattle, folliculogenesis begins during fetal life (Russe, 1983) and primordial, primary, secondary and tertiary follicles appeared at Days 90, 140, 210 and 250 of gestation, respectively (Russe, 1983).

The classification of the developmental stages of a follicle is based on the shape and configuration of granulosa cells around the oocyte and on oocyte and follicular diameters (Braw-Tal and Yossefi, 1997, Lundy et al., 1999). After activation of a primordial follicle, the flattened precursors to the follicular (granulosa) cells undergo transformation to cuboidal cells and form a primary follicle. The Primary follicle has a single layer of cuboidal granulosa cells without any antrum (Braw-Tal and Yossefi, 1997). The granulosa cells in the primary follicle then undergo proliferation. Based on increasing layers of granulosa cells in a follicle's largest cross-section, the follicle is defined as a secondary (2-6 layers of granulosa cells; no antrum) or tertiary (> 6 layers of granulosa cells; presence of fluid-filled antrum) follicle. A tertiary follicle is also referred as a vesicular or antral follicle. The oocyte continues to grow concurrent with the follicular growth (Eppig, 2001). Follicles as small as 0.25 mm in diameter are termed

as antral follicles, and well-developed pre-ovulatory antral follicles (14-16 mm) are called Graafian follicles. The Graafian follicle after the preovulatory gonadotropin surge is referred to as an ovulatory follicle (Braw-Tal and Yossefi, 1997). The classification of follicles is based on the relationship between the number of granulosa cells, the number of granulosa cell layers, follicle diameter, and the oocyte diameter, as well as other important morphological changes that occur at different follicular stages (Table 1.1).

Table 1.1 Characteristics of bovine ovarian follicles during development

Follicle stage	Follicle type	FSHr & LHr	Granulosa cell layers*	Granulosa cells per section *	Follicle diameter (mm)	Oocyte diameter (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	Absent	Absent
Secondary	3 Small preantral		2-3	41-100	0.08-0.13	50	Begins to form	Begins to form
	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	
	Graffian ovulatory			40×10^6	16-18	132		

(Lussier et al., 1987; Xu et al., 1995; Braw-Tal and Yosefi, 1997)
*Largest cross-section of the follicle is defined as the section where the nucleolus of the oocyte is present
§ Oocyte commenced growth when there were at least 40 granulosa cells in the largest cross-section (fourth generation of follicle cells)
§§ The beginning of an antrum formation was observed in follicles with at least 250 granulosa cells in the largest cross-section.

1.5 Role of FSH in follicular activation and early follicular development

The later stages (4-9 mm) of follicular growth require FSH (Adams et al., 1992a). The circulating concentrations of FSH during the estrous cycle display a pattern of recurrent surges, and each surge precedes the emergence of a cohort of 4-5 mm follicles (Adams et al., 1992a; Gong et al., 1995). Contrary to the definitive role of FSH in the development of large follicles, its role in the development of small follicles is controversial. In cattle, active immunization against GnRH resulted in anestrus

(Prendiville et al., 1995), reportedly due to a decrease in estradiol concentrations (Prendiville et al., 1996). Follicles did not grow to more than 5 mm in diameter, demonstrating a requirement for gonadotropins in the later stages of follicular growth. In other words, the study demonstrated that gonadotropins might not be required for the small size categories of follicles. It was also reported that follicles could grow up to 2 to 4 mm in diameter in cattle either in the absence of gonadotropins or in the presence of very low concentrations of gonadotropins (Driancourt et al., 1991; Webb and Armstrong, 1998). Similar studies were reported in the rat (Camp et al., 1991) and sheep (Campbell et al., 1995). In another study (Bao and Gavernick 1998), the intensity of mRNA expression for FSHr was reported to be similar in the granulosa cells of follicles from the primary stage up to 2 mm in size, thus raising question about the role of FSH during early stages of follicular growth.

In cattle, there is evidence indicating expression of FSH receptor mRNA in primary follicles (with only one layer of granulosa cells; Xu et al., 1995; Bao and Gavernick 1998), which suggests that FSH may have a role in the early stages of follicular development. Exogenous gonadotropins have been reported to stimulate the onset of follicular growth in the mouse (Lintern, 1977). Various studies of cultured preantral follicles (Hulshof et al., 1995; Gutierrez et al., 2000, Ralph et al., 1995, 1996) supported the involvement of FSH in growth and development of preantral follicles. Small follicle growth was retarded in hypophysectomized fetal monkeys (Gulyas et al., 1977) and rats (Hirshfield, 1985). The FSH along with LH and insulin plays a synergistic role in the growth and development of bovine large preantral follicles in vitro (Itoh et al., 2002). FSH has also been reported to suppress apoptosis in serum-free culture of rat preantral (Mcgee et al., 1997) and antral (Tilly and Tilly, 1995) follicles

suggesting that the physiological role of FSH may be to prevent atresia in preantral and antral follicles. 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 the fetus, as well as in adult cows, the number of follicles and stages of follicular development were associated with changes in the concentration of FSH (Tanaka et al., 2001). In rats, a correlation between elevated levels of FSH in the early neonatal life and the high rate of activation of primordial follicles (Dahl et al., 1988) was detected. The correlation was confirmed by injecting GnRH antagonists into rats, which suppressed high endogenous levels of FSH as well as suppressed the size of the total pool of growing follicles. The effect of FSH on small follicle development was tested in vivo, wherein ovaries of newborn rats were isografted to the kidney capsules of ovariectomized (gonadotropin-rich) or ovariectomized-hypophysectomized (gonadotropin poor) adult hosts (Arendsen, 1982). Morphometric examination of the isografts 15 days later revealed that the grafts in the gonadotropin-rich environment had more growing follicles and fewer primordial follicles than those in the gonadotropin-poor environment, leading to the conclusion that gonadotropins, especially FSH, seem to have a stimulatory effect on early follicle cell development and early oocyte growth. The number of small pre-antral follicles was increased in the hypophysectomized mice following administration of exogenous FSH (Wang and Greenwald, 1993 a, b). Similar studies were reported in humans (Abir et al., 1997) and sheep (Cecconi et al., 1999).

1.6 Growth rate of growing follicles

The growth rates of follicles that have entered the growing phase have been estimated at different stages of development in cows (Marion and Gier, 1971; Lussier et al., 1987). These studies calculated the number of granulosa cells in follicles of various size classes and the time required to double the number of granulosa cells within a follicle. According to a study in cattle, a follicle takes 27 days to grow from 0.13 mm to 0.67 mm, 6.8 days from 0.68 mm to 3.67 mm, and that 2 estrous cycles were required for preantral follicles to reach the mature antral stage (Lussier et al., 1987). Information about granulosa cell generation, as well as the time spent by a follicle in different size classes, is presented in Table 1.2. The total interval required by a bovine follicle to reach an ovulatory diameter from initial activation has been estimated to be 80-100 days (Britt, 1991). The time required to double the number of granulosa cells visible in the largest cross section through a follicle (termed generation interval) indicated that the initial stages (follicle <0.5 mm in diameter) of follicular growth proceed slowly (Scaramuzzi et al., 1980) compared with stages after antrum formation (Lussier et al., 1987; Scaramuzzi et al., 1980). In cattle, the formation of a preovulatory follicle requires more than 10 generations of granulosa cells and the incidence of atresia is highest (>30%) after 8^{th} generation (Table 1.2) of granulosa cells compared with only 6-7% up to 7^{th} generation (Lussier et al., 1987). The growth rates of follicular development also were estimated in rodents (Pedersen, 1970; Oakberg and Tyrell, 1975; Hirshfield, 1984) and sheep (Cahill and Mauleon, 1980), as well as in women (Gougeon, 1982). However, these estimations do not provide information of day-to-day follicular dynamics at early stages of development.

Table 1.2 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)

1.7 Developmental pattern of follicles

It has been well documented in cattle that the final stages of follicular development (follicles ≥4 mm) occur in a wave-like pattern (Pierson and Ginther, 1988b; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a,b,c; Knopf et al., 1989). The wave pattern refers to periodic, synchronous growth of a group of antral follicles. The majority (>95%) of bovine estrous cycles consist of 2 or 3 waves of follicular development (reviewed in Adams, 1999) each of which is characterized by a preceding surge in circulating concentration of FSH (Adams et al., 1992a) followed by a sudden (within 1 to 2 days) appearance of several follicles 4-6 mm in diameter, as detected by serial ultrasonography (Ginther et al., 1989a). Follicles of the cohort grow at a similar rate for about 2 days, followed by preferential growth of one (dominant) follicle over the others

(subordinates) in a process referred to as selection. The dominant follicle suppresses growth of its subordinates (Adams 1993 a, b; Bodensteiner et al., 1996; Mihm et al., 1997; Bigelow and Fortune, 1998) and continues to grow for about 6 days (Ginther et al., 1989a). The dominant follicle stops growing in the presence of a functional CL and enters a static phase followed by a regressing phase. If luteal regression occurs during the growing phase, the dominant follicle will go on to ovulate (Kastelic et al., 1990; Bergfelt et al, 1991; Lucy et al., 1992). In either instance, a new follicular wave starts (Adams et al., 1992b; Ginther et al., 1996; Gibbons et al., 1997).

The wave-like developmental pattern of follicles ≥4 mm in cattle was documented through the use of transrectal ultrasonography (reviewed in Adams, 1999). The experimental approach involved daily examination of the ovaries (serial data) to identify and monitor the diameter of individual follicles recognized by their position within the ovary (the serial identity method; Knopf et al., 1989). The wave-like developmental pattern of follicles ≥4 mm was also characterized by a method involving repeated examination of the ovaries without a necessity of individual follicle identification (the serial non-identity method; Ginther, 1993).

The mechanisms controlling recruitment of primordial follicles into the growing pool, and controlling the stage at which growing follicles may join follicular waves are unknown. However, based on the well-documented developmental pattern of large follicles (≥ 4 mm), we hypothesized that small follicles (< 4 mm) develop in a wave-like manner. The fact that the number of follicles in successive waves is similar (Boni et al., 1997; Singh et al., 2003) suggests that follicular development is organized in to waves before follicles become ultrasonographically detectable. The impetus to test the stated hypothesis was derived from observations that 1) FSH receptors are present in small

follicles shortly after entering the growing pool (Xu et al., 1995; Bao et al., 1997), 2) the development of primary follicles to secondary follicles in the developing fetus at Day 120 of gestation was associated with an increase in the serum concentration of FSH (Tanaka et al., 2001), and 3) circulating concentrations of FSH surge in a rhythmic and periodic manner during the estrous cycle (Adams et al., 1992a). We are unaware of any reports on the effect of superovulatory treatment on small follicle development in cattle.

The developmental pattern of large (≥4 mm) follicles is well established; however little is known about the developmental dynamics of small (<4 mm) follicles. The follicles <4 mm in diameter in cattle includes tertiary (antral follicles: >0.25 mm), secondary (large preantral: 0.13-0.25 mm and small preantral: 0.08-0.13 mm), primary (0.04-0.08 mm) and primordial (0.04 mm) follicles (Braw-Tal and Yossefi, 1997). It is now feasible to image follicles as small as 1 mm in diameter using the high-resolution ultrasonography. However, no such non-invasive tool is available to study the dynamics of follicles <1 mm in diameter. The only approach to study developmental dynamics of such follicles is histological examination of the excised ovaries. The limitation with the histology is the difficulty in making inference about small follicle dynamics using non-serial histologic data. A model was proposed nearly 2 decades ago to demonstrate follicular dynamics in women using previously derived histomorphometric data of the follicular population of normal ovaries obtained at various stages of the menstrual cycle (Gougeon, 1986). However, changes in follicular populations were not documented quantitatively, and the concept remained hypothetical.

The objectives of the present study were to monitor and record daily changes in 1-3 mm follicles and to design and validate a new method to study the dynamics of follicles <1 mm using non-serial histologic data.

The knowledge about the developmental pattern of small follicles will provide insight on endogenous control of folliculogenesis and methods to manipulate it. The understanding of the developmental pattern of small follicles may also provide efficient artificial management over ovarian activity for clinical and biotechnological purposes.

1.8 Hypotheses

Experiments were designed to test the hypotheses that small antral (1-3 mm) follicles develop in a wave-like manner in cattle (Study 1; Experiments 1 and 2), and that a new method for studying follicle dynamics using non-serial data will enable characterization of the developmental pattern of follicles too small to be examined by serial ultrasonography, e.g., histologic examination of excised ovaries (Study 2).

2. DEVELOPMENTAL PATTERN OF SMALL (1-3 mm) ANTRAL FOLLICLES IN THE BOVINE OVARY

2.1 Abstract

The study was designed to characterize the developmental pattern of 1-3 mm follicles and to determine the stage at which the future dominant follicle first attains a size advantage among its cohorts. In Experiment 1, heifers (n = 18) were examined every 24 h by transrectal ultrasonography for one interovulatory interval. In Experiment 2, cows (n = 9) were examined and bled every 6 h from Days 5 to 13 (Day 0 = ovulation) to monitor precisely the diameter changes of individual follicles ≥1 mm with regard to changes in circulating concentrations of FSH during emergence of Wave 2. Results revealed a day effect ($P < 0.05$) on the number of 1-3 mm follicles, with a peak ($P < 0.05$) 1 or 2 days before wave emergence (conventionally defined as when the dominant follicle was first detected at 4 mm), followed 3 to 4 days later by a peak ($P < 0.05$) in the number of ≥4 mm follicles. The number of 1-3 mm follicles detected in anovulatory waves did not differ ($P = 0.53$) between 2- versus 3-wave interovulatory intervals. More 1-3 mm follicles ($P < 0.05$) were detected at the emergence of the ovulatory wave than anovulatory waves in 3-wave interovulatory intervals, but not ($P = 0.63$) in 2-wave interovulatory intervals. The future dominant follicle was first identified at a diameter of 1 mm and emerged 6-12 h earlier than the first subordinate follicle ($P < 0.01$).

Emergence of the future dominant ($r = 0.71$) and first subordinate ($r = 0.73$) follicles was temporally associated ($P < 0.01$) with a rise in circulating concentrations of FSH. It was concluded that 1-3 mm follicles develop in a wave-like manner in temporal association with rising concentrations of FSH, and selection of the dominant follicle was manifest much earlier than previously documented.

2.2 Introduction

The wave-like developmental dynamics of follicles ≥4 mm have been well documented in cattle (Pierson et al., 1988a; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a,b,c; Knopf et al., 1989). The majority of bovine estrous cycles (>95%) consist of 2 or 3 waves of follicular development (Adams 1999) each of which is characterized by a surge in circulating concentration of FSH (Adams et al., 1992a) followed by a sudden (within 1 to 2 days) appearance of several follicles 4-6 mm in diameter, as detected by serial ultrasonography (Ginther et al., 1989a). Follicles of the cohort grow at a similar rate for about 2 days, followed by preferential growth of one (dominant) follicle over the others (subordinates) in a process referred to as selection. The dominant follicle suppresses growth of its subordinates (Adams et al., 1993a,b; Bodensteiner et al., 1996; Mihm et al., 1997; Bigelow et al., 1998) and continues to grow for about 6 days (Ginther et al., 1989a). In the presence of a functional CL, the dominant follicle stops growing and enters a static phase followed by a regressing phase like its subordinates. If luteal regression occurs during the growing phase, the dominant follicle ovulates (Kastelic et al., 1990; Bergfelt et al., 1991; Lucy et al., 1992). In either instance, a new follicular wave starts (Adams et al., 1992a; Ginther et al., 1996; Gibbons et al., 1997).

As opposed to the known wave-like pattern of follicles ≥4 mm, the dynamics of follicles <4 mm is not well understood. Primordial follicles in the fetal bovine ovary constitute the lifetime reservoir of follicles (approximately 68000 at birth); a reservoir that is progressively depleted throughout the life span of a cow (Erickson 1966b). The mechanism controlling recruitment of primordial follicles into the growing pool, and the stage at which growing follicles conform to the wave pattern of development, are unknown. However, consistency in the number of follicles ≥4 mm recruited into a follicular wave from one wave to the next (Boni et al., 1997; Singh et al., 2003) suggests that follicular development may be organized into waves before follicles become ultrasonically detectable. Mean growth rates of follicles from early to ovulatory stages of development have been estimated in cows (Marion and Gier, 1971; Lussier et al., 1987), rodents (Pedersen 1970; Oakberg et al., 1975; Hirshfield 1984), sheep (Cahill and Mauleon, 1980) and women (Gougeon 1982). However, these estimations do not shed light on the dynamics of small follicle development relative to wave emergence, or the relationship to changes in circulating concentrations of gonadotropins.

Granulosa cells of follicles as early as the primary stage of development (i.e., immediately after activation from the primordial pool) possess FSH receptors (Xu et al., 1995; Bao et al., 1997), and in vivo and in vitro studies have documented the stimulatory effects of FSH on the growth of small follicles (Wang and Greenwald, 1993a,b; Xu et al., 1995; Ralph et al., 1996; Bao et al., 1997). These observations, plus the known phenomenon of periodic surges in the circulating concentrations of FSH during the estrous cycle (Adams et al., 1992a), provide rationale for the hypothesis that small follicles (<4 mm) develop in a wave-like manner.

The objective of this study was to characterize the developmental pattern of small antral follicles (1-3 mm) in cattle using high resolution real-time, B-mode ultrasonography. Our hypotheses were (1) small antral follicles (1-3 mm) develop in a wave-like manner; (2) the dominant follicle of a wave has a size advantage over its subordinates at its detection at around 1 mm; and (3) emergence of dominant and subordinate follicles at the diameter of 1 mm is associated with rising plasma concentrations of FSH.

2.3 Materials and Methods

2.3.1 *Experiment 1: Developmental pattern of 1-3 mm follicles during one interovulatory interval*

Animals. Sexually mature Hereford-cross heifers (n = 18) 18 to 24 months of age and weighing 450 to 550 kg were selected from a group of 28 on the basis of physical fitness and normal cyclicity as judged by two ultrasound examinations 10 days apart. The heifers had not been treated with hormones that may be expected to influence ovarian function (e.g. growth promotants, ovarian synchronization or superovulation treatment) during the previous six months. 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.

Ovarian ultrasound examinations. Ovarian follicular development was monitored every 24 h (daily) by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SDD-900 Co. Instruments for Science and Medicine, Vancouver, BC). The scanner provided lateral and axial resolution of 1 mm. Ultrasound examinations of heifers commenced irrespective of the day of the estrous cycle and continued until two successive ovulations were recorded, so as to encompass one complete interovulatory interval All follicles ≥4 mm were identified based on the previous day's records of the topographic location and diameter of follicles and corpora lutea (Knopf et al., 1989), and attempts were also made to identify individual follicles 1-3 mm in size. In addition, the number of follicles in the 1-3 mm and ≥4 mm categories was recorded during each examination.

2.3.2 *Experiment 2: Developmental pattern of 1-3 mm follicles at the time of wave emergence*

In Experiment 1, follicles ≥4 mm could be individually identified on a daily basis; however, identification of individual 1-3 mm follicles was difficult owing to (i) their comparatively large numbers, (ii) similarity in shape, and (iii) insufficiently frequent ultrasound examinations. Experiment 2 was, therefore, designed to overcome difficulties encountered during Experiment 1 by incorporating following specific animal selection criteria, the use of alternative methods of identifying individual follicles, and more frequent ultrasound examinations.

Animals. Hereford-cross cows (n = 24), 3-4 years of age and weighing 600 to 650 kg were selected during the fall (September) from a group of 37 using the same criteria as in Experiment 1. The previous studies indicate that the total number of follicles within the ovaries differs widely between cows (Erickson 1966), but that within cows, the number of follicles ≥4 mm recruited into a follicular wave remains consistent from one wave to the next (Boni et al., 1997; Singh et al., 2003). In Experiment 1, we experienced that examining more animals at a time was laborious; and that in animals wherein, a large number of follicles recruited into a wave, confounded the identification of small follicles on a daily basis. To minimize the complexity of monitoring small follicles and to reduce inter-animal variation, cows at the upper and lower extremes of follicle numbers were excluded. To this end, the cows were given 2 luteolytic doses of cloprostenol 12 h apart (500 µg Estrumate®, i.m., Schering-Plough Animal Health, Canada) and the number of ovarian follicles ≥1 mm in diameter was recorded during daily ultrasound examinations from the day of prostaglandin treatment until one day after ovulation. Ovulation was detected in 21 cows within 5 days of cloprostenol treatment. The Cows (n = 21) were ranked according to the cumulative total number of follicles detected in both ovaries –1, 0 and +1 days from ovulation. The median value of the cumulative number of follicles ≥1 mm was 119, and cows (n = 9) nearest to the upper and lower sides of the median rank (range 77-154 follicles) were selected for detailed ultrasound examinations.

Ovarian ultrasound examinations. The ovaries of each cow were examined by transrectal ultrasonography at 6 am, 12 noon and 6 pm from 5 to 13 days after ovulation so as to encompass the emergence of the second follicular wave of the estrous cycle.

Ultrasound examinations were done using the equipment described in Experiment 1, and a routine was established to optimize follicle enumeration and minimize errors. The right ovary was examined first, followed by the left, and the ultrasound transducer was moved from the lateral to medial aspect of the ovary and back again. The transducer was moved slowly and kept steady for a few seconds when a follicle was resolved at its full diameter. The ovaries were then scanned a second time in a similar fashion except that follicular images were frozen on the screen and measured using the integrated electronic calipers. Two values, measured at right angles to each other, were recorded and averaged to obtain an estimate of follicle diameter (Pierson et al., 1987a).

Methods of follicle data recording. The conventional method of profiling daily changes in individual follicles by ultrasonography involves retrospective evaluation of serial ovarian sketches that provide topographical and dimensional information of follicles ≥4 mm. Such sketches are usually rendered as a 3-dimensional impression of the amalgamated series of 2-dimensional images (Figure 1). In Experiment 1, we used this conventional amalgamation method of sketching daily changes in the small follicles; however, retrospective tracking of individual small follicles remained difficult. In particular, we noticed that as small follicles grow, they tended to change planes within the ovary, which made it difficult to follow them retrospectively. To circumvent the problem of tracking individual small follicles, we employed a sectional method of sketching follicles in which multiple ovarian maps (Figure 2.1a) were used to record images of follicles in sequential sections of each ovary while moving the transducer from the lateral to the medial aspect of the ovary. This sectional method of sketching follicles was very laborious. To simplify it, we followed the procedure of sectional

sketching (Figure 2.1a) only for the first ultrasound examination of the day. On subsequent ultrasound examinations, changes in individual follicle diameters were recorded against the respective first sectional sketch (Figure 2.1b), without the necessity of re-drawing ovarian structures. To minimize the error in monitoring daily changes in the follicular diameter, we recorded each ultrasound examination on S-VHS video-tapes (Video Cassette Recorder Model PV-VS4821-K, Panasonic, PT Matsushita Kotobuki Electronic Industries, Indonesia). A separate video-tape cassette was used for each animal. Individual small follicles were identified by retrospective analysis of ovarian sketches and recorded ultrasound images.

Plasma sampling and radioimmunoassay for FSH. Jugular blood samples were collected in heparinized tubes (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes NJ, USA) at 6 am, 12 noon and 6 pm from 5 to 13 days after ovulation, and were centrifuged for 15 minutes at 1500 g within 30 to 60 min of collection. 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 8% and 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.

2.3.3 *Data analysis*

In Experiment 1, ovarian follicular data of heifers were grouped into two categories based on the number of follicular waves displayed during the interovulatory interval (IOI) i.e., 2-wave IOI and 3-wave IOI. The day of wave emergence (Day 0) was determined by retrospective analysis of follicular data and defined as the day on which the dominant follicle of a wave was first detected at a diameter of 4-5 mm (Ginther et al., 1989a,b; Adams, 1999). The dominant follicle was defined as the largest follicle of a wave and subordinate follicles were defined as those that appeared to originate from the same pool of follicles (Ginther et al., 1989a; Knopf et al., 1989). For the statistical analysis and the preparation of figures, individual heifers' follicle data for each wave were centralized to begin on the day of wave emergence (Ginther et al., 1989b; Adams

et al., 1992a). The numbers of small and large follicles were analyzed from Day -3 to Day 5 (Adams et al., 1993a,b). The repeated measures data were analyzed by PROC MIXED (Littell et al., 2000) of the Statistical Analysis System software package (SAS version 8.2 for MS Windows; SAS Institute Inc. Cary, North Carolina, 2002). Five covariance structures [Compound Symmetry (CS), Autoregressive, order 1 (AR1), Unstructured (UN), Unstructured 1 (UN 1) and Huynh-Feldt (HF)] were fitted to the data and the best model was selected based on the smallest Akaike's Information Criterion (AIC) values. Data were analyzed for the effect of day (Day -3 to Day 5), follicle type (small and large follicles), IOI type (2-wave versus 3-wave IOI), and wave type (wave 1 versus wave 2 versus wave 3; anovulatory versus ovulatory wave). If main effects or their interaction were statistically significant ($P < 0.05$), multiple comparisons were made using Tukey's post-hoc test. Correlation between the profiles of small and large follicles was estimated using Pearson's correlation analysis.

In Experiment 2, data were analyzed for the effects of day (Day 5 to Day 13 after ovulation) and follicle type (dominant, first and second subordinates). Data were analyzed in two ways: (1) by centralization to the day of wave emergence (i.e., when dominant follicle was first detected at 4-5 mm) to determine the time of emergence of the dominant and subordinate follicles relative to the conventional definition of wave emergence, (2) by centralization to the detection of dominant follicle at 1 mm to compare detection time of first and second subordinates relative to the dominant follicle, and (3) by centralization to the day of detection of all three follicles (dominant, first and second subordinates) at 1 mm to compare their growth rates. Centralization of the data to the wave emergence or to the emergence of all three follicle types at 1 mm rendered a 6 h profile of follicles during a 24 h period. If main effects or their interaction were

significant ($P < 0.05$), multiple comparisons were made using Tukey's post-hoc test. The association between FSH and emergence of dominant and subordinate follicles was estimated using Pearson's correlation analysis after centralizing the data from -48 to +48 h of detection of the future dominant follicle at 1 mm (0 h).

2.4 Results

2.4.1 *Experiment 1*

One heifer was found missing from the herd from 2 to 5 days after the first ovulation and therefore, it was excluded from the statistical analyses due to missing data. Data from the remaining 17 heifers were divided into two groups based on the number of follicular waves observed during the IOI; 9 heifers displayed 2 follicular waves and 8 heifers displayed 3 follicular waves. Wave emergence (i.e., when the prospective dominant follicle was first detected at 4-5 mm in diameter) was detected, on average, 0 and 9 days after ovulation for 2-wave IOI, and 0, 9 and 17 days after ovulation for 3-wave IOI. It was feasible to detect and count small follicles (1-3 mm), but individual identity of small follicles could not be traced on the basis of daily examinations. Therefore, only data pertaining to follicle numbers were used to investigate the developmental pattern of small follicles in 2-wave and 3-wave IOI.

In 2-wave IOI (Figure 2.2), the number of small (1-3 mm) and large ($\geq$ 4 mm) follicles changed over days ($P < 0.05$). A peak in the small follicle population was noticed on Day -1 of wave emergence (defined conventionally as the day on which the dominant follicle of a wave is 4-5 mm in diameter) for both Wave 1 (anovulatory wave)

and Wave 2 (ovulatory wave), whereas a peak in large follicles was noticed between Day 1 and Day 2 after wave emergence. There was an inverse relationship between the number of small and large follicles during Wave 1 ($r = -0.66$; $P = 0.05$) and Wave 2 ($r = -0.62$; $P = 0.04$). The pattern of peaks and troughs in the number of small and large follicles in 3-wave IOI (Figure 2.3) were similar to those in 2-wave IOI except that the day effect was statistically non-significant for large follicles ($P = 0.18$) during Wave 1 and for small follicles ($P = 0.49$) during Wave 3. An inverse relationship between the number of small and large follicles existed for Wave 1 ($r = -0.79$; $P = 0.01$) and Wave 3 ($r = -0.90$; $P = 0.001$), but not for Wave 2 ($r = -0.57$; $P = 0.14$).

In 2-wave IOI (Figure 2.4a), there was no difference in follicle number profiles between anovulatory (Wave 1) and ovulatory (Wave 2) waves (wave effect, $P = 0.63$; wave-by-day interaction, $P = 0.80$). Similarly, there was no difference ($P = 0.23$) in follicle number profiles between the anovulatory waves in 3-wave IOI (i.e., Wave 1 versus Wave 2). However, greater numbers of small follicles were detected during emergence of the ovulatory wave (Wave 3) of 3-wave IOI (Figure 2.4b) than either of the anovulatory waves (Waves 1 and 2; $P < 0.05$) and the ovulatory wave of 2-wave IOI ($P = 0.06$).

Except the ovulatory wave in 3-wave IOI, no differences in follicle number profiles at wave emergence were detected among waves in 2- and 3-wave IOI (IOI type, $P = 0.81$; IOI type-by-day interaction, $P = 0.39$; Figure 2.5). Therefore, data for all but Wave 3 of 3-wave IOI were combined to characterize the relationship between the number of small (1-3 mm) and large (≥4 mm) follicles during wave emergence (Figure 2.6). The significant category-by-day interaction ($P < 0.01$) and Pearson's correlation

coefficient ($r = -0.79$, $P = 0.01$) documented an inverse relationship between the number of small and large follicles.

2.4.2 *Experiment 2*

Sketching of follicles using the sectional method (Figure 2.1a) provided information about the location and number of small follicles in the ovary, but was labor and time intensive. Modification of the sectional method (Figure 2.1b) was helpful, but not entirely effective because the plane in which small follicles were detected within the ovary changed as they grew and regressed. Hence, data tabulated using the sectional sketching method were systematically compared with video recordings of each examination to enable individual identification of follicles as small as 1 mm. Data centralized to the time the dominant follicle was detected at 4-5 mm (conventional definition of wave emergence; Figure 2.7a) revealed that the follicle destined to become dominant was first detected at a diameter 1 mm 66 h earlier. The first subordinate follicle was first detected at 1 mm 48 to 54 h earlier (i.e., 6 to 12 h later than the future dominant follicle). The prospective dominant follicle was larger than the first subordinate ($P < 0.05$) 1 day after detection of the former at 1 mm (Figure 2.7a). Similarly, when data were centralized to the emergence of the dominant follicle at 1 mm size (Figure 2.7b), a significant difference in size ($P < 0.01$) was detected between dominant and subordinate follicles.

FSH and small follicle emergence. Data centralized to the peak in FSH (Figure 2.7c) revealed a change ($P < 0.01$) in circulating concentrations of FSH over time. Data also revealed a significant ($P = 0.01$) positive correlation between follicle diameter and plasma FSH concentration (dominant follicle $r = 0.71$; first subordinate follicle, $r = 0.73$; second subordinate follicle, $r = 0.76$) from the time of follicle detection at 1 mm to the

time at which FSH concentrations peaked (0 h). A significant ($P < 0.01$) negative correlation was detected thereafter (dominant follicle $r = -0.90$; first subordinate follicle, $r = -0.68$; second subordinate follicle, $r = -0.78$). Furthermore, growth of the three largest follicles began before the peak in circulating concentration of FSH (Figure 2.7 a,b,c).

Growth rates of dominant, first and second subordinate follicles. Data of the dominant and subordinate follicles centralized to their respective day of detection at 1 mm (Figure 2.8), revealed a difference in the diameter (divergence in growth rate) between the dominant and the second subordinate follicle at 84 h ($P < 0.01$) and between the dominant and first subordinate follicle at 90 h ($P = 0.06$).

2.5 Discussion

The mechanisms controlling recruitment of primordial follicles into the growing pool, and the stage at which growing follicles join follicular waves are unknown. It has been well documented that large follicles (≥4 mm) develop in a wave-like fashion. A previous study reported an inverse relationship between the number of 2-3 mm and ≥4 mm follicles (Pierson and Ginther, 1987a), which is indicative of a wave-like developmental pattern. Consistency in the number of follicles ≥4 mm recruited into a follicular wave from one wave to the next (Boni et al., 1997; Singh et al., 2003) suggests that the follicular development is organized into waves before follicles become ultrasonographically detectable. We, therefore, hypothesized that small follicles (1-3 mm) develop in wave-like manner. The impetus to test the hypothesis was derived from observations that 1) FSH receptors are present in small follicles shortly after entering the growing pool (Xu et al., 1995; Bao et al., 1997), and 2) the development of primary follicles to secondary follicles in the developing fetus at Day 120 of gestation was associated with an increase in the serum concentration of FSH (Tanaka et al., 2001). These observations, plus the knowledge that circulating concentrations of FSH surge in a rhythmic and periodic manner during the estrous cycle (Adams et al., 1992a); provide rationale for the hypothesis that small follicles (1-3 mm) develop in a wave-like manner

Results of Experiment 1 support our first hypothesis that small antral follicles develop in a wave-like pattern. A significant inverse relationship was detected in the profiles of the number of small follicles (1-3 mm) and large follicles (≥ 4 mm), consistent with a wave-like developmental pattern (Pierson et al., 1987b). The observed periodic shift in the peak number of small follicles to a peak number of large follicles

resulted when smaller follicles grew as a cohort to a larger diameter and were not immediately replaced by another set of smaller follicles. Results of the present study are consistent with those of a previous study (Pierson and Ginther, 1987a) in which an inverse relationship was found between the number of 2-3 mm and ≥4 mm follicles. The statistical rigor of this inverse relationship is exemplified by its detection in the previous study, despite that data were tabulated and analyzed irrespective of wave emergence. In an early study (Choudary et al., 1968), no cyclic changes in the number of vesicular follicles up to 5 mm in diameter were detected; however, statistical inference was not possible because only one cow was used for each day of the estrous cycle. In a later study, wherein the ovaries of cows were examined by laparotomy on Days 3, 8, 13 and 18 (Matton et al., 1981), an increase in the number of small follicles was noted on Day 3. The timing of the peak in the number of small follicles observed in the present study (i.e., 1 day before ovulation, or 1 day before detection of the dominant follicle at 4 mm in diameter) was earlier than that reported in the laparotomy study; however, in the latter, the point of reference (i.e., estrus or ovulation) was not clear and follicle enumeration was done by examining only the superficial surface of the ovary. In addition, follicular measurements in the laparotomy study were made using vernier calipers from the ovarian surface, in contrast to that of the present study in which electronic calipers were used on freezed ultrasound images of follicles throughout the depth of the ovary.

The pattern of ovarian follicular development remained uncertain until serial ultrasonography became possible (Pierson and Ginther, 1984; Quirk et al., 1986; reviewed by Adams, 1999). The elusiveness of the dynamics of follicles too small to be monitored by ultrasonography persists for the same reason. The difficulty experienced in

Experiment 1 in serial identification of individual follicles < 3 mm was perhaps not surprising because the small diameter was near the limit of image resolution, and smaller follicles grew more slowly and were greater in number than larger follicles. In addition, daily changes were more difficult to track because the tendency of small follicles to change plane within the ovarian tissue during growth confounded the use of topographic landmarks. To address these issues, Experiment 2 incorporated special criteria for animal selection to minimize variation, more frequent ultrasonography to detect subtle changes among small follicles, and modifications to data recording and tabulation. Critical comparison of methodically recorded videotape images with previous section-by-section sketches of serial ultrasound images permitted individual profiling of follicles as small as 1 mm. With this approach, the dominant follicle was initially identified at a diameter of 1 mm, 66 h before it reached a diameter of 4 to 5 mm i.e., previously stated time of wave emergence (Adams, 1999).

Exquisite sensitivity of small antral follicles to FSH was reflected in concurrent increases in circulating FSH and follicular development at the time of wave emergence at 1 mm. The temporal relationship between the surge in FSH and the growth of small follicles in the present study confirms the results of an earlier study (Adams et al., 1992a). In the earlier study, the surge in FSH was reported to begin 2 to 4 days (~ 48 to 96 h) before ultrasonographic detection of a dominant follicle at 4-5 mm (conventionally defined as wave emergence). The ultrasonographic detection of a dominant follicle at 1 mm in the present study, 66 h earlier than previously detected, was coincident with the beginning of the surge in FSH. In addition, the peak in the number of 1-3 mm follicles in the present study and the peak in the circulating concentrations of FSH in the earlier study are concurrent; i.e., 1-2 days before detection of a dominant follicle at 4-5 mm.

Emergence of the dominant follicle at 1 mm occurred 6 to 12 h earlier than that of subordinate follicles in the same wave. In an earlier study (Bodensteiner et al., 1996; Ginther et al., 1996), the future dominant follicle was identified 6 h earlier than the future first subordinate follicle, at a diameter of 3 mm. Hence, the observation that the selected dominant follicle often has a size advantage at the time of its earliest detection (Ginther et al., 1996) is in agreement with the present study. The size advantage of the dominant follicle from its earliest detection at 1 mm may be implicated in its ability to first reach a critical diameter of ≥8.5 mm (termed selection). At a diameter of ≥8.5 mm, the developing dominant follicle attains a greater capacity to suppress circulating FSH concentrations to below that necessary to sustain smaller follicles of the wave; hence, the subordinates regress. In addition to an enhanced FSH-suppressing ability, the largest follicle also develops the ability to utilize the reduced concentrations of FSH for its continued growth. Expression of mRNA for LH receptors in the granulose cells of follicles ≥8.5 mm and elevations of circulating concentrations of LH around the time of selection, suggests role of LH for the sustained growth of the dominant follicle (Ginther, 2000).

The design of the present study permitted critical comparison of follicle dynamics between waves within and among 2-wave and 3-wave IOI. The similarity in the number of follicles recruited into all but the ovulatory wave of 3-wave IOI may be attributed to the even spacing (~10 days) of wave-eliciting FSH surges (Adams et al., 1992a) and consequent even spacing (~10 days) of wave emergence (Ginther et al., 1989d). A shorter interval (i.e., ~7 days) between the second and third FSH surges in 3-wave cycles (Adams et al., 1992a) may be implicated in the increase in the number of follicles recruited into the ovulatory wave in 3-wave IOI. The addition of FSH has been reported

to suppress apoptosis in serum-free culture of rat preantral (McGee et al., 1997) and antral (Tilly and Tilly, 1995) follicles suggesting that a physiological role of FSH may be to prevent atresia. The smaller, shorter-lived dominant follicle of the second wave in 3-wave IOI (Ginther et al., 1989a; Adams et al., 1992b; Adams et al., 1993b) may be responsible for less profound follicular and gonadotropin suppression than other dominant follicles. Perhaps less interwave suppression and an early surge in circulating concentrations of FSH preceding emergence of the third (ovulatory) wave is responsible for rescuing more follicles from atresia causing recruitment of more follicles into the final wave in 3-wave IOI. In earlier studies, number of follicles recruited in to a wave did not differ between anovulatory and ovulatory waves in 2-wave IOI (Ginther et al., 1989a); and between anovulatory waves and an ovulatory wave in 3-wave IOI (Fortune et al., 1988). These results are in agreement with the present study, except that the ovulatory wave in 3-wave IOI had higher number of follicles than anovulatory waves. However, in a recent study, there appear to be more larger follicles (>6 mm) in 2-wave cycles on Days 3 and 4 from ovulation in association with higher circulating concentrations of inhibin A (Parker et al., 2003).

In summary, small antral follicles (1-3 mm) developed in a wave-like manner, and the characteristics of follicular development were similar among all waves in 2- and 3-wave interovulatory intervals except the ultimate wave in 3-wave intervals. Ovarian follicles 1 to 3 mm in diameter were acutely sensitive to changes in circulating concentrations of FSH, and physiologic selection of the future dominant follicles was associated with a size advantage over all other follicles of the wave much earlier than previously documented.

3. A METHOD OF CHARACTERIZING THE DEVELOPMENTAL PATTERN OF SMALL OVARIAN FOLLICLES IN CATTLE USING NON-SERIAL DATA

3.1 Abstract

The wave-like developmental pattern of follicles ≥4 mm has been documented through the use of daily ultrasound examinations (i.e., serial method). However, the developmental pattern of follicles not detectable by ultrasound (<1 mm) can only be assessed histologically by examining the ovaries from different animals on different days of an interovulatory interval (i.e., non-serial method). A study was designed to validate a new non-serial method of characterizing follicle dynamics using the conventional serial method for comparison. A data set (n = 56 heifers) of follicles ≥4 mm collected for the purpose of a previous study was tabulated in a serial (data from the same set of heifers each day; n = 7/day, N = 7) and non-serial (data from a different set of heifers each day; n = 7/day, N = 56) manner to compare follicle dynamics based on diameter and number profiles around emergence of the first follicular wave of an interovulatory interval. Both serial and non-serial methods revealed a change in the number of follicles (4-5 mm, 6-8 mm, ≥9 mm) over days ($P < 0.01$), but the effect of method ($P = 0.53$; $P = 0.50$; $P = 0.28$) and the day-by-method interaction ($P = 0.64$; $P = 0.97$; $P = 0.84$) were not significant. The diameter of the dominant and first two subordinate follicles changed over days ($P < 0.0001$), but again the effect of method ($P =$

0.06; P = 0.39; P = 0.42) and the day-by-method interaction (P = 0.11; P = 0.85; P = 0.99) were not significant. We concluded that the new non-serial and conventional serial methods detected similar patterns of follicular development, and that the new method offers a valid approach for the study of follicle dynamics using non-serial (e.g. histologic) data.

3.2 Introduction

Although the developmental pattern of antral follicles ≥4 mm in diameter has been well documented (Adams, 1999), little is known about the developmental pattern of follicles <1 mm in diameter. During an interovulatory interval (IOI), cattle exhibit 2 or 3 distinct follicular waves (Pierson and Ginther, 1988a; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a,b,c; Knopf et al., 1989), each characterized by a sudden rise in the number of 4-6 mm follicles followed within a few days by selection of a dominant follicle and regression of the subordinates. Each wave is elicited by a surge in peripheral concentrations of FSH 1 or 2 days before wave emergence is detected (Adams et al., 1992a).

Mean growth rates of follicles from primary to ovulatory stages of development have been estimated in cows (Marion and Gier, 1971; Lussier et al., 1987), rodents (Pederson, 1970; Oakberg and Tyrell, 1975; Hirshfield, 1984), sheep (Cahill and Mauleon, 1980) and women (Gougeon, 1982). However, these estimates do not shed light on the dynamics of small follicle development or the relationship to changes in circulating concentrations of gonadotropins and follicular wave emergence. Although a model was proposed nearly 2 decades ago to demonstrate follicular dynamics in women

using previously derived histomorphometric data of the follicular population of normal ovaries obtained at various stages of the menstrual cycle (Gougeon, 1986), changes in follicular populations were not documented quantitatively and the concept remained hypothetical.

The wave-like developmental pattern of follicles ≥4 mm in cattle was documented through the use of transrectal ultrasonography (Adams, 1999). The experimental approach involved repeated examination of the ovaries (i.e. serial data) on a day-to-day basis to identify and monitor the diameter of individual follicles according to their topographic location in the ovary (i.e. serial identity method; Knopf et al., 1989). The wave-like developmental pattern of follicles ≥4 mm was also characterized by a method involving repeated examination of the ovaries (i.e. serial data) but without the necessity of individual follicle identification (i.e. serial non-identity method; Ginther, 1993). Whereas, ultrasonography may be used to monitor changes in follicles ≥4 mm, no method is available to study the temporal dynamics of smaller follicles. The only method available to study small follicles is histological examination of excised ovaries. The limiting factor with this technique is the difficulty in making inference of a dynamic process using static non-serial data.

The objective of the present study was to validate an alternate method of documenting the developmental pattern of follicles based on non-serial data (i.e. simulating histomorphometric data). To design and validate such a non-serial method, we used a data set previously used to detect wave dynamics in follicles ≥4 mm using serial method. Our hypotheses were (1) a follicular wave pattern can be detected using non-serial data; and (2) the pattern of follicular growth detected by non-serial methods is similar to that detected by conventional serial methods.

3.3 Materials and Methods

3.3.1 *Data set*

A data set collected by ultrasonography from 56 beef heifers, and previously analyzed using the serial identity method to detect waves in follicles ≥4 mm in diameter, was used to compare the results of conventional serial analysis versus analysis of the subset of the same data arranged to simulate non-serial data. Information about the heifers as well as their housing, maintenance, and ultrasound examination procedure have been published previously (n = 28, Singh et al., 1998; n = 28, Singh and Adams, 2000).

3.3.2 *Serial and Non-serial Methods*

The developmental pattern of follicles has been studied by using follicle number (Pierson and Ginther, 1987b) or diameter (Knopf et al., 1989) profiles. The diameter profile of follicles is conventionally used for studying the dynamics of follicles ≥4 mm. Whereas the diameter profile reveals the growth pattern of individual follicles, the follicle number profile provides the pattern of growth of follicle populations. If ovarian structures of an individual are examined repeatedly over time (Figure 3.1a), serial data are obtained (possible only through the use of a non-invasive tool like ultrasonography) and the method to characterize such data may be analyzed using a serial method. However, with follicles <1 mm a non-invasive tool to monitor changes over time is not available and characterization requires ex situ examination of tissues from different

animals on different days (e.g., histological technique) (Figure 3.1b). Such non-serial data require the use of a non-serial method of analysis.

In the present study, serial and non-serial methods of characterizing follicle dynamics were compared using a common data set. Serial data collected during previous studies (Singh et al., 1998; Singh and Adams, 2000) were analyzed for the period encompassing emergence of the first follicular wave of the IOI (Day –2 to Day 5; Day 0 = ovulation) using serial and non-serial methods. For non-serial methods, data were obtained by randomly selecting 7 heifers for each examination day (i.e., 7 observations on each of Day -2 to Day 5). Data from each heifer were used for only one day; hence, data from all heifers were used (8 days x 7 heifers per day = 56). For serial methods, 7 heifers were randomly selected from the 56 heifers, and daily data from the same 7 heifers were used from Day -2 to Day 5 (i.e., n = 7 observations per day). The number of follicles detected each day in the 4-5 mm, 6-8 mm and ≥9 mm categories was compared between conventional serial and new non-serial methods. Similarly, diameters of the dominant and two largest subordinate follicles were compared between conventional and new methods (for summary, refer Figure 3.2).

3.3.3 *Comparison of follicular dynamics using follicle number profile by serial and non-serial methods*

Serial method. In this method, ovaries of the same animals(s) are examined repeatedly during the period of wave emergence using ultrasonography, and the identified follicles were sketched according to their topographic position within the ovary. The diameter of each follicle was recorded against its respective sketch (Knopf et al., 1989). Follicles in various size classes (i.e., 4-5 mm; 6-8 mm; and ≥9 mm) were counted in both ovaries for each examination and tabulated as described (Pierson and Ginther, 1987b). The mean daily number of follicles in various size classes was plotted against time to reveal follicular dynamics.

Non-serial method. Ovaries of a different set of animals were examined during each time period using ultrasonography and the identified follicles are sketched according to their topographic position within ovary. The diameter of each follicle was recorded against its respective image in an ovarian sketch. Follicles in various size classes (i.e., 4-5 mm; 6-8 mm; and ≥9 mm) were counted in both ovaries for each examination and tabulated (Table 3.1). The mean daily number of follicles in various size classes was plotted against time to reveal follicular dynamics. This method was designed for the purposes of histological approaches for studying the dynamics of follicles <1 mm.

3.3.4 *Comparison of follicular dynamics using follicle diameter profile by serial and non-serial methods*

Serial identity method. In this conventional method of studying follicular dynamics, ovaries of the same animal(s) were examined repeatedly over time (i.e., serially) using ultrasonography, and visible follicles were individually identified (i.e., identity scheme) during each examination. Hence, the method is defined as the serial identity method. The day-to-day diameter of each ultrasonographically visible follicle were simultaneously sketched and recorded (Knopf et al., 1989). To analyze the diameter changes in follicles over time, follicles of interest in the last sketch were assigned a letter code e.g., a, b, c to designate the dominant, the first, and the second subordinate follicles. Using the topographic position within the ovary, the marked follicle was retrospectively identified from the preceding sketches and marked with the assigned letter code. For each follicle type (a, b, or c) of each animal(s), the diameter for respective day was tabulated. The mean daily diameter of each follicle type was plotted against time to reveal follicular dynamics.

Serial non-identity method. In this method, ovaries of the same animal(s) were examined over time (i.e., serially) using ultrasonography, but without identifying individual follicle (i.e., non-identity scheme) during each ultrasound exam. Hence, the method is referred to as a serial non-identity method (Ginther, 1993). For each examination day, diameters of largest to smallest follicles were tabulated in a decreasing order in a row. Over the time, each constructed column represented a follicle type e.g., first three columns from left to right represent the largest, the second largest and the 3rd largest follicles, respectively. The mean daily diameter of each follicle type was plotted against the time to reveal follicular dynamics.

Non-serial-non-identity method. This method was designed with a purpose to use it for histological technique of studying dynamics of follicles <1 mm. In histological technique, ovaries are excised from different animals on different days (i.e., non-serially), which do not reveal identity of individual follicles (i.e., a non-identity scheme). Thus the non-identity scheme described previously for the serial-non-identity method simulates with histological approach to study follicle dynamics except that the data in the later approach would be non-serial. Hence, the method to characterize follicular dynamics using non-serial data with non-identity scheme was defined as the non-serial-non-identity method. In this non-serial-non-identity method, follicle diameter data were he tabulated in a similar fashion as described for serial-non-identity method except that a new animal was used for each new examination (Table 3.1).

Table 3.1 A new non-serial method of tabulating follicle number and diameter profiles in cattle using non-serial data

		Number of follicles in size groups (mm)			Follicle diameters in descending order (mm)		
Day	Heifer #	4-5	6-8	≥9	Largest	2nd largest	3rd largest
0	2	12	0	1	9	5	5
0	3	10	1	0	7	4	4
0	421	7	3	0	6	6	6
.		.	.	.	.	.	.
	Mean	8.4	1.1	0.4	8.1	4.9	4.9
	SEM	2.30	0.40	0.20	0.80	0.40	0.40
1	8	24	2	0	7	6	4
1	19	2	7	0	10	6	6
1	412	4	6	0	8	7	7
.		.	.	.	.	.	.
	Mean	7.9	3.3	0.6	7.9	6.7	6.0
	SEM	3.37	0.97	.57	0.77	0.61	0.65
2	9	12	0	1	9	5	5
2	29	5	4	2	10	9	8
2	422	4	4	1	9	7	6
.		.	.	.	.	.	.
	Mean	4.9	3.1	1.0	9	7.3	6.3
	SEM	1.55	0.77	0.22	0.38	0.47	0.52

The mean ±SEM was calculated using data from different heifers on each day (n = 7 per day; N = 56 over 8 days).

3.3.5 *Data analysis*

The data for the profile of number of follicles in 4-5 mm, 6-8 mm and ≥9 mm size categories were tabulated using both serial and non-serial methods and were analyzed for the period from Day -2 to Day 5. The data for diameter profiles of the dominant and first two subordinate follicles were tabulated using (1) the serial identity method, (2) the serial non-identity method, and (3) the non-serial non-identity method. Follicle

diameters data were profiled for Day -2 to Day 5 for graphical completeness, but statistical analysis was confined to the period from Day 0 to Day 5. The effects of day, method, and day-by-method interaction were analyzed by PROC MIXED (Littell et al., 2000) of the Statistical Analysis System software package (SAS version 8.2 for MS Windows; SAS Institute Inc. Cary, North Carolina, 2002). Multiple analyses for each endpoint were performed to select the best covariance model based on the smallest Akaike's Information Criterion (AIC) value. If the main effects or interaction terms were significant ($P < 0.05$), multiple comparisons were made using Tukey's post-hoc test for the selected covariance model. The diameter profile of one follicle from one heifer was excluded from the study because the follicle persisted into the next wave (follicular cyst) and was identified as an outlier (>3 standard deviations above the mean diameter).

3.3 Results

3.4.1 *Follicle number profiles*

A significant day effect ($P < 0.01$) on the number of follicles in each size category (4-5 mm, 6-8 mm and ≥9 mm) was detected using both serial and non-serial methods (Figure 3.3). No difference in follicle number profiles within each category were detected between serial and non-serial methods (method: 4-5 mm, $P = 0.53$; 6-8 mm, $P = 0.50$; ≥9 mm, $P = 0.28$; day-by-method: 4-5 mm, $P = 0.64$; 6-8 mm, $P = 0.97$; ≥9 mm, $P = 0.84$). Peaks and troughs in the number of follicles within each category were detected on the same days using serial and non-serial methods. Both methods revealed a peak in the number of 4-5 mm follicles on Day 0 followed by a peak in 6-8 mm follicles on Day 2,

and a peak in ≥9 mm follicles on Day 4. Consistent with a wave pattern of follicle development, both methods revealed a subsequent decrease in numbers within the lower size category of follicles; e.g., with an increase in 6-8 mm follicles there was a decrease in 4-5 mm follicles, and with an increase in ≥9 mm follicles there was a decrease in 6-8 mm follicles.

3.4.2 *Follicle diameter profiles*

A significant day effect ($P < 0.01$) on the diameter profiles of the dominant and first 2 subordinate follicles was detected using serial and non-serial methods (Figure 3.4). The diameter of the dominant and first two subordinate follicles changed over days ($P < 0.01$). No differences in the diameter profiles of the first ($P = 0.39$) and second ($P = 0.42$) subordinate follicles were detected among serial identity, serial-non-identity and non-serial non-identity methods; however, the profile of the dominant follicle tended to differ ($P = 0.06$) among methods (Figure 3.4). Direct comparison of serial data (identity and non-identity methods combined) versus non-serial data revealed no differences in follicle diameter profiles (dominant follicle, $P = 0.78$; first subordinate follicle, $P = 0.84$; second subordinate follicle, $P = 0.31$).

3.5 Discussion

The interval from entry into the growing pool (i.e., growing primary follicle) to entry into ultrasonographically detectable waves (late tertiary follicles ≥4 mm) in cattle has been estimated to take about 80-100 days (Britt, 1991). Little is known of the dynamics of the preceding lifespan (> 80%) of ovarian follicles, while much has been learned by characterization of follicle dynamics during the final 10 days of development (ovulatory and anovulatory follicular waves). A method of characterizing the developmental dynamics of small follicles would allow us to determine if and when primary, secondary and early tertiary follicles conform to the wave pattern of development, and would provide new avenues of research into the mechanisms controlling follicle recruitment. A better understanding of follicular recruitment may lead to more effective diagnosis and manipulation of ovarian function and dysfunction in cattle as well as in species for which the bovine model has been validated, including women (Adams and Pierson, 1995; Baerwald et al., 2003). However, this topic remains largely unexplored because technology is not yet available to perform repeated examinations of individual follicles smaller than 1 mm. At present, study of the development of small follicles is limited to non-serial data generated by histomorphometric evaluation of excised ovarian tissues from different animals on different days. The objective herein was to investigate the possibility of detecting changes in follicular development (diameter and numbers) using non-serial data.

A data set of follicles ≥4 mm collected by ultrasonography, in which a wave-like pattern of development had already been documented by repeated examination of the same ovaries over days (Singh et al., 1998; Singh and Adams, 2000), was used to

compare the results of conventional serial analysis and analysis of a subset of the same data arranged to simulate non-serial data. The supposition was that if a wave-like pattern of follicular development could be detected using a non-serial method of profiling non-serial data, then it would be valid to apply the same method to histomorphometric data and test the wave theory of development of follicles <1 mm. A critical feature of the non-serial approach is the use of a point of reference about which data may be centralized (e.g., wave emergence, ovulation, gonadotropin surge).

Follicle numbers detected by ultrasonography were subdivided into size categories to mimic histomorphometric arrangement of data (Choudary et al., 1968; Hirshfield and Midgley, 1978; Gougeon, 1982, 1986; Wandji et al., 1996; Braw-Tal and Yossefi, 1997; Cushman et al., 1999). A chronological shift in the population of follicles from one size category to the next represents either the growth of smaller follicles into the larger size category or regression of larger follicles (Pierson and Ginther, 1987a). It may also be possible that smaller follicles grew as a cohort to a larger diameter and were not immediately replaced by another set of smaller follicles, and the pattern may be considered a wave-like growth of a single cohort of follicles (Pierson and Ginther, 1987a; Ginther et al., 1989a). Such a shift was detected in the present study using serial and non-serial data, and supported the hypothesis that a follicular wave pattern can be detected by using non-serial data.

Tracking diameter changes of individually identified follicles by ultrasonography has become a conventional method of characterizing wave dynamics (Knopf et al., 1989); however, the non-identity method (Ginther, 1993) was developed to circumvent the necessity of making detailed sketches of the topographic arrangement of individual follicles. The later method revealed similar dynamics without specifying the individual

identity of follicles. The non-identity approach lends itself to histomorphometric data because data are not serial and identification of individual follicles <1 mm is not feasible. Diameter profiles of the dominant and first two subordinate (or among the 3 largest) follicles were compared using the conventional serial identity method, the serial non-identity method, and a new non-serial-non-identity method. The purpose was to compare the follicular dynamics using the serial non-identity and the conventional serial-identity methods; and using the tested serial non-identity and the new non-serial-non-identity methods. The diameter profile of the dominant follicle was similar in all the three methods; however, the diameter on the day of ovulation appeared to be slightly larger using serial and non-serial non-identity methods compared to the serial identity method. This difference was attributed to areas of ambiguity in follicle tracking using the non-identity method (Ginther, 1993). Ambiguity at the time of ovulation results from lingering follicles of the previous wave (i.e., regressing dominant follicle of an anovulatory wave or subordinates of the ovulatory wave) assuming the largest or second largest positions in the new wave emerging at the time of ovulation. The effectiveness of the approach was further documented by direct comparison of serial and non-serial methods (irrespective of identity versus non-identity); no differences in diameter profiles of the dominant and largest two subordinate follicles were detected between methods.

The impetus to develop a method of studying small follicle dynamics was derived from the desire to test the hypothesis that small follicles develop in a wave-like fashion, similar to the wave pattern observed in large follicles. This hypothesis is based on the observations that 1) the presence of FSH receptors in small follicles shortly after entering the growing pool (Xu et al., 1995; Bao et al., 1997), 2), the association between an increase in the serum concentration of FSH and the development of primary follicles

to secondary follicles in the developing fetus at Day 120 of gestation (Tanaka et al., 2001), and 3) wave pattern of development of follicles ⩾4 mm is temporally associated with the changes in the circulating concentrations of FSH (Adams et al., 1992a).

In conclusion, results supported the stated hypotheses: (1) a follicular wave pattern can be detected using non-serial data; and (2) the pattern of follicular growth detected by non-serial methods is similar to that detected by conventional serial methods. The non-serial method described herein may be useful to study small follicle dynamics by histomorphometry.

4. GENERAL DISCUSSION

The developmental pattern of large follicles (≥4 mm) has been accepted as "wave-like", in temporal association with periodic surges in circulating concentrations of FSH (Adams et al., 1992a). With this understanding, new treatment protocols have been developed to improve the efficacy of estrus control and to optimize the superovulatory response to gonadotropin treatments (Adams, 1998). However, imprecision in the degree of estrus synchrony, and variability in the response to superovulatory treatments remain the limiting factors to widespread implementation of advanced reproductive technologies in mammals, including humans and cattle. These limitations may be attributed to the lack of knowledge about the dynamics of small follicles <4 mm. A set of studies was therefore, planned to understand the dynamics of small follicles with a general hypothesis that small (<4 mm) follicles develop in a wave-like fashion.

The impetus to test the stated hypothesis was derived from observations that 1) follicles ⩾4 mm develop in a wave-like fashion, in temporal association with changes in circulating concentrations of FSH (Adams et al., 1992a), 2) there is an inverse relationship between the number of follicles 2-3 mm vs ≥4 mm (Pierson and Ginther, 1987a), 3) the number of follicles ≥4 mm recruited into a follicular wave from one wave to the next is consistent (Boni et al., 1997; Singh et al., 2003), suggesting that the follicular development might organize into waves during early stages of development, 4) FSH receptors are present in small follicles shortly after entering the growing pool

(Xu et al., 1995; Bao et al., 1997), indicating a functional role of FSH on small follicle development, and 6) the development of follicles from primary to secondary stages in the developing fetus at Day 120 of gestation was associated with an increase in serum concentration of FSH (Tanaka et al., 2001), thus suggesting functional role of FSH in small follicle development.

The signal that triggers a specific primordial follicle to start growing from the resting pool is not clearly understood (Chapter 1). Once a primordial follicle is activated, it grows continuously until it either regresses or ovulates. The role of FSH is unequivocally implicated in the development of a follicle after it reaches a diameter of 4 mm; however, controversy persists regarding the role of FSH during early stages of the follicular development. Immediately after activation of a primordial follicle from the resting pool, the expression of mRNA was detected for FSH receptors in the granulosa cells (Xu et al., 1995; Bao et al., 1997) is indicative of a possible functional role of FSH in small follicle development. In contrast, another study (Bao and Gaverick, 1998) refuted this possibility, as changes were not detected in the intensity of mRNA expression for FSHr in granulosa cells of follicles from the primary stage up to 2 mm in diameter. However, the latter conclusion may be challenged because during growth, the number of granulosa cells increases in a follicle (Lussier et al., 1987), and with that, an increase in the total number of FSHr in a follicle. Hence, even though the intensity of mRNA for FSHr remains constant in a follicle until it attains a diameter of 2 mm size, the increase in the number of FSHr due to increase in the number of granulosa cells with follicle growth may be able to fulfill the increased requirement of FSH for a developing small follicle. Small follicles may thus be able to grow at a basal concentration of FSH,

but with an increase in their size, they may become increasingly sensitive to stimulation by FSH.

Studies have revealed involvement of FSH in the increase in the number of large follicles recruited during superovulatory regimes in women and domestic animals (Fortune, 1994, Driancourt, 2001). Superovulatory treatment in cattle doubles follicular growth rates of large follicles from 1.6 mm/day without artificial stimulation (Ginther et al., 1989a) to 3 mm/day (Driancourt et al., 1991). In cattle, the growth rate of follicles during early stages of development (<0.5 mm size) is comparatively slower (Scaramuzzi et al., 1980) than the final stages follicular development (Scaramuzzi et al., 1980; Lussier et al., 1987). The reason as to why the effect of endogenous or exogenous FSH on the early stages of follicular development is not noticeable, may be due to their slow rate of growth. Moreover, the elusiveness of demonstrating a temporal association between small follicular development and recurrent peaks in circulating FSH concentrations may be due to the unavailability of a non-invasive tool like ultrasonography to monitor daily changes in the diameters of small follicles.

Small follicles <4 mm are classified into 1-3 mm and < 1 mm categories based on the type of tool used to visualize them. Follicles 1-3 mm can now be identified on a conventional daily basis using high resolution ultrasonography (Chapter 2). However, a non-invasive tool like ultrasonography is not available to visualize follicles <1 mm. The only approach available to study the dynamics of follicles <1 mm is to excise ovaries from different animals on different days and visualize follicles by microscopy. However, such approaches generate non-serial data as opposed to serial data as obtained by ultrasonography. The dynamics of follicles <1 mm has remained unaddressed as no method is available to analyze non-serial histological data. The discourse of this thesis

was therefore to study the dynamics of 1-3 mm follicles using ultrasonography (Study 1; chapter 2), and to design and validate a method to study the dynamics of follicles using non-serial data (Study 2; Chapter 3).

Results of Experiment 1 revealed that small antral (1-3 mm) follicles develop in a wave-like pattern. The dynamics of 1-3 mm follicles in Experiment 1 was inferred by using profile of the number of follicles. A significant inverse relationship was detected in the profiles of the number of small follicles (1-3 mm) and large follicles (≥ 4 mm), consistent with a wave-like developmental pattern (Pierson and Ginther, 1987a). The chronological shift in the population of follicles from one size category to the next represents the growth of smaller follicles, as a cohort, to a larger diameter without immediate replacement by another set of small follicles.

The dynamics of 1-3 mm follicles between waves within and among 2- and 3-wave interovulatory intervals (IOI) were compared in Experiment 1 (chapter 2). The profile of 1-3 mm follicles was found to be similar among waves in 2- and 3-wave IOI, except the ovulatory wave in 3-wave IOI. A higher number of follicles was detected at the emergence of the ovulatory wave (Wave 3) in 3-wave IOI. In earlier studies, the number of follicles recruited into a wave did not differ between anovulatory and ovulatory waves in 2-wave IOI (Ginther et al., 1989a); and between anovulatory and ovulatory waves in 3-wave IOI (Fortune et al., 1988). These results are in agreement with the present study, except that the ovulatory wave in 3-wave IOI had a greater number of follicles than anovulatory waves. However, in a recent study, there appeared to be more larger follicles (>6 mm) in 2-wave cycles on Days 3 and 4 from ovulation in association with higher circulating concentrations of inhibin A (Parker et al., 2003).

Reproductive senescence in bovine females is associated with the depletion of gametes, which occurs between 15 to 20 years of age, and is manifested by irregular estrus or complete anestrus (Erickson, 1966a). Similar studies in mice revealed that the average reproductive life span was related to germ cell numbers (Jones and Krohn, 1961). In women, only several hundred follicles remain between the ages of 45 and 55, and is associated with the onset of menopause (Richards, 1980; Richardson et al., 1987). Based on this information, we speculated that cows with 3-wave IOI might reach reproductive senescence earlier than cows with 2-wave IOI because of the emergence of one extra wave of follicles per IOI. Studies on the repeatability of the 2- or 3-wave IOI pattern within cows have not been reported; however, over 95% of IOI are composed of 2 or 3 waves (reviewed by Adams, 1999). A preponderance of 2-wave IOI has been reported by some (Ginther et al., 1989d; Ahmad et al., 1997), whereas others report a preponderance of 3-wave IOI (Sirois and Fortune, 1988) or a uniform distribution of 2- or 3-wave pattern (Evans et al., 1994). Recent studies in women (Baerwald et al., 2003) reported a wave-like developmental pattern of follicles and prevalence of 2- or 3-wave pattern within IOI. Further, the number of follicles ≥4 mm detected during successive waves in an individual cow is consistent (Boni et al., 1997; Singh et al., 2003). Reports are conflicting regarding the pool of follicles present in ovaries of a bovine female at birth (i.e., 68000, Erickson, 1966b; 16200, Tanaka et al., 2001). However, assuming consistency in 2- or 3-wave pattern and the activation of an average of 43 follicles during each wave based on the average number of ultrasonographically detected follicles ≥4 mm during wave emergence (Ginther et al., 1996) and the described rate of atresia in preceding size follicles (Table 1.2), the estimated rate of gamete depletion may be compared between cows with 2- versus 3-wave IOI (Table 4.1). On average, cows

exhibiting 2-waves of follicular emergence have an IOI of 20 days versus 23 days in cows with 3-waves of follicular emergence (Adams, 1999). Mathematically (Table 4.1), cows with 3-wave IOI exhibit 210.9 extra waves of follicles during 20 years of life, equivalent to activation of approximately 9068.7 extra follicles or 5.76 years of reproductive life compared to cows with 2-wave IOI. A difference of 10,000 follicles represents approximately 15% (Erickson, 1966b) or 61% (Tanaka et al., 2001) of the total pool at birth. This implies that gametes are more rapidly depleted in cows with 3-wave IOI, leading to the early onset of reproductive senescence. Perhaps a similar association exists in women with a 2- versus 3-wave pattern IOI and early onset of menopause may be investigated. In women, the onset of menopause occurs half way through life and is of major concern to the general well being of a woman.

Table 4.1 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

Mathematics		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% (refer Table 1.2)[3]		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 to Table 1.2), 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

A higher number of follicles detected at the emergence of the ovulatory wave in 3-wave IOI may be associated with shorter intervals between successive FSH peaks. The addition of FSH has been reported to suppress apoptosis in serum-free culture of rat preantral (McGee et al., 1997) and antral (Tilly and Tilly, 1995) follicles. A small increase in FSH allowed the development of follicles beyond the normal stage of atresia (Zeleznik and Kubik, 1986). The higher mitotic activity of 0.68 to 1.52 mm follicles (Lussier et al., 1987) indicates a higher potential for growth in this size category of follicles. The study also revealed that the atresia is much higher (40%) in this size category of follicles compared to preceding small size categories (Lussier et al., 1987). It is therefore, implied that 0.68 to 1.52 mm follicles are more sensitive to the deprivation of the FSH, and the shorter interval between successive FSH peaks preceding emergence of Wave 3; i.e., the ovulatory wave in 3-wave IOI might provide a timely thrust to rescue more of the 0.68 to 1.52 mm follicles from atresia. Therefore, a shorter interval between second and third wave-eliciting FSH surges (Adams et al., 1992a) and less inter-wave suppression by short-lived dominant follicle of the second wave (Ginther et a., 1989d; Adams et al., 1992b; 1993a) in 3-wave IOI may account for the higher number of follicles detected at the emergence of the third wave in 3-wave IOI. In addition, detection of an increased number of follicles subsequent to an early peak in FSH is consistent with the stimulatory role of FSH on small follicles. It would be interesting to compare the superovulatory response during different waves between and among 2-wave and 3-wave IOI cows.

The inter-peak intervals of circulating FSH, as well as inter-wave interval in 2-wave IOI animals, are evenly spaced (Ginther et al., 1989d; Adams et al., 1992a). Hence, follicles in IOI with 2-waves are exposed to uniform changes in hormonal milieu

over time. The longer inter-wave interval in IOI with 2-waves may also ensure complete nuclear and cytoplasmic maturation of an oocyte. Conversely, FSH peaks in 3-wave IOI are not evenly spaced, and are frequent compared to 2-wave IOI (Ginther et al., 1989d; Adams et al., 1992a). Thus, the short-lived ovulatory follicle of cows with 3-wave IOI may not be as mature as the ovulatory follicle of cows with 2-wave IOI. Alternatively, the ovulatory follicle of 3-wave IOI may be healthier than that of 2-wave IOI due to frequent exposure to FSH as can be evidenced by reports (Ahmad et al., 1997; Townson et al., 2002) indicating greater fertility in cows with 3-wave IOI. Assuming repeatability within animals, it seems unlikely that fertility of 2-wave or 3-wave IOI would differ because the frequency of 2-wave and 3-wave IOI is not skewed. However, the repeatability of wave pattern and influence on fertility deserve further critical evaluation.

In the present study (Chapter 2), we used follicle number profile to infer the dynamic process in 1-3 mm follicles as it was difficult to identify individual small follicles and to record diameter changes in them on a daily basis. Similar findings were reported earlier (Fortune et al., 1988; Ginther, 1993), but no reasons other than the machine inefficiency were given therein. The difficulty experienced in Experiment 1 in serial identification of individual 1-3 mm follicles was not surprising because the small diameter was near the limit of image resolution, and smaller follicles grew slower and were greater in number than larger follicles. In addition, daily changes were difficult to track because small follicles tended to change plane within the ovarian tissue during growth, thus confounding the use of topographic landmarks. To address these issues, the design of Experiment 2 incorporated special criteria for animal selection to minimize variation and to allow for more frequent ultrasonography to detect subtle changes among small follicles and for modifications to data recording and tabulation.

Results of Experiment 2 indicate that the selection of dominant follicle occur much earlier than previously reported, and allowed us to monitor growth of individual follicle beginning at 1 mm in diameter. The dominant follicle initially identified at a diameter of 1 mm, 66 h before it reached a diameter of 4 to 5 mm (previously stated time of wave emergence; reviewed in Adams, 1999) was coincident with the beginning of the surge in FSH (Adams et al., 1992a). It's detection at 1 mm, 6 to 12 h earlier than that of subordinate follicles is in agreement with earlier studies, wherein the dominant follicle was detected at 3 mm a mean of 6 h (Ginther et al., 1997) or at 4 mm a mean of 7 h (Kulick et al., 1999) earlier than the future largest subordinate follicle. Hence, the dominant follicle has a size advantage even before its detection at 1 mm, which is in agreement with studies indicating thaKulick et al., 1999t a follicle which has a size advantage during the development most likely (>90 %) become the dominant follicle of a wave (reviewed by Ginther et al., 2001). Perhaps, the process of selection of a follicle to become the dominant follicle of a wave is merely a consequence of the size advantage among follicles in the cohort. The functional dominance of a follicle is defined as the ability to inhibit the growth of its subordinate follicles through suppression of circulating FSH and to survive under low concentrations of FSH by acquiring LH responsiveness (reviewed by Adams, 1999). In studies wherein a dominant follicle was removed (Adams et al., 1993a; Gibbons et al., 1997) or exogenous FSH was administered (Adams et al., 1993b) subordinates follicles were able to achieve dominance.

The study described in Chapter 3 was carried out to validate a newly designed non-serial method to create a platform to study dynamics of follicles <1 mm using non-serial

histomorphometric data. A method was proposed earlier (Gougeon, 1986) to study the developmental pattern of follicles based on histomorphometric data; however, changes in follicular populations were not documented quantitatively, and the concept remained hypothetical. The understanding of the dynamics of follicles <1 mm would allow us to determine if and when primary, secondary and early tertiary follicles conform to the wave pattern of development, and would provide new avenues of research into the mechanisms controlling follicle recruitment. A better understanding of follicular recruitment may lead to more effective diagnosis and manipulation of ovarian function and dysfunction in cattle as well as in species for which the bovine model has been validated, including women (Adams and Pierson, 1995; Baerwald et al., 2003).

Ultrasound data of follicles ≥4 mm, in which a wave-like pattern of development had already been documented by repeated examination of the same ovaries over days (Singh et al., 1998; Singh and Adams, 2000), were used to compare the results of conventional serial analysis and analysis of a subset of the same data arranged to simulate non-serial data. The supposition was that if a wave-like pattern of follicular development could be detected using a non-serial method, then it will be valid to apply the same method to histomorphometric data and test the wave theory of development of follicles <1 mm.

Results of our study validated the non-serial method for characterizing the wave-like developmental pattern of follicles using non-serial data. When the developmental pattern of large follicles was compared by profiling their diameter data using serial and non-serial methods and by using identity or non-identity schemes, non-serial data revealed similar follicular dynamics to that found using serial data. At this stage it is not known whether the diameter profile of follicles using non-serial data will be useful for

studying small follicle dynamics, because the analysis of data using diameter profile may be feasible only when there is a limited number of follicles and day-to-day changes in the diameter of follicles are prominent. Further, the non-serial data may not reveal the growth rate of follicles, unlike the serial method of diameter profiling, due to the slow rate of their growth compared to large follicles (Lussier et al., 1987; Fortune, 1994). The slow rate of growth of small follicles and failure to identify them repeatedly over time may also render it difficult to identify the wave of their origin since it takes 80-100 days from activation of a primordial follicle to its ovulation (Britt, 1991).

The data tabulated for profiling the developmental pattern of large follicles based on number of follicles in different size categories were used to mimic histomorphometric data (Choudary et al., 1968; Hirshfield and Midgley, 1978a; Gougeon, 1982, 1986; Wandji et al., 1996; Braw-Tal and Yossefi, 1997; Cushman et al., 1999). A change in the population of different size follicles on different days of the cycle is indicative of a "wave-like" growth of the same cohort of follicles. The use of serial and non-serial data to characterize the follicular dynamics revealed a similar developmental pattern and thus validated the use of non-serial method to characterize follicular dynamics. Changes in the population of different size categories of <1 mm follicles on different days or during different endocrine milieu would reveal the basic developmental pattern of small follicles.

Present studies on the dynamics of 1-3 mm follicles have opened new avenues to improve ovarian control regimes and to improve superovulatory treatment protocols. The purpose of validating the new non-serial method was to create a platform to study developmental dynamics of follicles <1 mm in diameter using histomorphometric data.

In conclusion, Study 1 revealed 1) a wave pattern in follicles as small as 1 mm, 2) that wave pattern of small follicles is associated with surge in FSH, 3) that selection of dominant follicle manifest much earlier than previously reported, 4) that the largest follicle of a cohort is detected 6-12 earlier at 1 mm size than its subordinates. Study 2 validated new technique for the study of developmental dynamics of very small follicles (refer Figure 4.1 for thesis summary).

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