EFFECT OF PROGESTERONE ON GNRH-MEDIATED LH RELEASE, OOCYTE QUALITY AND FERTILITY IN CATTLE

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ABSTRACT

The objective was to investigate the effects of progesterone (P_4) on luteinizing hormone (LH) release, follicle development, and oocyte competence in cattle. We tested the general hypotheses that: 1) The suppressive effect of P_4 on gonadotrophin releasing hormone (GnRH)-mediated LH release can be overcome by increasing GnRH dose or pre-treatment with estradiol (E_2); and 2) a shorter period of P_4 exposure during the growing phase of the ovulatory follicle improves oocyte competence and fertility after fixed-time artificial insemination or superstimulation in cattle.

In the first experiment, heifers (n=22) were treated with 100 or 200 μg of GnRH or pretreated with E_2 prior to administration of GnRH during high or low circulating P_4 concentrations to characterize LH release (Chapter 2). Increasing the dose of GnRH did not alter LH secretion; however, E_2 pretreatment overcame the suppressive effect of high P_4 on LH secretion. Cattle with lower (n=11) P_4 concentrations had higher circulating LH concentrations than those with higher P_4 concentrations (n=11), and tended to have higher ovulation rates.

Two experiments were conducted to determine the effect of the duration of P_4 exposure during the ovulatory wave on fertility followed fixed-time artificial insemination or superstimulation. In the first experiment (Chapter 3), the dominant follicle was allowed to grow for 3 days (n=181) or 6 days (n=184). Six days of growth resulted in a larger dominant follicle, but in both groups, ovulatory follicles had similar capacities to ovulate and establish pregnancy. In the second experiment (Chapter 4), multiple follicles were allowed to grow for 3 or 6 days by 8 or 14 injections of FSH (at 12-hour intervals). There was no difference between groups for ovulation rate or total ova/embryo recovery rate. Although the 3-day group had higher embryo quality at slaughter (4 days after insemination), further development (7, 9, and 10 days after insemination) did not differ among groups. The effect of FSH starvation following 4 days of FSH treatment (Chapter 4) resulted in loss of ovulatory capability. Overall, a shorter duration of P_4 exposure during ovulatory follicle growth did not improve fertility after fixed-time AI or oocyte competence after superstimulation.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
CIDR	Controlled internal drug releasing device
CIDR-B	Controlled internal drug releasing device for bovine
CL	Corpus luteum
E_2	Estradiol
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
IM	Intramuscular
IOI	Interovulatory interval
LH	Luteinizing hormone
MHz	Megahertz
Mg	Microgram
mg	Milligram
mm	Millimeter
Ng	Nanogram
pg	Picogram
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGC	Primordial germ cells
SEM	Standard error of the mean
vs	Versus

CHAPTER 1

GENERAL INTRODUCTION

1.1 Reproductive life in cattle

The reproductive life of the heifer starts with the onset of puberty, which includes first ovulation and corpus luteum (CL) formation. The onset of puberty involves the ability of the hypothalamus to produce gonadotrophin releasing hormone (GnRH) in sufficient quantities to promote a luteinizing hormone (LH) peak and ovulation; it depends on factors such as breed or genotype, body weight and nutrition, social environment, and season [1]. Ovarian activity decreases with age (senescence) [2]; 55% of cows are considered infertile by 13 years of age [2].

During their reproductive life, females have many estrous cycles. An estrous cycle is defined as a series of changes which occur in most female mammals during the interval from one estrus to the next estrus [1]. In cattle, the duration of the estrous cycles averages 21 days [1, 3]. Each estrous cycle can be divided into four periods: proestrus, estrus, metestrus, and diestrus [1, 3]. Proestrus is the period that precedes estrus and is characterized by regression of the CL from the previous estrous cycle to the onset of estrus (average duration, 2 days) [1, 3]. Estrus is the period that the female is receptive to mating. Estrous behavior can last up to 20 hours; ovulation occurs approximately 28 hours after the onset of estrus, and is followed by development of a CL at the site where ovulation occurred [1, 3]. Metestrus starts immediately after cessation of estrus and is characterized by early development of the CL (average, 3 days) [1, 3]. Diestrus is the period when the CL is fully developed and active [3]; it can last up to 15 days in the absence of pregnancy, or the CL can be maintained until parturition if the animal becomes pregnant [1]. With the advent of ultrasonography, it has become possible to readily detect the day of ovulation [4, 5]. Therefore, in the past 20 years, the emphasis is slowly shifting to define the reproductive cycle as an interovulatory interval (from one ovulation to the next), rather than based on behavior (from one estrus to the next).

There are 2 basic phases of the ovarian cycle that are based on the structures in the ovary: follicular and luteal phases [1, 3]. The follicular phase (development of the preovulatory follicle) is the period from luteolysis to ovulation, whereas the luteal phase is the period when the CL is active [1, 3].

1.2 Ovarian follicular development

Folliculogenesis is the process of ovarian follicular development, with the growth of small follicles into large preovulatory follicles [6]. Ovarian follicles can be classified as primordial, primary, secondary, tertiary, or preovulatory [6, 7]. Primordial follicles contain an immature oocyte, surrounded by flat squamous (pre-granulosa) cells [6, 7]. The change in the shape of granulosa cells, from flat to cuboidal, characterizes the emergence of primary follicles [6, 7]. The follicles and oocytes continue growing, and the number of layers of granulosa cells surrounding the follicle increase [7]; secondary follicles have up to 6 layers of granulosa cells. The zona pellucida, a capsule of glycoproteins, is formed around the oocyte [7]. Also in secondary follicles, theca cells start to form and surround the basal lamina in the follicle [7]. With formation of a fluid-filled antrum, the follicle is designated as a tertiary follicle [7], also known as a vesicular, antral, or Graafian follicle. Tertiary follicles can be classified by their developmental stage into early or late tertiary, and preovulatory [7]; they can reach ≥15 mm in diameter and become the ovulatory follicle.

1.2.1 Follicular dynamics and wave theory

In the mid-1980's, researchers started using real-time, transrectal ultrasonography to monitor ovarian follicular dynamics in domestic livestock, especially cattle and horses [4, 5, 8]. With this technology, changes in ovarian biology could be sequentially monitored, with essentially no interference with function [9]. This provided considerable impetus for research in ovarian function and follicular dynamics; consequently, there were many discoveries in a short interval.

Monitoring ovaries with real-time ultrasonography confirmed that follicles develop in a wave-like pattern [4, 10, 11], with each wave characterized by periods of emergence, growth, dominance, and ultimately atresia or ovulation [3, 12]. Emergence of the first wave usually occurs on Days 0 or 1 of the reproductive cycle (Day 0 = ovulation), when up to 24 follicles (follicle cohort), approximately 4 to 5 mm in diameter, are detected [3, 12]. In the growth phase, follicles from the cohort continued growing for more than 2 or 3 days [3, 12]. Usually, a single follicle is selected which continue to grow (dominant follicle), whereas the remaining follicles (subordinate follicles) stop growing. Selection occurs when the dominant follicle is approximately 8.5 mm in diameter [13]. The dominant follicle grows to a diameter of approximately 12-20 mm (dominant phase) [3], followed by either atresia or ovulation. During mid-cycle, the CL is still active, so the dominant follicle undergoes atresia and a new follicular wave emerges. However, if the CL regresses, the dominant follicle continues to grow and ovulates, and the cells surrounding the follicular wall (thecal and granulosa cells) luteinize and become the CL [3].

In cattle, the estrous cycle is usually composed of 2 or 3 follicular waves [3, 5, 12, 14, 15]. Although the preponderance and repeatability of 2 or 3 wave cycles is still not clear [6], the number of waves does not appear to not be related to factors such as breed, age, or season [6]. Females with 3 waves have a longer interovulatory interval than females with 2 follicular waves [6]. In that regard, the interovulatory interval is approximately 18-20 days in animals with 2 waves, and 22-24 days in animals with 3 waves [1]. In the first wave, follicles emerge (Day 0), grow for 1-2 days, one follicle becomes dominant (at approximately Day 3), it grows until approximately Day 6, reaches a plateau (no change in diameter), and ultimately undergoes regression (starting at approximately Day 12) [3]. A second wave emerges at Day 10 in 2-wave cycles, and at Days 8 or 9 in 3-wave cycles; it becomes the ovulatory wave in 2-wave cycles [3]. In 3-wave cycles, a third wave emerges at approximately Day 16 or 17 (its dominant follicle becomes the ovulatory follicle), with the dominant follicle of Wave 2 become atretic shortly after emergence of the third wave [3]. Ovulatory follicles grow longer in 2-wave versus 3-wave cycles, and they have a larger diameter at ovulation [5]. Moreover, prior to luteolysis, ovulatory follicles grow under a high-progesterone environment for 6 versus 3 days in 2- and 3-wave cycles, respectively [5].

There are contradictory reports regarding the effect of the duration of the ovulatory wave on oocyte competence and fertility. Some reports suggested that with prolonged periods of follicular dominance, fertility decreases gradually until Day 9 of dominance; thereafter, any further prolongation of dominance results in very rapid declines in fertility [16]. In that regard, prolonged follicular dominance could result in an aged oocyte and decreased fertility [16]. Conversely, other studies failed to detect a difference in pregnancy rates between cattle with 2 or 3 waves [17]. Therefore, it remains unclear whether length of progesterone exposure during dominant follicle development affects fertility.

1.2.2 Hormonal influences during the estrous cycle

The advent of radioimmunoassays in the late 1960's provided a method to measure hormone concentrations and study their effects on follicular dynamics during the estrous cycle [18]. Reproductive hormones are produced and secreted by the hypothalamus, pituitary, ovaries, and uterus [1]. The hypothalamus produces and secretes gonadotrophin-releasing hormone (GnRH), a neurohormone which acts on the anterior pituitary, releasing follicle stimulating hormone (FSH) and luteinizing hormone (LH) [3]. In the ovaries, estradiol is produced by the dominant follicle, whereas progesterone is produced by the CL [3]. The main hormone produced and released by the uterus is prostaglandin ($PGF_{2\alpha}$) [3].

Understanding the pattern of reproductive hormones and the effect of those hormones on the reproductive process is of great interest to many researchers. The effects of the main reproductive hormones are reviewed in the following sections.

1.2.2.1 Effect of follicle stimulating hormone

In mammals, FSH is produced and released by the gonadotropes in the anterior pituitary gland [19]. It is a glycoprotein and contains 2 polypeptide subunits, alpha (α) and beta (β) [19]. The α subunit is species-specific and similar to other glycoproteins such as LH and TSH [19]. However, the β subunit is hormone-specific [19]. The primary action of FSH in the female is to promote follicular development; its receptors are present exclusively on granulosa cells [20].

An increase in peripheral FSH concentrations consistently precedes emergence of a new wave of ovarian follicular development [21]; furthermore, the growing cohort is dependent on FSH [20]. Differential growth of a dominant follicle normally occurs when the largest follicle reaches 8.5 mm in diameter [22, 23]. The production of inhibin and estradiol by the future dominant follicle suppress FSH concentrations [20]; when FSH reaches a nadir, subordinate follicles stop growing and become atretic. However, the dominant follicle acquires LH receptors on its granulosa cells, and therefore is no longer dependent on FSH [20]. It is clear that the dominant follicle suppresses FSH, since its removal results in increased FSH and emergence of a new follicular wave 1.5 days later [24, 25].

Maintenance of high concentrations of FSH prevents dominant follicle selection, therefore a prolonged effect of FSH may result in superstimulation and a superovulatory response [26]. Since FSH has a short half life (approximately 5 hours), multiple doses are required in superstimulation programs [26].

1.2.2.2 Effect of luteinizing hormone

Similar to FSH, LH is a glycoprotein which is released in response to GnRH [22]. The episodic LH release from the pituitary is essential for ovarian function [27]. The role of LH in follicular selection is not completely clear; however, several studies suggest that LH is involved with follicular deviation, or selection [22, 28, 29]. From the cohort of follicles that emerges and constitutes a wave, it appears that the first follicle to acquire LH receptors will become the dominant follicle [30]. Receptors for LH can be first detected on granulosa cells from 2 to 4 days after wave emergence, which is the expected time of follicular selection [31]. In addition, increased concentrations of LH at the time of deviation was reported in heifers [22, 31]. The acquisition of LH receptors on granulosa cells appears to be related to increased concentrations of estradiol and high molecular weight inhibin in follicular fluid of the dominant follicle [30]. In that regard, the dominant follicle uses mechanisms to prevent competition with other follicles.

It is well known that LH is associated with final growth and maturation of the dominant follicle [32]. The pre-ovulatory surge of this hormone is required for ovulation [3]. In mid-

cycle, P_4 is still high, suppressing LH pulses and preventing ovulation [3]. As a consequence, a new follicular wave begins [3]. At the end of the cycle, when P_4 is low, LH can reach a pre-ovulatory peak, which is characterized by high frequency, but low amplitude pulses and consequently the dominant follicle ovulates [3]. When LH concentrations are maintained high under a subluteal P_4 environment, the dominance of the follicle is prolonged and an oversized persistent follicle may be formed [33].

1.2.2.3 Effect of progesterone

Progesterone is a steroid hormone derived from cholesterol [1]. The CL is the principal source of progesterone and its main function is maintenance of pregnancy [12]. During the estrous cycle, blood P_4 concentrations influence several other hormones. On Day 5 (Day 0 = ovulation), plasma concentrations of P_4 increase (due to release from the CL) [12]. Blood P_4 concentrations remain high during much of the estrous cycle. Luteolysis usually occurs on Days 16 to 18 of estrous cycle, depending on the number of waves [12], and then P_4 decreases. Luteolysis occurs as a consequence of prostaglandin ($PGF_{2\alpha}$) release from the endometrium [12]. The CL and the dominant follicle stimulate oxytocin-receptor expression in the endometrium [34]. Oxytocin activates phospholipase activation and prostaglandin synthesis in the endometrium [34]. $PGF_{2\alpha}$ reaches the ipsilateral ovary by a countercurrent exchange mechanism from the utero-ovarian vein to the ovarian artery [34]. $PGF_{2\alpha}$ activates apoptosis and luteolysis occurs [34]. A clear understanding of the effects of P_4 and $PGF_{2\alpha}$ on the estrous cycle is crucial for estrous synchronization.

Progesterone exerts a negative feedback on the hypothalamic-pituitary axis, suppressing GnRH, LH, and FSH [12]. Progesterone has differential effects on LH and FSH. An increase in FSH is enable in the presence of elevated P_4 to initiate a new follicular wave [12]; however, LH pulsatility does not reach a peak under a high P_4 environment [12], suppressing ovulation of the dominant follicle.

1.2.2.4 Effect of estradiol

Estradiol-17β (E_2-17β) is also a steroid hormone, derived from cholesterol, and synthesized by theca and granulosa cells of the follicles [1]. Theca cells provide an androgen precursor which passes into granulosa cells and is subsequently aromatized to estradiol [1]. Estradiol is known as the female hormone, since it promotes the development of secondary sex characteristics in females and is also responsible for estrous behavior [1].

During the estrous cycle, estradiol can exert either a positive or negative feedback on the hypothalamic-pituitary axis, affecting gonadotrophin release [1]. Estradiol has been implicated in follicle deviation [22, 35, 36]. The concentration of estradiol in follicular fluid increases when the follicle diameter is 8-9 mm [35]. Blood estradiol concentrations also increase at follicular deviation [35]. Some studies suggested that synthesis of estradiol by the dominant follicle is associated with acquisition of LH receptors by granulosa cells at follicular deviation [35]. Thus, estradiol appears to be a facilitator for a transition in the responsiveness of FSH to LH by the dominant follicle [35]. Moreover, estradiol suppresses FSH release, preventing further development of subordinate follicles, ultimately resulting in their atresia, and selection of the dominant follicle [22, 35].

A pre-ovulatory peak of LH is induced by low progesterone and high estradiol concentrations [37]. After luteolysis, the dominant follicle produces high concentrations of estradiol, which induces high frequency and low amplitude LH pulses [37].

1.2.3 Manipulation of follicular development and ovulation in cattle

Since reproduction is critical to cattle production, there has been substantial investigation of reproductive technologies for cattle. An important aspect of reproductive technologies is the ability to pharmacologically control follicular development and ovulation [3]; this requires knowledge of ovarian function, hormone pathways, and also the effects of exogenous hormones on female physiology [3]. Over the past several years, there have been many studies and reports regarding the use of hormones to manipulate ovarian function. Treatments to induce emergence of a new follicular wave are based on the removal of a

negative effect of the dominant follicle on FSH. Furthermore, to induce ovulation, treatments have to promote luteolysis and induce an LH peak. Although much progress has been made, a major limitation of controlling ovarian function is the variability in treatment response, especially the lack of a highly synchronous ovulation [38].

1.2.3.1 Follicular wave synchronization

The ability to reliably induce emergence of a new wave, regardless of the stage of the cycle (even in the absence of cyclicity), was an important step in synchronization programs. As the dominant follicle is known to suppress the release of FSH, elimination of a dominant follicle will create a rise in FSH, followed by emergence of a new cohort of follicles. Dominant follicles can be eliminated by cautery [39] or by transvaginal, ultrasound-guided follicle ablation [25]. After the dominant follicle is removed, FSH is expected to rise 12 hours later, and a new follicular wave emerges 1.5 to 2 days after removal of the dominant follicle [20, 40].

Exogenous steroid hormones can also induce follicular wave emergence [41-43]. Administration of exogenous estradiol suppresses FSH and causes follicular atresia [41, 43] . However, under a low progesterone environment, estradiol will induce a pre-ovulatory LH surge, delaying emergence of a new wave [43]. Treatment with estradiol in a high-progestin environment (due to exogenous or endogenous progestins, or both) suppressed both LH and FSH, with a subsequent rise in FSH and synchronous emergence of a new follicular wave approximately 4 days later [44].

Treatments with GnRH have also been used to synchronize wave emergence. A single dose of GnRH will increase LH and FSH; the magnitude is dependent on the stage of the estrous cycle [45] and the dose injected [46]. In that regard, treatment with GnRH induced ovulation and a emergence of a new follicular wave when a follicle $\geq$10 mm in diameter was present in the ovary [41]. However, the probability of GnRH causing ovulation, followed by wave emergence, was 85% in cows, but only 56% in heifers [47]. When ovulation occurred, the interval from treatment to wave emergence averaged 2 days [41].

1.2.3.2 Synchronization of estrus and ovulation

The general objective of synchronization protocols is to have a fertile estrus or ovulation in a short, predictable interval [48]. Researchers have developed several protocols to synchronize estrus; all are based on either extending or shortening the lifespan of the CL [3, 48].

Endogenous $PGF_{2\alpha}$ is responsible for luteolysis; exogenous $PGF_{2\alpha}$ (and its analogs) is commonly included in estrus synchronization protocols to ensure that luteolysis occurs [48]. When it is administrated during mid-cycle (5-16 days after ovulation), $PGF_{2\alpha}$ will efficiently induce luteolysis [48]. However, $PGF_{2\alpha}$ has an effect exclusively on the CL [3], making its efficacy dependent on the stage of the estrus cycle [48]. If $PGF_{2\alpha}$ is given when a responsive CL is not present (Day -3 to +4 of ovulation), it will not synchronize estrus. A common solution to enhance the efficacy of $PGF_{2\alpha}$ is the use of double injections 11-14 days apart, with the ischiorectal fossa as an ideal site of injection [49, 50]. In the majority of cattle that undergo luteolysis in response to $PGF_{2\alpha}$ treatment, ovulation occurs 3 to 5 days after injection [3]. To increase pregnancy rates after AI, estrus detection should be included in the protocol [3]; in the absence of synchronization of ovarian follicular development, timed-AI will result in low to modest pregnancy rates.

Protocols using GnRH are also common for estrus synchronization. Ovsynch and Cosynch programs are based on 2 injections of GnRH, 9 days apart, with a single injection of $PGF_{2\alpha}$ on Day 7 [51]. The difference between those two programs is that AI is either performed approximately 16 hours after the second GnRH (Ovsynch) or concurrent with the second GnRH (Cosynch). Ovulation is expected approximately 28 hours after the second injection of GnRH [52]. When compared with a protocol using only $PGF_{2\alpha}$, GnRH-based protocols appear to have more synchronous ovulation [51] and improved pregnancy rates to timed-AI in cows [53]. The effect of 3 commercial gonadorelin products (gonadorelin diacetate tetrahydrate (Cystorelin® and Fertagyl®) and gonadorelin hydrochloride (Factrel®)) on LH release and ovulation was recently reported [54]. In that study, Cystorelin seemed to induce a higher LH peak concentration; however there was no difference in ovulatory response [54].

Progestins (progesterone and its analogs) can also be used to synchronize estrus. The use of oral progestins, eg. melengestrol acetate (MGA), for estrus synchronization, started approximately 4 decades ago [3, 55]. The minimal amount to suppress estrus and ovulation is approximately 0.5 mg per day; most MGA-based synchronization protocols use 0.5-1.0 mg per day [55]. Although MGA suppresses estrus, it suppresses development of subordinate follicles, but enhances development of a dominant follicle, that will ovulate following MGA withdrawal [3, 56]. Furthermore, prolonged treatment with MGA (in the absence of a CL) results in a persistent dominant follicle [3, 56]. The interval from withdrawal of MGA to ovulation can vary from 3 to 7 days [3]. Many studies were conducted to test fertility after various MGA-based protocols. However, in the absence of exogenous $PGF_{2\alpha,}$ MGA was fed for prolonged intervals, resulting in persistent follicles and poor fertility [57, 58].

A subcutaneous implant containing a synthetic progestin, norgestomet, was also intensively tested after the mid 1970's [3]. It was demonstrated that a short interval (5 days) of norgestomet exposure resulted in higher pregnancy rates compared with treatment for longer intervals [59]. However, fertility remained compromised when norgestomet programs were compared with controls. Subsequently, the combination of an implant containing 6 mg of norgestomet (removed after 9 days), and a single injection of 3 mg of norgestomet plus 5 mg of estradiol valerate at implant insertion, (Synchro-Mate-B) resulted in more efficient estrus synchronization and higher pregnancy rates [43]. It is noteworthy that the estradiol was originally included to induce luteolysis [43], but years later, it was shown that it also synchronized ovarian follicular development [43].

Progesterone-releasing silicone intravaginal devices were developed in the mid 1960's and so far are the most useful for commercial application [3]. Several devices were developed and tested. The CIDR-B is the most common intravaginal progesterone device in North America [3]. The CIDR-B contains 1.94 g of progesterone and is extensively used in various protocols for estrus synchronization, fixed-time AI, fixed-time embryo transfer, and superstimulation programs [3]. The use of those devices for 7 days, with an injection of $PGF_{2\alpha}$ at device removal, results in pregnancy rates that do not differ from non-treated animals [60]. However the effect of duration of P_4 exposure through the use of CIDR-B was not fully clear. Prolonged CIDR-B use could result in an aged oocyte and poor fertility [33]. When subluteal

(1-2 ng/ml) progesterone concentrations are maintained for a prolonged interval, ovarian follicular dynamics are altered, resulting in development of a large, persistent follicle [61], that has poor fertility [61]. Therefore, estrus synchronization programs must minimize the development of persistent follicles [62].

To synchronize an LH surge and thereby induce a synchronous ovulation, an additional dose of GnRH (100 μg), porcine LH (pLH, 10-12.5 mg), or estradiol (0.5-1.0 mg) can be given at the end of a synchronization protocol [63]. In that regard, all three hormones will induce a pre-ovulatory LH peak, that is followed by ovulation, when blood P_4 concentrations are low [3]. It is noteworthy that highly synchronous ovulation increases pregnancy rates, particularly to timed-AI [3].

In cattle with low peripheral P_4 concentrations, treatment with 100 μg of GnRH will induce an LH peak and ovulation [48, 51, 64]. Moreover, lower doses (50 μg) of GnRH after $PGF_{2\alpha}$ injection were equally efficient in inducing ovulation in Ovsynch programs [65]. However, it is unknown if a higher dose of GnRH could reliably induce ovulation under a high P_4 environment.

1.2.3.3 Fixed-time AI

Fixed-time AI programs use protocols to synchronize estrus and ovulation, permitting AI at a predetermined time. Highly synchronous and predictable ovulation has many advantages for cattle breeding programs. First of all, it facilitates planning AI and increases pregnancy rates [3, 66]. In traditional AI programs, estrus detection is required, which requires considerable time and experienced staff. With fixed-time AI, there is no need for detecting estrus [66], and more cattle can be inseminated at a specific time [66]. Consequently, the introduction of fixed-time AI programs has greatly increased the utilization of AI in the cattle industry [3, 67]. The biggest challenge of fixed-time AI is to maintain acceptable pregnancy rates [3]. Several protocols have been developed. The inclusion of a CIDR-B in Ovsynch or Cosynch programs is common in fixed-time AI programs [68]. Other protocols involve synchronization of follicular wave emergence with ultrasound-guided follicular ablation or a combination of estradiol and progesterone, with a CIDR-B for 5-9 days, $PGF_{2\alpha}$ at CIDR

removal, and 24-56 hours later, treatment with pLH, GnRH, or estradiol, to induce ovulation, followed by timed-AI [3, 26]. Other types of progestins or progesterone devices have also been used.

Although estradiol is highly efficacious for synchronizing wave emergence and ovulation, it is noteworthy that the use of exogenous estradiol in the commercial cattle industry is forbidden in the European Union, New Zealand and United States [69, 70], due to concerns regarding residues in milk and meat, and their potential to impact human health [69, 70]. Therefore, protocols which do not involve the use of estradiol for synchronization are heavily favored.

1.2.3.4 Superstimulation and superovulation

Superstimulation is based on the principle that subordinate follicles that would otherwise undergo atresia can be 'rescued', with continued development and ovulation [26]. The main objective is prevent atresia of many follicles and ultimately have many ovulate, to maximize fertilized ova and transferable embryos [26].

One of the major limitations of superovulation is the extreme variability in the response to superstimulatory treatments [15, 26]. In one study, 30% of 2048 cows produced 70% of the total embryos collected, whereas 24% of cows failed to produce an embryo [71]. Overall, the mean number of transferable embryos per donor is approximately 6 [71]. Heritability of the response to superstimulation treatments is low [72]; the variability in response has been attributed to environmental factors related to reproductive, sanitary or nutritional conditions, and the type and duration of gonadotrophin used [26, 72].

Gonadotrophin treatments provide FSH activity, resulting in superstimulation [26]. Historically, equine chorionic gonadotrophin (eCG) was commonly used in superstimulation protocols, whereas more recently, purified porcine pituitary extract (pFSH) is more widely used [26].

It is well known that eCG has primarily FSH activity in cattle, with varying degrees of LH activity. A single injection of the recommended dose (2500 IU) can remain in circulation for up to 10 days and cause excessive superstimulation [26], resulting in few embryos of low

quality [73]. However, the use of pFSH increased rates of ovulation and transferable embryos when compared with eCG treatments [74]. When purified pFSH which has low LH activity is used, the superstimulatory response is increased [75-77]. In that regard, the maximum acceptable LH contamination is 20% [77].

Since FSH has a half life of approximately 5 hours, multiple injections need to be given [26]. Usually twice daily injections (12-hours intervals) given IM for 4-5 days [78] induces an optimal stimulatory response [26].

The major determinants of superstimulatory response are the number of follicles available at the beginning of a wave [9] and the timing of the onset of treatments in relation to wave emergence [79]. When gonadotrophin treatments are initiated before follicular selection, the superstimulatory and superovulatory response is greater [80]. Therefore, synchronization of wave emergence, followed by initiation of superstimulation treatment at expected wave emergence, is a very popular means of superovulation. One protocol used in superstimulation programs is follicular ablation and insertion of a CIDR-B, followed by the initiation of pFSH treatment 1 day later and continued for 4 days, with $PGF_{2\alpha}$ injection and CIDR removal on the last day of FSH treatment [81].

The interval from the end of FSH treatment to ovulation or collection of oocytes influences oocyte competence [82]. In one study, blastocyst rates were increased when follicle stimulation included 48 hours of FSH starvation [83]. However, Barros et al. [84] reported that FSH starvation at the end of superstimulation treatment had no advantage over conventional protocols for in vitro embryo production. Therefore, studies regarding a short period of FSH starvation are contradictory and further investigation is required.

1.3 Oocyte competence

Oocyte competence is defined as the ability of oocytes to be fertilized, develop into a blastocyst, and result in successful pregnancy [26, 85, 86]. In most in vitro embryo production programs, oocyte competence is assessed by visual evaluation of oocyte quality. Although evaluation of oocyte quality is subjective, oocytes evaluated as Grade 1 are likely to be competent. Oocytes acquire competence during folliculogenesis and oocyte development [86].

However, the two major factors that influence oocyte competence are the origin of the follicle and hormonal influences [86]. It has also been suggested that follicular size and health may influence oocyte competence [86]. Moreover, appropriate communication between the oocyte and the surrounding cummulus complex within the follicle is required for competent oocytes [86]. It is known that the hormonal environment also plays a role in oocyte competence.

1.3.1 Oocyte development

Oogenes is the process of oocyte formation and development; it can be divided in two phases: oocytogenesis, which is the development of primordial germ cells (PGC) into primary oocytes; and ootidogenesis, which is the development of primary oocytes into mature oocytes.

In cattle, during the embryonic stage, PGCs migrate from the endoderm of the yolk sack to gonadal ridges at approximately Day 40 of gestation [87, 88]. The factors that attract PGCs to gonadal ridges are not clear [87, 88]. From the start of migration, PGCs undergo mitosis; PGCs that arrive early also mature early. On Day 80, the most mature oogonia start to undergo meiosis; they enlarge and are termed primary oocytes [87]. Primary oocytes do not progress beyond the pachytene stage of prophase I (first meiotic arrest) [87, 88]. A layer of epithelial cells surrounds primary oocytes and form primordial follicles [87]. Primordial follicles form the follicular reserve, which are capable of developing into all other stages of follicular growth [88]. Females are born with a reserve of approximately 133,000 primordial follicles [89].

The activation of primordial follicles has not been well characterized. The development of greater communication, through gap junction formation, between granulosa cells and oocyte is critical for the activation of primordial follicles [87, 90]. Nutrients, hormones and growth factors may also have a role in initiating growth of primordial follicles [87, 90]. Furthermore, germ cells may secrete substances that inhibit development of some primordial follicles, thereby preventing the reserve to grow simultaneously [87].

After primordial follicle activation, follicles continue developing and oocytes increase in volume [32, 91]. When the follicular antrum is formed, oocytes are meiotically competent [32, 91]. The preovulatory LH surge overcomes the first meiosis arrest; however, meiosis is

again stopped at metaphase II (second meiosis arrest) until fertilization, when meiosis is finally completed [32, 91].

Oocyte nuclear maturation occurs in response to a pre-ovulatory LH peak [32, 91]. Furthermore, LH also plays a key role in the expansion of cumulus cells, which is crucial for fertility [32, 91]. The mechanism by which LH induces oocyte nuclear maturation is unclear. However two hypotheses have been proposed [92]. The first one is that granulosa cells produce meiosis-inhibiting substances and that the LH peak interferes with communication between oocyte and granulosa cells. The second hypothesis is that the LH peak induces a maturation signal on the granulosa cells, which transport this signal to the oocyte, inducing it to mature (reviewed by Buccione et al. [92]).

The competence of an oocyte to undergo fertilization and pre-implantation is termed cytoplasmic maturation [92]. Although granulosa cells are suspected to have a role [92], the mechanisms regulating cytoplasmic maturation are not well characterized.

1.3.2 Influence of progesterone on oocyte competence

The effect of progesterone on female endocrinology and ovarian function in cattle has been intensively studied. However, there is a paucity of data regarding the effect of progesterone on the oocyte microenvironment. In most studies regarding the effect of progesterone on the oocyte, the outcomes were reproductive responses, including rates of blastocyst formation or pregnancy.

It is known that a high preovulatory LH peak is crucial for oocyte maturation [92]; this is believed to disrupt the gap junction between the oocyte and granulosa cells [92]. Granulosa cells are suspected to produce substances, e.g. cAMP, which arrest meiosis [92]. However, with reduced communication between the oocyte and granulosa cells, less of the meiosis inhibitor substances are carried to the oocyte, and meiosis is reactivated [92].

Moderate concentrations of progesterone at the end of the growing phase of the dominant follicle increase LH pulse frequencies [92], resulting in a prolonged growth and maintenance of the dominant follicle [20], termed a persistent follicle [20]. It has been suggested that oocytes from persistent follicles undergo premature activation (resumption of

meiosis), which leads to abnormal development, early embryonic mortality, and ultimately poor fertility [93].

1.4 General objectives and general hypotheses

The overall goals of the research conducted for this thesis were to improve estrus synchronization in cattle and to improve reproductive performance after fixed-time AI or superstimulation. The general hypothesis was that different concentration of progesterone and different duration of progesterone exposure during the growing phase of ovulatory wave affects GnRH-mediated LH release, oocyte quality and fertility in cattle.

Specific Objectives and Specific Hypotheses

Objective 1: (Chapter 2): To determine if higher doses of GnRH can overcome the suppressive effect of progesterone on LH release, and to determine the effect of exogenous estradiol on LH release (and ovulation) in response to GnRH treatment in cattle with elevated P_4 concentrations.

Hypothesis 1: (Chapter 2): The suppressive effects of P_4 on GnRH-induced LH release can be overcome by increasing the dose of GnRH, or by pretreatment with estradiol

Objective 2: (Chapter 3): To determine the effect of the duration of P_4 exposure during the growing phase of the ovulatory follicle on pregnancy rate.

Hypothesis 2: (Chapter 3): A short interval of P_4 exposure during the growing and early-static phases of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long interval (analogous to the ovulatory wave of 2-wave cycles).

Objective 3: (Chapter 4): To determine the effects of the duration of P_4 exposure during the growing phase of pre-ovulatory follicles on oocyte competence after superstimulatory treatment.

Hypotheses 3: (Chapter 4): 1) A short interval of P_4 exposure during follicular growth under ovarian superstimulation improves oocyte competence; and 2) FSH starvation at the end of superstimulatory treatment does not affect oocyte competence.

CHAPTER 2

EFFECT OF PROGESTERONE AND ESTRADIOL ON GNRH-MEDIATED LH SECRETION IN HEIFERS

2.1 Abstract

High circulating progesterone (P_4) concentrations during the ovulatory wave in cattle can decrease circulating estradiol (E_2) concentrations, luteinizing hormone (LH) pulsatility, diameter of ovulatory follicles, and ovulation rates. However, it is unknown whether increasing the dose of GnRH or pretreatment with E_2 can overcome the suppressive effects of elevated P_4 concentrations on LH release and ovulation in response to GnRH treatment. We hypothesized that increased doses of GnRH and exogenous E_2 would enhance LH release, particularly in heifers with high plasma P_4 concentrations. Two experiments were conducted using 22 crossbred postpubertal heifers. In Experiment 1, all heifers had the two largest follicles ablated on Day 5 after ovulation; they received a once-used P_4-releasing device (CIDR) and were monitored ultrasonographically for follicle wave emergence. They were randomly assigned to receive two injections of prostaglandin $F_{2\alpha}$ (PGF), 12 hours apart (Low- P_4), or no treatment (High- P_4) starting at the time of follicle ablation. Six days after follicle wave emergence, half of the heifers in each group received either 100 or 200 μg of GnRH, and blood samples were collected every hour for 3 hours; there was an effect of time ($P<0.0001$) but no effect of treatment on LH release. Heifers were handled similarly in Experiment 2, except that when a 5.5-day dominant follicle was expected, half the heifers in each group received 0.25 mg estradiol benzoate IM, and 8 hours later, all heifers received 100 μg GnRH. Blood samples were collected every hour for 4 hours (for LH concentrations) and ultrasonography was used to detect ovulation. The two groups that received E_2 (low- and high- P_4) and the low P_4 without E_2 had higher peak plasma LH concentrations ($P<0.04$) compared to the group with high P_4 without E_2 (12.6±1.8, 10.4±1.8, 8.7±1.3, and 3.9±1.2 ng/mL, respectively; mean±SEM). High plasma P_4 concentrations tended to reduce ovulation rates ($P=0.09$). For both experiments combined, heifers with low circulating

concentrations of P_4 had higher LH concentrations (P=0.039) following GnRH treatment. In conclusion, the hypothesis that higher doses of GnRH will be more efficacious in inducing LH release was not supported. However, the hypothesis that exogenous estradiol will increase LH release following treatment with GnRH was supported. Moreover, high concentrations of P_4 decreased LH secretion and tended to decrease ovulation rates.

2.2 Introduction

Gonadotrophin-releasing hormone (GnRH) is a neurohormone synthesized and released by the hypothalamus, which acts on the anterior pituitary to induce FSH and LH release [19, 94-96]. In clinical veterinary practice, GnRH is commonly used in estrus synchronization protocols (e.g. Ovsynch and Cosynch); 2 injections of GnRH are given 9 days apart, and prostaglandin $F_{2\alpha}$ is given 2 days before the second GnRH [51, 97]. The first GnRH treatment is intended to induce ovulation, and synchronize emergence of a new ovarian follicular wave [98]. However, only 2 of 3 (approximately 66%) of cattle ovulated in response to a single GnRH treatment [99]); this has been attributed to the diameter of the viable dominant follicle and blood progesterone concentration at the time of treatment [98]. Follicles achieve ovulatory capability at 10 mm in Bos taurus cattle [99], and there is an inverse relationship between circulating P_4 concentrations and the probability of ovulation [37].

The secretion of LH during the estrous cycle in cattle is mediated by peripheral P_4 concentrations [12, 100]. Elevated P_4 concentrations suppress pre-ovulatory LH pulses [12, 100]; following luteolysis, P_4 is low during the final stages of follicle development, enabling ovulation to occur [37]. However, the effects of high circulating concentrations of P_4 on LH pulsatility and ovulation in response to exogenous GnRH have not been well characterized. In that regard, it is unknown whether a high dose of exogenous GnRH can overcome the suppressive effect of elevated P_4 concentrations on LH release. A high pre-ovulatory peak is important in increasing diameter of the ovulatory follicle, inducing granulosa cell proliferation before ovulation, and inducing ovulation [99]. The number of granulosa cells and their associated LH receptors are directly associated with the amount of P_4 that will be produced by the CL after luteinization [99].

Consequently, a lower P_4 concentration near the time of ovulation may result in a CL that produces more P_4, which may enhance pregnancy rates [101].

Estradiol benzoate is a useful tool in estrus synchronization protocols in cattle [37, 102]. Estradiol can be used to suppress peripheral FSH concentrations, followed by an FSH surge, resulting in emergence of a new follicular wave. Similarly, in vivo, E_2 is involved with the suppression of FSH during follicular selection [37, 102]. Steroid hormones regulate gonadotrophin release by both positive or negative feedbacks on the hypothalamus and/or anterior pituitary [103]. Progesterone suppresses LH release, whereas E_2 has a positive effect on LH surges [103]. In that regard, E_2 is often given when P_4 concentrations are subluteal to induce an LH surge and synchronize ovulation. Administration of E_2 24 hours after PGF administration and progesterone device removal improved synchrony of both estrus and ovulation [48, 104]. However, the effects of exogenous E_2 on LH pulses in cattle with high P_4 concentrations have apparently not been reported.

The objectives of the present study were to determine, in cattle with elevated blood progesterone concentrations, whether LH release and ovulation in response to GnRH treatment is enhanced by: 1) an increased dose of exogenous GnRH; and 2) by pretreatment with estradiol. We hypothesized that higher doses of GnRH and exogenous E_2 will enhance LH release and ovulatory response to exogenous GnRH in cattle with high blood P_4 concentrations.

2.3 Materials and methods

2.3.1 Cattle

Crossbred, postpubertal, nulliparous heifers (n=22) were used in Experiments 1 and 2, which were conducted from October to December, 2006. These heifers were housed in open-air corrals at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). All procedures were performed in accordance with the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

2.3.2 Experiment 1

Heifers at random stages of the estrous cycle were given two doses of a prostaglandin $F_{2\alpha}$ analog im (500 µg of cloprostenol; Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada), 13 days apart, to synchronize estrus and ovulation. To detect ovulation, heifers were examined by transrectal ultrasonography, once daily for 4 days, with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). Five days after ovulation, the two largest follicles were ablated by transvaginal ultrasound-guided follicle puncture, to induce emergence of a new follicular wave (1.5 days later [25]). Concurrently, all heifers received a once-used (7 days) CIDR (Pfizer Canada Inc., Montreal, QC, Canada) and were randomly assigned to receive two injections of $PGF_{2\alpha}$ im (Lutalyse®; Pfizer Canada Inc.; Montreal, QC, Canada), 12 hours apart (Low- P_4 group; n=11), or no treatment (High- P_4 group; n=11). It was expected that the Low- P_4 and High- P_4 groups would have sub-physiological (i.e. <2.5 ng/ml; [21]) and high-physiological plasma P_4 concentrations, respectively, during dominant follicle development. Six days after follicle wave emergence, heifers received either 100 µg GnRH (Cystorelin, Merial Canada Inc., Victoriaville, QC, Canada; n=6 from Low- P_4 and n=6 from High- P_4 group), or 200 µg of GnRH (n=5 from Low- P_4 and n=5 from High- P_4 group).

Blood samples were collected by coccygeal venipuncture into 10 ml heparinized, vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at CIDR insertion and at 0, 1, 2, and 3 hours after GnRH treatment. Samples were centrifuged at 1500 x g for 15 minutes, and plasma was separated and stored at -20 °C until assayed for P_4, E_2, and LH.

2.3.3 Experiment 2

Similar to Experiment 1, heifers were given two doses of $PGF_{2\alpha}$ im (Lutalyse; Pfizer Canada Inc.), 13 days apart, to synchronize estrus and ovulation. Ovulations were detected using daily transrectal ultrasonography for 4 days, starting 1 day after the second $PGF_{2\alpha}$ injection. Follicular ablations were performed 5 days after ovulation (as in Experiment 1). Heifers received

a once-used CIDR (Pfizer Canada Inc.) and were randomly assigned to receive two injections of, $PGF_{2\alpha}$ at 12-hour intervals (Low- P_4) or no treatment (High- P_4). On Day 5.5-day (wave emergence = Day 0), half the heifers in each group received 0.25 mg im estradiol benzoate (0.2 ml of 2.5 mg/mL of estradiol benzoate; Sigma Chemical Co., St. Louis, MO, USA), prepared in canola oil), and 8 hours later, all heifers received 100 μg GnRH. Transrectal ultrasonography was performed daily with a 7.5 MHz linear-array transducer (Aloka SSD-900) until ovulation and emergence of the new follicular wave (after ovulation) was determined. Blood samples were collected, processed, and subsequently assayed, as described in Experiment 1.

2.3.4 Experiments 1 and 2 combined

Data from both experiments were combined to evaluate LH concentrations following treatment with 100 μg of GnRH in heifers with low versus high plasma P_4 concentrations. The analysis included heifers which received 100 μg of GnRH (Low- P4 and High- P_4 groups in Experiment 1) and those which did not receive estradiol (Low- P4 and High- P4 in Experiment 2).

2.3.5 Radioimmunoassays

Plasma LH concentrations were measured in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) [105]. The range of the standard curve was 0.06 to 8.0 ng/ml. For the low and high reference sera (means, 0.55 and 1.44 ng/ml, respectively), intra-assay coefficients of variation were 9.7 and 6.6%, and inter-assay coefficients of variation were 8.8 and 6.9%.

Plasma P_4 concentrations were measured in a single assay (for both Experiments 1 and 2) using commercial solid-phase kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/ml. The intra-

assay coefficients of variation were 4.1 and 1.8% for low and high reference sera (means, 1.77 and 16.51 ng/ml).

Estradiol was determined using a modified commercial RIA human kit (DPC Coat-a-Count; Diagnostic Products Corporation). Samples from both experiments were measured in the same assay. Intra-assay coefficients of variation were 10.1 and 3.3% for the low and high reference sera (means, 18.51 and 53.37 ng/ml).

2.3.6 Statistical analyses

For both experiments, statistical analyses were performed using the Statistical Analysis System software package (SAS Learning Edition 4.1, 2006; SAS Institute Inc., Cary, NC, USA). Time-series hormone data were analyzed by repeated measures, using a MIXED procedure. The main effects for Experiment 1 were progesterone (low vs high), GnRH dose (low vs high), time, and their interactions. For Experiment 2, main effects were P_4 (low vs high), estradiol (yes vs no), time, and their interactions.

Plasma concentrations of P4 and E_2 at CIDR insertion and at GnRH treatment were analyzed by one-way ANOVA, where the main variable was low vs high P_4. Follicle diameter at GnRH treatment, in Experiments 1 and 2, and prior to ovulation (only in Experiment 2) were analyzed by two-way factorial ANOVA. The main effects were P_4 concentrations and GnRH dose (Experiment 1) and estradiol (Experiment 2) and their interactions.

For Experiment 2, ovulation rates were analyzed by Fisher's Exact test.

Data from Experiments 1 and 2 were also combined and analyzed to determine the effect of P_4 concentration on LH profile. Heifers with low vs high P_4 given 100 μg of GnRH (Experiment 1) and those with high and low P_4 that were not given exogenous E_2 (Experiment 2), were analyzed to determine the effects of P_4 on plasma LH concentrations; a repeated-measures procedure (MIXED model) was used.

All values are expressed as mean ± SEM. If there were significant main effects or interactions ($P \leq 0.05$), Tukey's post-hoc test for multiple comparisons were used to locate differences.

2.4 Results

2.4.1 Experiment 1

Plasma P_4 concentrations were not significantly different between groups at CIDR insertion (Table 2.1); however, at GnRH treatment, plasma P_4 concentrations were lower in the Low-P_4 group than in the High-P_4 group ($P<0.01$). Furthermore, the Low-P_4 group was characterized by higher plasma E_2 concentrations ($P=0.002$) and a greater diameter of the largest follicle at GnRH treatment ($P<0.01$).

For plasma LH concentrations, there was only an effect of time ($P<0.0001$; Figure 2.1). In that regard, LH concentrations peaked 2 hours after GnRH treatment; concentrations at that time were significantly different from those at 0 and 3 hours (with no other significant differences).

Table 2.1 Mean (±SEM) plasma concentrations of progesterone (P_4) and estradiol (E_2) and diameter of the largest follicle in heifers with low (Low- P_4) or high (High- P_4) plasma P_4 concentrations and treated with 100 or 200 μg of GnRH.

	Low- P_4	**High- P_4**	**P value**
P_4 at CIDR insertion (ng/ml)	3.2 ± 0.3	3.0 ± 0.2	0.14
P_4 at GnRH treatment (ng/ml)	2.4 ± 0.3	6.0 ± 0.3	0.01
E_2 at GnRH treatment (pg/ml)	19.9 ± 0.4	12.5 ± 0.4	0.002
Diameter of largest follicle at GnRH treatment (mm)	11.7 ± 0.9	8.2 ± 0.5	0.01

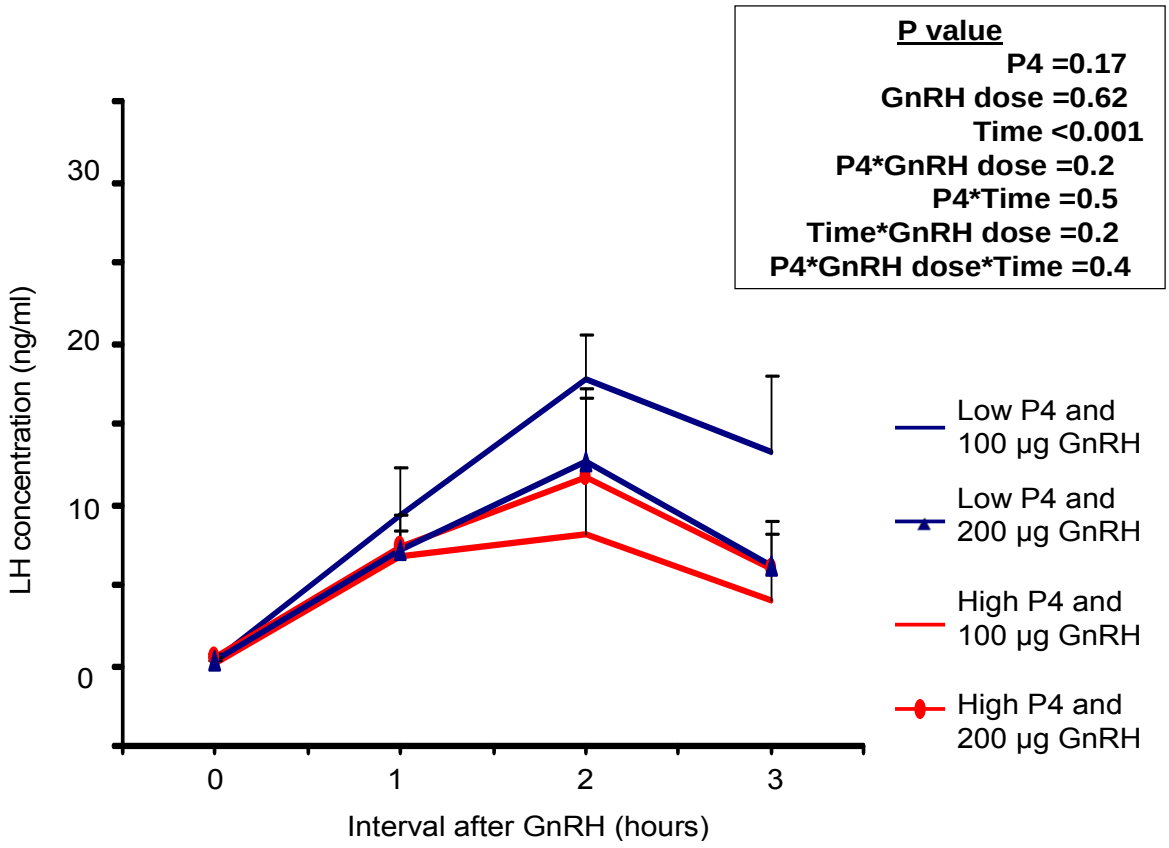

Figure 2.1 Mean (±SEM) plasma concentrations of luteinizing hormone (LH) in heifers with low (Low- P_4) or high (High- P_4) plasma progesterone concentrations and treated with 100 or 200 µg GnRH.

2.4.2 Experiment 2

Plasma P_4 concentrations did not differ significantly at the time of CIDR insertion (Table 2.2). However, at GnRH treatment, plasma P_4 concentrations were significantly lower and plasma E_2 concentrations were significantly higher in the Low- P_4 group than in the High- P_4 group. There was an effect of progesterone ($P \leq 0.05$), but no effect of E_2 ($P > 0.21$), nor an interaction ($P > 0.36$) on the diameter of the dominant follicle at GnRH treatment, or at the last examination prior to ovulation.

Table 2.2 Mean (±SEM) plasma concentrations of progesterone (P_4) and estradiol (E_2), diameter of the largest follicle, and ovulatory response in heifers with low (Low- P_4) or high (High- P_4) plasma P_4 concentrations and treated with 100 or 200 μg of GnRH.

End point	**Low- P_4**	**High- P_4**	**P value**
P_4 at CIDR insertion (ng/ml)	5.2 ± 0.6	4.3 ± 0.5	0.21
P_4 at GnRH treatment (ng/ml)	1.8 ± 0.2	8.7 ± 0.8	0.01
Estradiol at GnRH treatment (pg/ml)	17.8 ± 0.8	9.5 ± 0.9	0.05
Diameter of largest follicle at GnRH treatment (mm)	13.6 ± 0.6	11.1 ± 0.6	0.027
Diameter of largest follicle prior to ovulation (mm)	14.0 ± 0.5	11.2 ± 0.4	0.002
Number of heifers ovulating	11 of 11	7 of 11	0.09
Interval from GnRH treatment to ovulation (days)	1.0 ± 0.2	1.6 ± 0.3	0.13

There were significant effects of progesterone, estradiol treatment, time, and interactions on plasma LH concentrations after GnRH treatment (Figure 2.2). Plasma LH concentrations were higher in the Low- P_4 than in the High- P_4 group, and overall, LH concentrations were higher at 1 and 2 hours after GnRH, then at 0, 3, or 4 hours. Both E_2-treated groups (High- P_4 and Low- P_4) had higher LH concentrations than the High- P_4 and no E_2 group 2 hours after GnRH treatment, whereas the Low- P_4 and no E_2 groups did not differ from other groups.

All heifers (11/11) ovulated in the Low- P_4 group, whereas only 7/11 heifers in the High- P_4 group ovulated (P=0.09). Regardless of progesterone concentrations, E_2 did not have a significant affect on ovulation rates (9 of 12 and 9 of 10 heifers with or without E_2 ovulated, P=0.6).

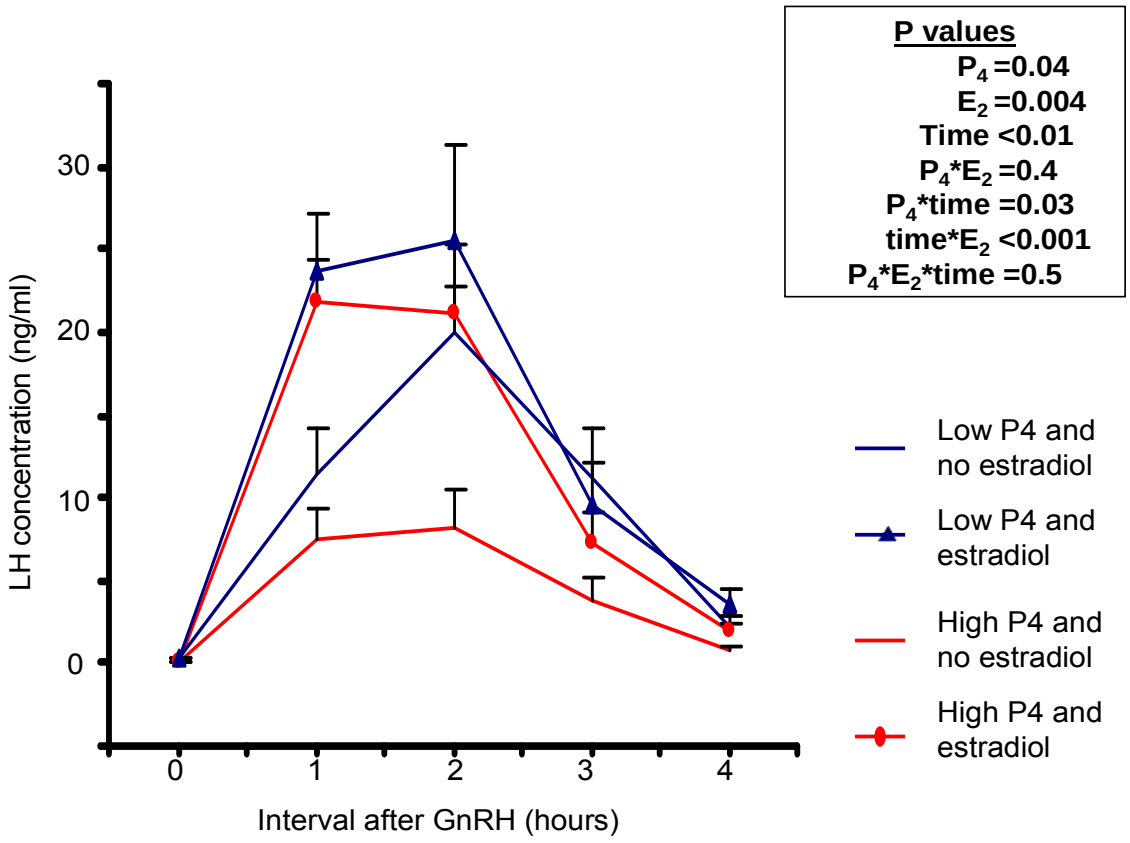

Figure 2.2 Mean (±SEM) plasma concentrations of luteinizing hormone (LH) in heifers with low (Low- P_4) or high (High- P_4) circulating progesterone concentrations and treated with or without estradiol benzoate 8 hours before receiving 100 μg GnRH.

2.4.3 Experiments 1 and 2 combined

When LH data following GnRH treatment were combined for the two experiments, there was an effect of time ($P<0.0001$) and an interaction between time and progesterone concentrations ($P<0.01$; Figure 2.3). Heifers with low progesterone concentration responded to GnRH with a higher LH surge than those with high progesterone concentrations (Figure 2.3).

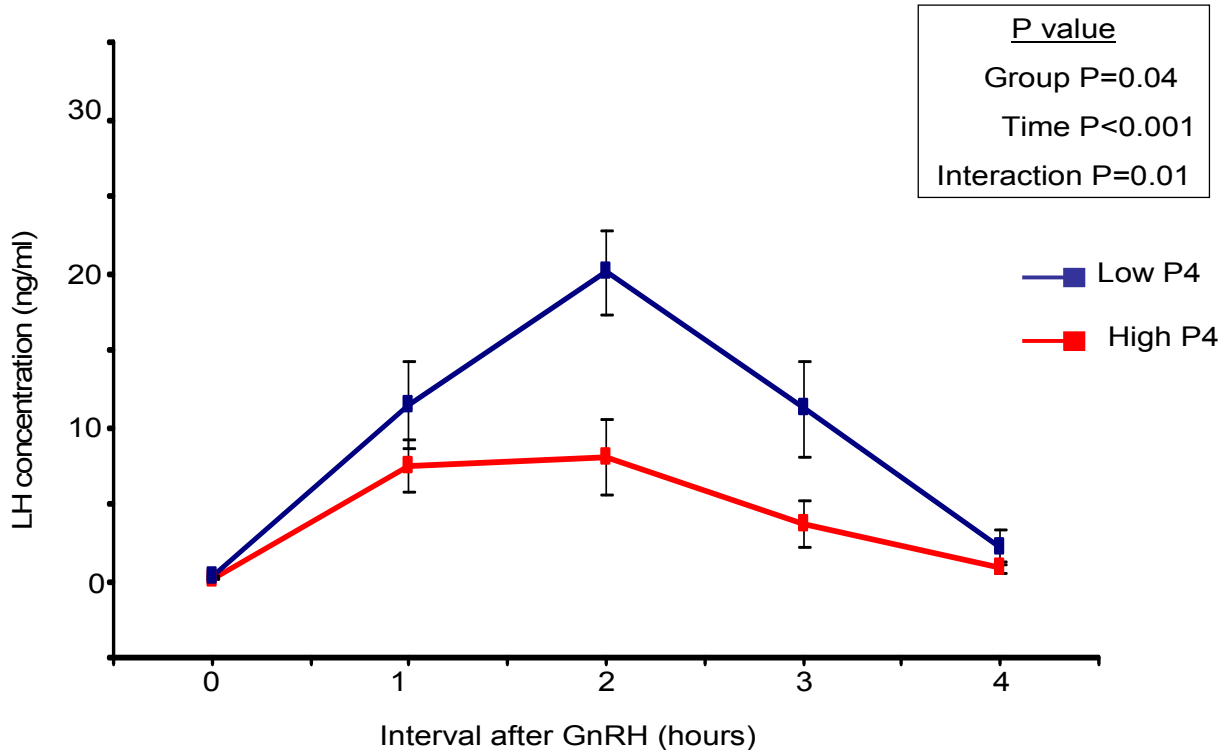

Figure 2.3 Mean (±SEM) plasma concentrations of luteinizing hormone (LH) in heifers with low (Low- P_4) or high (High- P_4) circulating progesterone concentrations and treated with 100 µg GnRH (data combined for Experiments 1 and 2).

2.5 Discussion

The hypothesis that higher doses of GnRH will be more efficacious in inducing LH release was not supported. However, the hypothesis that exogenous E_2 will increase LH release following the administration of GnRH was supported. Moreover, high concentrations of P_4 suppressed LH release following the administration of GnRH, and tended to decrease ovulation, whereas heifers in the High- P_4 groups had lower plasma E_2 concentrations prior to ovulation in both experiments. As expected, plasma P_4 concentrations did not differ between groups prior to CIDR treatment in both experiments, but circulating P_4 concentrations were significantly lower

in the Low- P_4 group at the time of GnRH treatment. A new follicular wave emerged approximately 1 day after follicular ablation in both the high and low P_4 groups, consistent with a previous report that this procedure increased plasma FSH concentrations and resulted in wave emergence approximately 1.5 days after follicle ablation [35]. Therefore, treatments to induce different P_4 environments and to synchronize emergence of follicular waves were highly successful.

In the present study, high plasma P_4 concentrations resulted in a smaller dominant follicle at GnRH treatment and prior to ovulation, decreased serum E_2 concentrations, and tended to reduce ovulation rates. Similarly, elevated P_4 concentrations 3 days prior to ovulation resulted in a smaller diameter follicle, reduced E_2 concentrations, and decreased fertility in other studies [31, 99]. The effect of P_4 on follicle diameter was likely due to suppression of LH secretion; once a dominant follicle emerges, its continued growth is largely dependent on LH secretion [6, 12, 35]. The suppression of plasma E_2 concentrations by P_4 has been previously reported and was probably associated with a suppression in gonadotrophin secretion [6, 106].

Although there was no significant effect of P_4 concentration on the LH profile in response to GnRH treatment in Experiment 1, there was an effect of P_4 on LH release in Experiment 2. Unfortunately, prior to Experiment 1, the heifers were not accustomed to frequent handling, examinations, and blood collection procedures. Despite efforts to minimize stress, the subjective impression was that the heifers were very anxious; high blood cortisol concentrations could have suppressed LH release [107, 108]. In contrast, when Experiment 2 was conducted, the heifers were much more accustomed to handling, and subjectively appeared to be much less stressed; in that experiment, there was a significant effect of P_4 on LH release in response to GnRH. Unfortunately, stress and elevated cortisol concentrations may have masked an effect of dose of GnRH on LH release in Experiment 1. Therefore, the hypothesis that higher doses of GnRH will be more efficacious in inducing LH release was not rigorously tested, and should be re-examined, ideally with cattle that are well accustomed to handling, examinations, etc.

Exogenous estradiol benzoate given 8 hours prior to GnRH mimicked the natural increase in E_2 that occurs prior to the onset of estrus [109], and enhanced LH release in heifers with both low and high P_4 concentrations. In particular, E_2 treatment of heifers with high P_4 significantly

increased LH release. However, despite higher LH concentrations, ovulation rate was not increased.

When LH data in the two experiments were combined, the effects of P_4 concentrations on LH release and ovulation rates were more obvious. Lower concentrations of progesterone during growth of the ovulatory follicle increased LH pulse frequency, follicle size and ovulation rates [6]. Follicles acquire LH receptors at deviation (at 8.5 mm of diameter) [29, 31], and LH secretion drives dominant follicle growth. However, when the dominant follicle developed in a high P_4 environment, the pre-ovulatory follicle diameter was smaller and required a higher LH peak to ovulate [52]. Other studies have demonstrated the suppressive effect of P_4 on pituitary LH release and ovulation [21, 52]. However, based on the current data, we inferred that a small dose of estradiol benzoate 8 hours prior to treatment with GnRH will increase LH release, although it may not improve ovulation rates.

In conclusion, increasing the dose of GnRH from 100 to 200 μg did not overcome the suppressive effect of progesterone on LH release. However, this should be reconfirmed, since stress may have confounded the results. Although increased plasma P_4 concentrations during the growth of the dominant follicle suppressed the release of LH and tended to decrease ovulation rates, the administration of estradiol benzoate prior to the administration of GnRH promoted LH release in heifers with elevated plasma P_4 concentrations. Therefore, to maximize the LH response, GnRH treatment should be given when P_4 concentrations are low, or alternatively, following pretreatment with estradiol benzoate. However, estradiol treatment is unlikely to increase ovulation rate.

CHAPTER 3

DURATION OF PROGESTERONE EXPOSURE DURING DEVELOPMENT OF THE OVULATORY FOLLICLE DOES NOT AFFECT PREGNANCY RATE IN CATTLE

3.1 Abstract

The objective was to determine the effect of length of P4 exposure on the ovulatory wave on fertility (pregnancy rate) in beef cattle. We tested the hypothesis that short-duration progesterone exposure during the growing and early-static phase of the ovulatory follicle is associated with higher fertility than a longer duration of progesterone exposure. Three to 5 days after ovulation, beef heifers (n=172) and suckled beef cows (n=193) received an intravaginal progesterone-releasing device (CIDR) and 2.5 mg estradiol-17β + 50 mg progesterone im to induce a new follicular wave. Cattle were allocated to two groups, analogous to the ovulatory wave of 3- and 2-wave estrous cycles (short and long progesterone exposure groups, for 3 and 6 days after wave emergence, respectively), after which prostaglandin $F_{2\alpha}$ was administered and CIDR were removed. Forty-eight hours later, all cattle were given 12.5 mg pLH, and artificially inseminated concurrently with frozen-thawed semen. Diameter of the two largest follicles and the CL were measured by transrectal ultrasonography at the time of CIDR removal, insemination, and 36 hours after insemination. Ultrasonographic pregnancy diagnosis was done 38 and 65 days after AI. There was no difference in pregnancy rates following short- versus long-duration progesterone exposure in heifers (53 vs 47%, P=0.44) or cows (63 vs 58%, P=0.51). However, the diameter of the ovulatory follicle at CIDR removal and AI was smaller in short- than in long-duration groups (P<0.02), and larger in cows than in heifers (P<0.006). In conclusion, although long-duration progesterone exposure during growth of the ovulatory follicle resulted in a larger diameter on the last examination before ovulation, fertility was not significantly different than in the short-progesterone exposure group.

3.2 Introduction

Progesterone suppresses the growth of the dominant follicle [12, 110]. In that regard, high circulating progesterone concentrations suppress LH pulse frequencies [111]. However, maintenance of prolonged low progesterone concentrations results in prolonged growth and maintenance of the dominant follicle in cattle [101, 110] and sheep [112].

Follicles develop in a wave-like pattern [12, 112, 113], with most cattle having either 2 or 3 follicular waves during each estrous cycle [112]. However, the preponderance and repeatability of 2- or 3-wave cycles remain unclear [6]. The duration of exposure of ovulatory follicles to high-progesterone concentrations is substantially different between 2- and 3-wave cycles. The duration of the ovulatory wave in 2-wave cycles is approximately 9 days, whereas in 3-wave cycles, the ovulatory wave lasts only 6 days [12]. Although progesterone concentrations are consistently elevated when the ovulatory wave emerges, the dominant follicle grows in a high-progesterone environment for approximately 6 days in 2-wave cycles, but only 3 days in 3-wave cycles. Thereafter, progesterone concentrations are low (for both 2- and 3-wave cycles) for approximately 3 days following luteolysis, culminating in ovulation of the dominant follicle [12, 114]. Hence, the interval from wave emergence to ovulation and the duration of growth in a high-progesterone milieu are longer in 2-wave than 3-wave cycles [115]. It has been suggested that follicles which grow for a longer interval in a high-progesterone environment (analogous to 2-wave cycles) may produce an aged oocyte [115]. Although fertility was higher in 3-wave than in 2-wave cycles in one report [115], others have failed to detect a difference [116], suggesting that further study is required.

The objective was to determine the effect of the duration of progesterone exposure, during the growing phase of the ovulatory follicle on pregnancy rate. We hypothesized that a short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).

3.3 Materials and methods

3.3.1 Cattle and treatments

The experiment was conducted between May and September 2006 in pubertal cross-bred beef heifers (n=172) and suckled, post-partum beef cows (n=193), maintained in outdoor corrals at three locations: University of Saskatchewan Goodale Research Farm, University of Alberta Research Farm, and Brandon Research Centre.

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee or the University of Alberta Animal Welfare Committee.

Animals at random stages of the estrous cycle were given a prostaglandin $F_{2\alpha}$ analog, 500 μg of cloprostenol im (Estrumate®, Schering-Plough Animal Health, Pointe-Claire, QC, Canada), on two occasions, 11 days apart, to synchronize estrus and ovulation (ovulation = Day 0). Eight days after the second cloprostenol treatment (Days 3 to 5), all cattle received an intravaginal, progesterone-releasing device (CIDR , Pfizer Canada Inc., Montreal, QC, Canada) and they were injected im with 1 ml of canola oil containing 2.5 mg estradiol-17β + 50 mg progesterone (E_2+ P_4; Sigma Chemical Co., St. Louis, MO, USA); a new ovarian follicular wave was expected to emerge 4 days later [44, 102]. The CIDR and functional CL were expected to raise plasma progesterone concentrations to mid-luteal values, analogous to the time of emergence of the ovulatory wave in both 2- and 3-wave cycles.

Heifers and cows were randomly allocated into two groups. The short duration of progesterone exposure (short progesterone) group was given 25 mg of prostaglandin $F_2\alpha$ im ($PGF_2\alpha$; Lutalyse, Pfizer Canada Inc.) 3 days after expected emergence of the new follicular wave (i.e., 7 days after E_2+P_4). The long duration exposure of progesterone (long progesterone) group was given $PGF_2\alpha$ 6 days after expected emergence of the new follicular wave (i.e., 10 days after E_2+P_4 injection). The CIDR devices were removed at the time of $PGF_2\alpha$ injection. Forty-eight hours later, all cattle received 12.5 mg porcine

luteinizing hormone im (Lutropin-V; Bioniche Animal Health, Belleville, ON, Canada) and were concurrently inseminated with frozen-thawed semen.

3.3.2 Ultrasonographic examination

Transrectal ultrasonography was performed using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan); diameter of the two largest follicles and all visible corpora lutea were measured at CIDR removal at all three locations. Follicle and CL diameter were also measured at the time of AI and 36 hours after AI to confirm ovulation in a subset of animals at the University of Saskatchewan Goodale Research Farm (n=37 heifers; n=57 cows). Ultrasonographic pregnancy diagnosis was performed at all three locations 38 days after AI. An additional ultrasonographic examination was conducted in cows and heifers from the University of Saskatchewan Goodale Research Farm at 65 days after AI to determine pregnancy loss.

3.3.3 Blood sampling and radioimmunoassay

Blood samples were collected from the coccygeal vessels into 10 ml heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at CIDR removal and at AI to determine plasma concentrations of LH and progesterone. Blood samples were centrifuged (1500 x g for 15 minutes), and plasma was stored at -20 °C until analyzed.

Plasma LH concentrations were measured in duplicate samples in a single assay, using a validated, double-antibody radioimmunoassay [105]. The range of the standard curve was 0.06 to 8.0 ng/ml and the intra-assay coefficients of variation were 6% for the low-reference serum (1.02 ng/ml) and 7% for the high-reference serum (2.18 ng/ml).

Plasma progesterone concentrations were measured in a single assay using commercial radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40 ng/ml. The intra-assay coefficients of variation were 6% for low-reference serum (1.84 ng/ml), 6% for medium-reference serum (3.5 ng/ml), and 6% for high-reference serum (17.06 ng/ml).

3.3.4 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System software package (SAS Version 9.0; SAS Institute Inc., Cary, NC, USA). The effect of treatment (long and short progesterone) and parity was compared for diameters of the largest follicle and CL and for plasma concentrations of LH and progesterone, by 2-way ANOVA using Proc MIXED. Normality of residuals was tested with a Shapiro-Wilk test ($P>0.05$ was considered a normal distribution). Values are expressed as mean ± SEM.

Pregnancy data were analyzed by 2-way factorial with randomized complete block design (Fixed factors: length of progesterone exposure, parity, length progesterone exposure*parity interaction; Random factor: location). Data collected at one location (follicle diameter at AI) were analyzed by 2x2 factorial design (length of progesterone exposure, parity and length of progesterone exposure *parity interaction). Tukey's post-hoc test for multiple comparisons was used if main effects or their interaction were significant ($P \leq 0.05$).

Dominant follicle growth during the pre-ovulatory period was determined by the difference in diameter of dominant follicle between CIDR removal and AI, and was also analyzed by 2-way ANOVA.

For plasma LH data, concentrations 3 times higher than the standard deviation of the mean LH concentration at the time of AI (i.e., cattle with an endogenous LH peak) were excluded from the analysis.

Animals which had CL <15 mm in diameter and plasma progesterone <1.5 ng/ml at CIDR removal were considered outliers; two analyses of pregnancy rates were performed (excluding and including this group of cattle).

All dichotomous variables (pregnancy rates at 38 days post-AI; pregnancy loss; diameter CL < 15 mm, and progesterone concentrations <1.5 ng/ml; ovulation rate; and detection of early ovulation) were analyzed using logistic regression with the Gen Mod procedure.

3.4 Results

3.4.1 Dominant follicle size and growth

The diameter of the ovulatory follicle at CIDR removal and AI were larger in long-progesterone than in short-progesterone cows (Figure 3.1), but did not differ in heifers (Figure 3.1). The diameter of the ovulatory follicle at CIDR removal and AI were larger in long-progesterone cows than long-progesterone heifers (Figure 3.1). Dominant follicle growth during the pre-ovulatory period did not differ between progesterone treatment groups (P=0.61), but was higher in cows than heifers (P=0.0005).

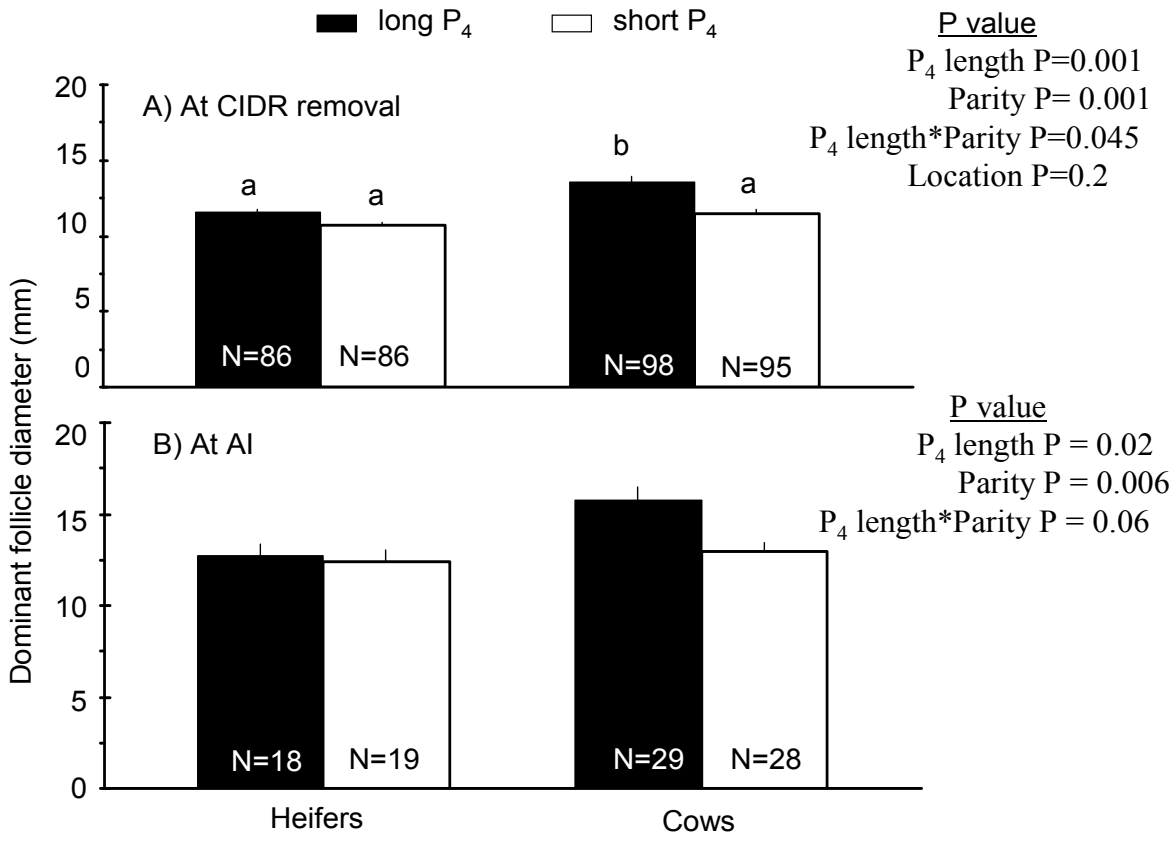

Figure 3.1 Mean (± SEM) follicle diameter at CIDR removal and AI in cows and heifers exposed to long- or short-duration progesterone environments during development of the ovulatory wave. [a,b]- Values without a common superscript were different (P<0.05).

3.4.2 Diameter of CL and plasma progesterone concentration

Diameter of the CL at CIDR removal was greater in cows in the short progesterone group than in cows and heifers in the long progesterone groups, but not in heifers in the short progesterone group (Figure 3.2). There was no difference in CL diameter at CIDR removal between heifers in the short and long progesterone groups (Figure 3.2).

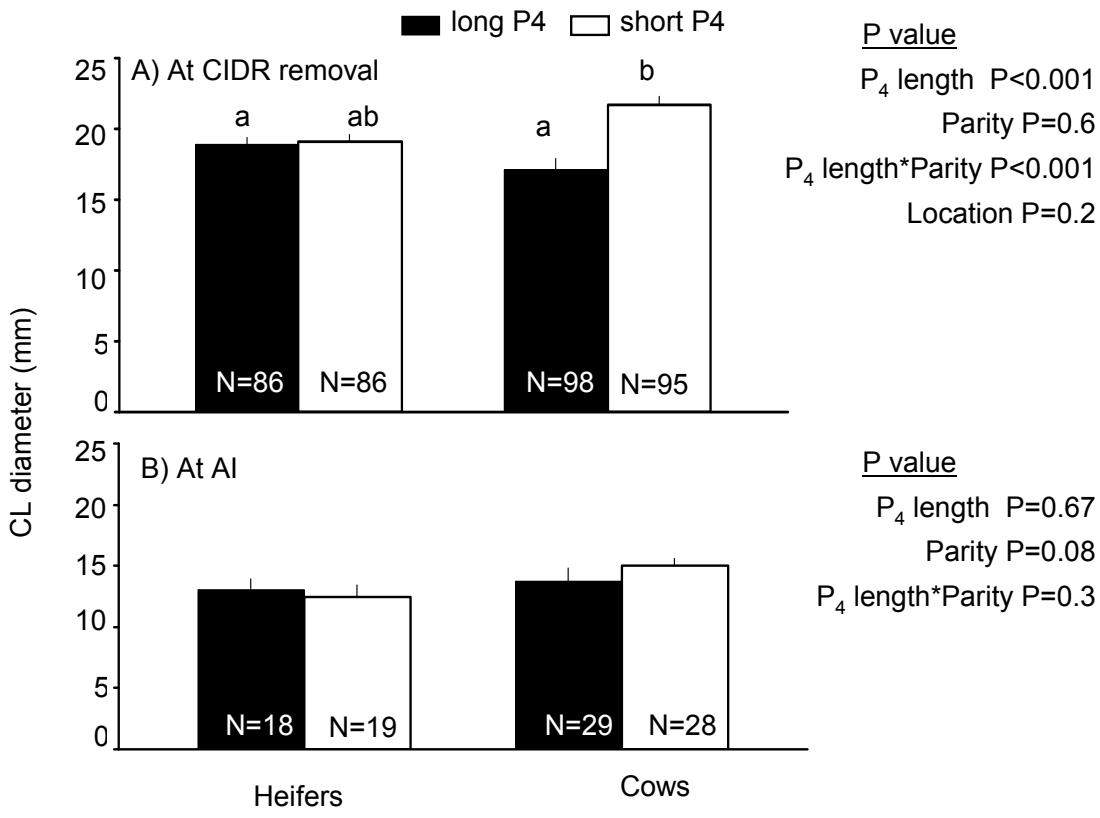

Figure 3.2 Mean (± SEM) diameter of the CL at CIDR removal and at AI in heifers and/or cows exposed to long- or short-duration progesterone environments during

development of the ovulatory wave. [a,b]- Values without a common superscript were different (P<0.05).

Plasma progesterone concentrations at CIDR removal were not different between groups (4.8 ± 0.8 in short progesterone heifers, 4.9 ± 0.8 in long progesterone heifers, 4.3 ± 0.6 in short progesterone cows and 3.2 ± 0.6 in long progesterone cows; P=0.3, 0.3 and 0.2 for group, location, and their interaction, respectively). However, progesterone concentrations at CIDR removal were higher in heifers (4.9 ± 0.7 ng/mL) than in cows (3.8 ± 0.6 ng/mL; P=0.02). Progesterone concentrations at AI were less than <0.5 ng/mL in all groups, and did not differ (0.4 ± 0.2 ng/mL in short progesterone heifers, 0.3 ± 0.2 ng/mL in long progesterone heifers, 0.4 ± 0.1 ng/mL in short progesterone cows and 0.3 ± 0.1 ng/mL in long progesterone cows, P=0.29, 0.64, 0.7, and 0.3 for group, parity, interaction, and location).

A greater proportion of cows in the long progesterone group had a CL <15 mm in diameter and plasma progesterone concentrations <1.5 ng/mL at CIDR removal (21.4%; 21/98) than cows in the short progesterone group (8.4%; 8/95), heifers in the long progesterone group(3.5%; 3/86), and heifers in the short progesterone group (1.2%; 1/86; P=0.005, 0.0006, and 0.9 for group, parity, and their interaction, respectively).

3.4.3 Plasma LH concentrations and ovulation rates

Plasma LH concentrations at CIDR removal did not differ by group, parity or interaction. However, LH concentrations at AI were higher in heifers in the long progesterone group than in the other groups (Figure 3.3).

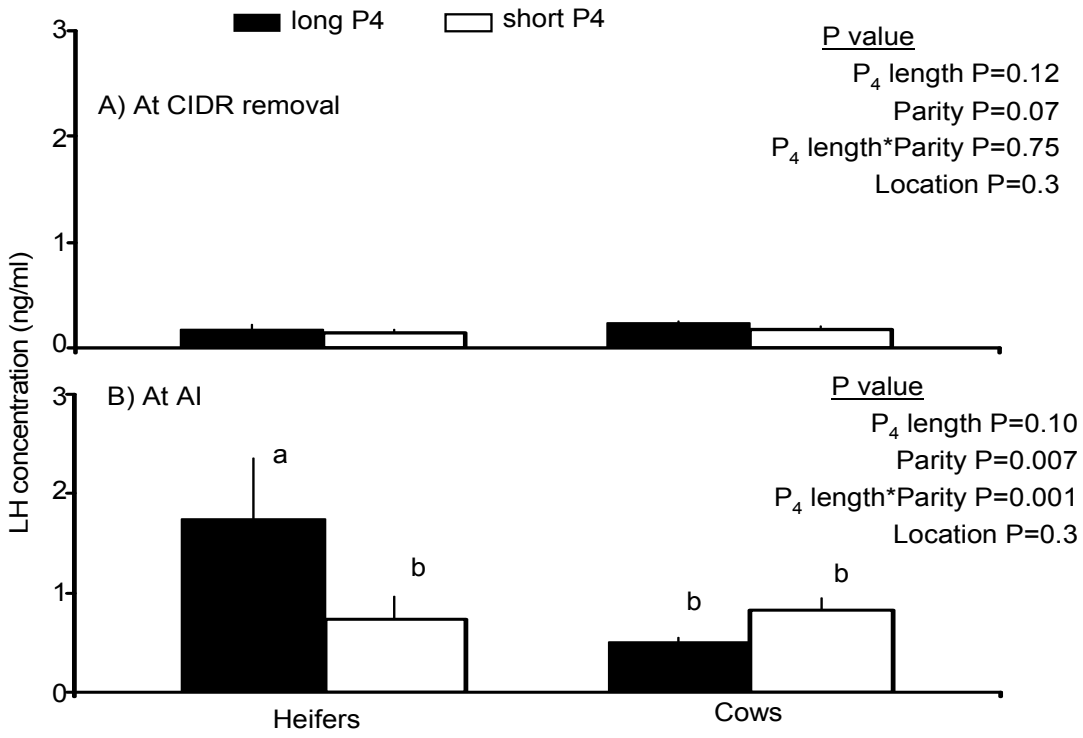

Figure 3.3 Mean (± SEM) plasma concentrations of LH at CIDR removal and at AI in heifers and cows exposed to long- or short-duration progesterone environments during development of the ovulatory wave. [a,b]- Values without a common superscript were different ($P<0.05$)

There were no significant differences between groups, parity or interaction, for the proportion of cattle that ovulated by 36 hours after pLH treatment (P=0.89, 0.23 and 0.6, respectively). Ovulation rates were 87% (41/47) in the long progesterone group (3 cows and 4 heifers did not ovulate by 36 hours after AI), Ovulation rates were 88.9% (16/18) in long progesterone heifers, 84% (16/19) in short progesterone heifers, 89.6% (26/29) in long progesterone cows and 89.3% (25/28) in short progesterone cows. For early ovulations (i.e., ovulations between CIDR removal and AI, P=0.32, 0.19, 0.9, respectively), there were no difference due to group, parity, or their interaction. One cow ovulated before AI in the short progesterone group and 3 heifers ovulated before AI in the long progesterone group.

3.4.4 Pregnancy rates

There were no differences in pregnancy rates between treatment groups (P=0.32, Table 3.1), parities (P=0.6, Table 3.1), or interactions (P=0.9). Location did not affect pregnancy rates at 38 days (P=0.6), and there was no difference in pregnancy loss between groups, parities or interactions (P=0.7; 0.85, 0.8) at the University of Saskatchewan Research farm. The overall incidence of pregnancy loss was 7.4% (1/19 in short progesterone heifers, 1/19 in long progesterone heifers, 3/28 in short progesterone cows, and 1/29 in long progesterone cows).

When pregnancy rates at 38 days were analyzed, excluding cattle with <1.5 ng/ml of progesterone and CL diameter <15 mm at CIDR removal, there were no differences due to group (P=0.5), parity (P=0.3), interaction (P=0.3), or location (P=0.9).

Table 3.1 Pregnancy rates in cows and heifers exposed to a short or long progesterone environment during development of the ovulatory wave.

Pregnancy rate	**Short progesterone**	**Long progesterone**
Cows	59/94 (63%)	58/99 (58%)
Heifers	45/85 (53%)	41/87 (47%)
Total	104/179 (58%)	99/186 (53%)

3.5 Discussion

The duration of progesterone exposure during ovulatory wave development did not significantly affect pregnancy rates. In that regard, the dominant follicle of both short progesterone (analogous to the ovulatory wave of 3-wave cycles) and long progesterone groups (analogous to the ovulatory wave of 2-wave cycles) ovulated, resulting in acceptable pregnancy rates after fixed-time AI, with a low incidence of subsequent pregnancy loss. Therefore, the hypothesis that short-duration progesterone exposure during

the growing and early-static phase of the ovulatory follicle is associated with higher fertility than long-duration exposure was not supported.

Although diameter of the ovulatory follicle at CIDR removal and AI were larger in cows in the long progesterone groups, pregnancy rates did not differ between groups, or between parities. Thus, follicles from heifers and from short progesterone cows were smaller, but they had the same potential for ovulation, fertilization, and maintenance of pregnancy. Cows have been reported to have larger ovulatory follicles than heifers [117, 118], and cattle from the long progesterone group had a follicular growing phase 3 days longer than those in the Short progesterone groups. Ovulatory follicles <11 mm in diameter at AI have had reduced pregnancy rates when ovulation was induced with GnRH [99]. In that regard, larger follicles contained more granulosa cells, which after luteinization resulted in a larger CL that produced more progesterone [99, 119]. However, in the present study, it was noteworthy that preovulatory follicles from all cattle in all groups exceeded 11 mm at AI; this may account for our inability to detect an effect of follicle diameter at AI on pregnancy rates.

The duration of follicle dominance has also been reported to affect fertility [16, 120]. In beef heifers, 4 days of follicle dominance resulted in the highest pregnancy rates; with increasing periods of follicular dominance, fertility decreased gradually until 9 days of dominance and thereafter, fertility declined rapidly [16]. In that regard, prolonged follicular dominance results in formation of a persistent dominant follicle, with an aged oocyte and compromised developmental competence [16]. When LH release was delayed in a superstimulation protocol by prolonging high progesterone concentrations to allow smaller follicles to fully mature, the number of ova/embryos retrieved was increased, but embryo production was either not affected or it was decreased [111]. Conversely, it has been suggested that prolonged maintenance of the oocyte in the follicular environment could be beneficial to developmental competence, since it could accumulate more maternal mRNA, which is important for early embryonic development [111, 121]. However, in the present study, shortening or extending the follicular growing phase (and dominance) with exogenous progesterone did not significantly affect pregnancy rates.

Cows in the Long progesterone group had lower progesterone concentrations and smaller CL diameters at CIDR removal, suggesting that some of these cattle were undergoing luteolysis. This may have been an effect of postpartum interval, since short cycles (approximately 8 days) are observed in suckled beef cows after parturition [97]. To avoid the effect of short cycles on results, animals which had progesterone concentrations <1.5 ng/mL at time of CIDR removal were removed from analyses of pregnancy rates. However, pregnancy rates still did not differ between groups. Animals from all groups responded to PGF treatment and progesterone concentrations were baseline at the time of AI.

In the present study, plasma LH concentrations at CIDR removal were not significantly affected by group or parity. However, LH concentrations at AI in heifers in the long progesterone group were significantly higher than in the other groups, suggesting that an LH surge was already occurring at that time. In other studies in heifers [41] and cows [37] in which treatments were similar to the short progesterone group in this study, estrous behavior was observed 52 hours after CIDR removal . Animals usually initiate estrus approximately 2 hours after their LH peak; therefore animals from that study might have an LH peak 50 hours after CIDR removal. Similarly, when norgestomet ear implants were left in place for 8 days after wave emergence, beef heifers showed estrous behavior 39 hours after ear implant removal (an LH peak approximately 37 hours after norgestomet removal) [16]. Therefore, a longer duration of progesterone/progestin exposure during growing phase of ovulatory follicle can result in a short interval to LH release in heifers.

Most pregnancy loss occurs in the first 45 days of pregnancy [120]. The incidence of pregnancy loss between 38 and 65 days of pregnancy in the current study was 7.4%, similar to previous reports [122], with no significant difference between groups or parities.

In summary, although there were differences in follicular, CL and hormone profiles when ovulatory wave was extended by 3 days with exogenous progesterone, there was no apparent affect on oocyte competence or pregnancy rates.

CHAPTER 4

EFFECT OF DURATION OF PROGESTERONE EXPOSURE DURING THE GROWING PHASE OF OVULATORY FOLLICLES ON OOCYTE COMPETENCE IN SUPERSTIMULATED COWS

4.1 Abstract

The objective was to determine the effect of duration of progesterone exposure, during the growing phase of ovulatory follicles, on oocyte competence in superstimulated cows. We tested the hypotheses that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth, but is not affected by FSH starvation at the end of superstimulatory treatment. Forty cross-bred beef cows (weight, 515 to 795 kg) were used. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation, and a progesterone-releasing device (CIDR) was placed intravaginally. Cows were allocated randomly to three groups; those in the short P_4 and FSH starvation groups received 8 injections of FSH (each was equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals, whereas the long P_4 group received 14 injections of FSH. In all groups, FSH treatment started on the day of wave emergence (Day 0). Cows in the short P_4 group were given 25 mg of PGF twice, (12 hours apart), on Day 3, whereas cows in the 2 other groups received two injections of PGF on Day 6. In all cows, the CIDR were removed at the time of second PGF treatment; 25 mg pLH was given im 24 hours after CIDR removal and cows were inseminated 24 and 36 hours later. Reproductive tracts were collected at slaughter, 4 days after insemination and ova/embryos were evaluated and cultured for 10 days to determine developmental competence. Data from cows that failed to ovulate (6 of 13 and 1 of 13 in the FSH starvation and long P_4 groups, respectively), were excluded from the analyses. When compared to the short P_4 or long P_4 groups, the FSH starvation group had fewer CL (i.e. ovulations; mean±SEM, 11.6±2.2, 15.4±3.0, and 2.5±1.2, respectively; $P=0.001$), and fewer ova or embryos (5.9±1.3, 6.7±1.5, and 0.9±0.6, respectively; $P<0.05$) at slaughter.

However, the proportion of Grades 1 and 2 embryos 4 days after insemination was higher in the short P_4 and FSH starvation groups than in the long progesterone group (60 of 69, 9 of 11, and 31 of 82, respectively; $P<0.001$), with no significant difference in embryo quality between long and short P_4 groups 7, 9, and 10 days after AI (there were too few embryos in the FSH starvation group to evaluate). In conclusion, the hypothesis that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth was not supported, whereas the hypothesis that oocyte competence is not affected by FSH starvation at the end of superstimulatory treatment was not adequately tested. Notwithstanding, FSH starvation substantially reduced the ability of superstimulated follicles to ovulate.

4.2 Introduction

Ovarian follicles in cattle develop in a wave-like pattern, with 2 or 3 follicular waves in each estrous cycle [12, 123]. The major difference between 2- and 3-wave cycles is the length of growing phase of the ovulatory follicle [5]; the duration of the ovulatory wave is 3 days longer in 2-wave cycles (6 versus 9 days [5]). Therefore, the ovulatory oocyte is 3 days older (relative to wave emergence) in 2-wave cycles [14].

In cattle with 2 waves, the ovulatory follicle is exposed to high progesterone concentrations (from a functional CL) for 3 days longer than the ovulatory follicle in cattle with 3 waves [14]. Progesterone suppresses GnRH and LH in a dose-specific manner [12]. A pre-ovulatory peak of LH is required for the oocyte to reactivate nuclear maturation and achieve developmental competence; however, the LH peak could be delayed 3 days in females with 2-wave cycles (relative to wave emergence) [92].

Although pregnancy rates in 2- versus 3-wave cycles have been compared [17, 115], the results were contradictory, and the effect of number of waves on fertility remains unclear. In addition, the effect of the duration of the growing phase of the ovulatory follicle in superstimulated cows has not been reported. Jaiswal and co-workers (2006) [2, 6] studied oocyte competence after superstimulatory treatment under the influence of various progesterone concentrations. However, in both 2- and 3-wave cycles, circulating

progesterone concentrations declined and remained subluteal for approximately 3 days prior to ovulation [14]. Therefore, the difference between 2- and 3-wave cycles is in the duration of progesterone exposure and not in progesterone concentrations [14].

For many years, gonadotrophin treatments have been used in multiple ovulation and embryo transfer programs [81, 111]; however, a major limitation is the extreme variability in the superovulatory response [26, 113]. On average, five to six transferable embryos are produced per donor [71, 72]. In one study, 30% of 2048 cows produced 70% of the embryos, whereas 24% cows failed to produce any embryos [71]. It has been postulated that one of the major determinants of the superstimulatory response is the number of follicles available at the beginning of a wave [9], and when FSH treatment is initiated in relation to wave emergence [79]. However, in superstimulated cows, it is not known whether a shorter duration of progesterone exposure during the growing phase of the ovulatory wave will increase the superovulatory response or embryo quality.

Superstimulatory response may also be affected by treatment protocol, total dose, batch, purity (i.e. LH content), and type of gonadotrophin [26]. In one study, increasing the interval from the end of gonadotrophin treatments to removal of a progesterone-releasing device resulted in a decreased ovulatory response [6]. However, this process, referred to as FSH starvation, was done in a prolonged, low-progesterone environment and it is unclear whether the loss of the capacity to ovulate was due to a lack of FSH, or to the formation of large, persistent follicles.

The objective of the present study was to determine the effect of duration of progesterone exposure, during the growing phase of ovulatory follicles, on oocyte competence in superstimulated cows. We tested the hypotheses that oocyte competence in superstimulated cows: 1) is improved by short-duration progesterone exposure during follicular growth; but 2) is not affected by FSH starvation at the end of a superstimulatory treatment protocol.

4.3 Materials and methods

4.3.1 Animals and treatments

The experiment was conducted at the University of Saskatchewan Goodale Research Farm (52°N and 106°W), during February and March, 2007. Forty cross-bred beef cows, weighing 515 to 795 kg, and maintained in outdoor pens, were used. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

Cows at random stages of the estrous cycle were given 2 doses of prostaglandin $F_{2\alpha}$ im (500 μg of cloprostenol; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada), at 14-day intervals, to synchronize estrus and ovulation. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation, to synchronize emergence of a new follicular wave 1 day later [25]. An intravaginal, progesterone-releasing device, CIDR-B (Pfizer Canada Inc., QC, Canada) was placed in the vagina immediately after follicle ablation.

The cows were allocated randomly to three groups (Figure 4.1): short P_4 (n=14), FSH starvation (n=13), and long P_4 (n=13). Starting 1 day after follicle ablation, i.e. on the expected day of wave emergence (Day 0), cows in the short P_4 and FSH starvation groups received 8 im injections of FSH (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada; each equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals over 4 days, whereas the long P_4 group received 14 im injections of FSH, over 7 days. Cows in the short P_4 group were given 2 im injections of 25 mg of prostaglandin-$F_2\alpha$ (Lutalyse®; Pfizer Canada Inc.) at 12-hour intervals on Day 3, whereas the FSH starvation and long P_4 groups received 2 injections of 25 mg im of prostaglandin-$F_2\alpha$ on Day 6. In all cows, the CIDR were removed at the time of the second $PGF_2\alpha$ treatment. Cows were given 25 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 hours after CIDR removal, and artificially inseminated with frozen-thawed bull semen 24 and 36 hours later. Ovulations were

monitored by transrectal ultrasonography, and a third AI was done 48 hours after LH treatment if ovulations were not completed at that time.

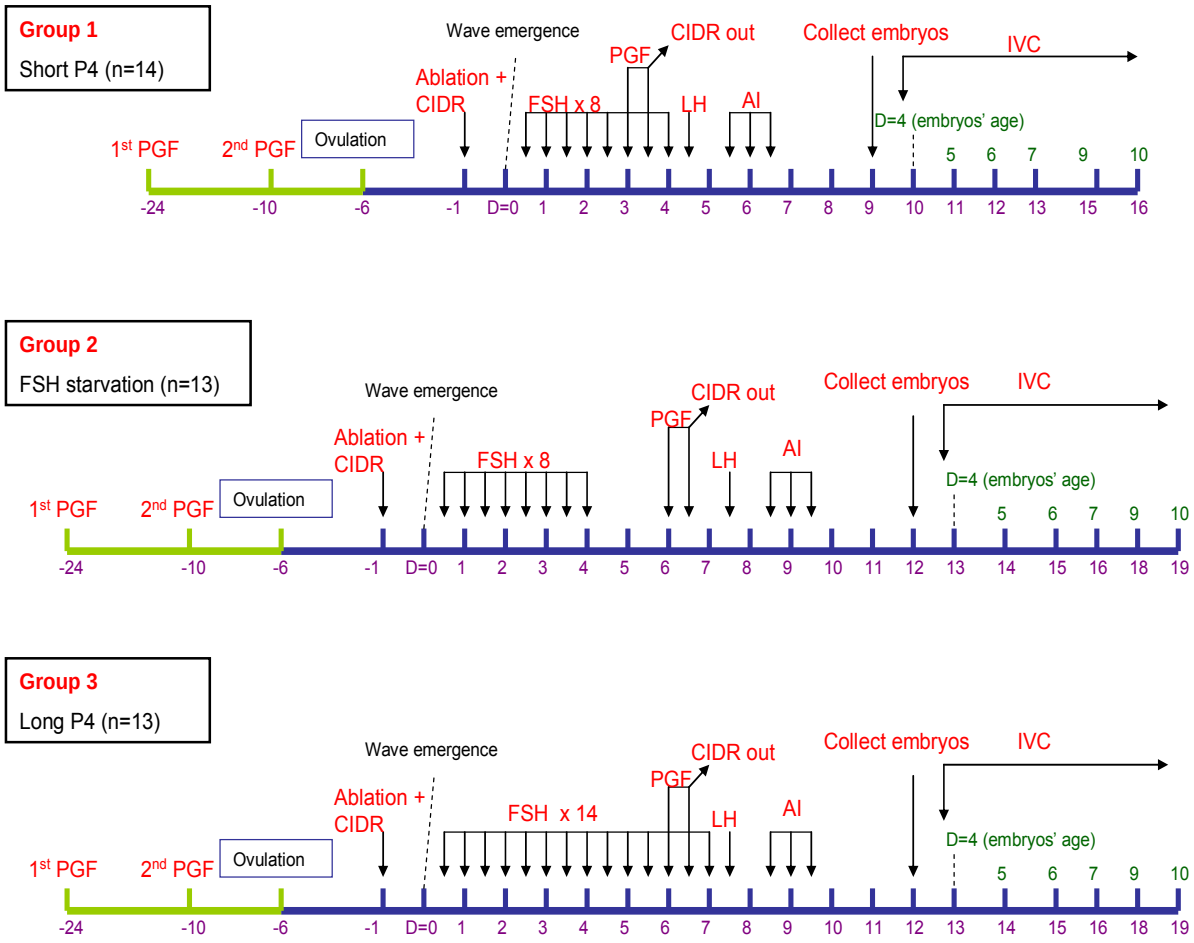

Figure 4.1 Experimental protocols used to test the effect of length of growing phase of the ovulatory follicles and FSH starvation on oocyte competence after superstimulatory treatment in cows. Five to 8 days after ovulation, follicles ≥5 mm were ablated and a CIDR was placed intravaginally. FSH treatment started at wave emergence (Day 0). Short P_4 and FSH starvation groups received 8 injections of FSH, whereas the long P_4 group received 14 injections of FSH. At Day 3, the short P_4 group received PGF, whereas the other groups received PGF on Day 6. CIDR were removed at the time of PGF, LH was given 24 hours

after CIDR removal, cows were inseminated 24 and 36 hours later, and reproductive tracts were collected at slaughter, 4 days after AI.

4.3.2 Ultrasonographic examination

Transrectal ultrasongraphic imaging was performed using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). To evaluate the number and size of ovulatory follicles, ultrasonography was done at the time of 8^{th} FSH injection, and at CIDR removal (for the short P_4 group, those events were concurrent). Cows were also examined 12 hours after the second AI to detect ovulation; those with at least 2 follicles ≥7 mm in diameter were re-inseminated 48 hours after LH treatment.

The differences in number of follicles at CIDR removal and at 12 hours after the second AI were used to estimate the number of ovulations that occurred during this interval.

4.3.4 Embryo recovery

Cows were transported to an abattoir in Moose Jaw, SK, Canada, (approximately, 250 km from the location of the experiment) 3 days after the first AI. The cows were killed 4 days after AI; reproductive tracts were collected after slaughter, placed in polyethylene bags, kept at 30 °C, and transported to the laboratory at University of Saskatchewan within 3 hours after collection. The numbers of corpora lutea and persistent (unovulated) follicles were counted to estimate number of ovulations. Oviducts and the uterine horns were flushed separately to recover embryos. The oviduct was dissected free from the mesosalpinx and separated from the uterus, leaving approximately 1 cm of the tip of the uterine horn. An 18 gauge, 1.5 inch blunt needle was attached to the infundibulum with tissue forceps and 20 mL of collection medium was injected slowly; the fluid was collected from the uterine tip in a Petri dish for direct searching. The collection medium was comprised of Delbecco's phosphate buffer saline (dPBS, Invitrogen Corporation, catalog

14190-144, Burlington, ON, Canada) and 2% fetal calf serum. To flush the uterus, the base of each uterine horn was incised and a 60 ml catheter-tipped syringe filled with collection medium was placed in the incision. A large tissue clamp was placed just behind the syringe, preventing medium from flowing into the body of the uterus. Collection medium (60 ml for each uterine horn) was passed through the tip of each uterine horn and filtered through a 70 micron embryo filter (Emcon filters; Veterinary Concepts; Spring Valley, WI, USA). Filters were washed with collection medium and sprayed with 20 mL dPBS using a syringe and a 25 gauge needle (to wash embryos into two petri dishes) and 0.2 ml fetal calf serum was added to each of the two petri dishes. Petri dishes were labeled and ova/embryos were searched using a stereo-microscope at 10X magnification. Recovered ova/embryos were evaluated for stage and quality at 50X magnification using International Embryo Transfer Society (IETS) criteria [124]. All recovered embryo/ova were placed in drops of embryo culture medium covered with mineral oil. Embryo culture medium primarily contained NaCl, KCl, sodium pyruvate, NaHCO3, BME essential amino acids, MEM nonessential amino acids, L-glutamic acid, bovine serum albumin and gentamicine (CR1aa + 5% fetal calf serum) [125]. From 1 to 8 embryos were cultured per drop, using 5 µl of medium per embryo. The petri dishes with embryo in culture medium were placed in an incubator with 5% CO2, 5% O2 and 90% N2 at 38.5 ^{0}C. Cleavage was evaluated 4 days after AI (immediately after recovery from the reproductive tract), and following in vitro culture, blastocyst and hatching rates were evaluated 7, 9, and 10 days after the first AI (approximately 6, 8, and 9 days after fertilization).

4.3.3 Blood sampling and radioimmunoassays

To estimate progesterone and LH concentrations, blood samples were collected (by venipuncture) from the caudal coccygeal vessels into 10 ml heparinized, evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA), concurrent with the first and sixth FSH treatments, first PGF, and pLH treatments. Blood samples were centrifuged at 1500 x g for 15 minutes, and plasma was separated and stored at -20 °C until assayed.

Plasma LH concentrations were measured in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) [105]. The range of the standard curve was 0.06 to 8.0 ng/ml. Intra-assay coefficients of variation were 3.4 and 5.6% for the low and high reference sera (means of 0.94 and 2.08 ng/ml, respectively).

Progesterone concentrations were measured in a single assay using solid-phase commercial kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/ml. The intra-assay coefficients of variation were 4.3, 3.3, and 3.4% for low, medium, and high reference sera (means, 1.79, 3.64, and 16.81 ng/ml, respectively).

4.3.5 Statistical analyses

Statistical analyses were performed using Statistical Analysis System software package (SAS Learning Edition 4.1; SAS Institute Inc., Cary, NC, USA). Numerically dependent variables (number of follicles at the 8th dose of FSH; number of follicles ≥7 mm at the 8th dose of FSH; number of follicles at CIDR removal; number of follicles ≥7 mm at CIDR removal; number of follicles 12 hours after second AI; number of follicles ≥7 mm at 12 hours after second AI; difference between number of follicles ≥7 mm at CIDR removal and at 12 hours after AI; and number of CL at slaughter) were analyzed by one-way ANOVA, using a general linear model procedure (GLM). Normality of residuals was tested basing on Shapiro-Wilk test (P values >0.05 were considered nonsignificant). Numbers of CL at time of slaughter were used to determine ovulation rates.

Progesterone and LH concentration were analyzed by repeated measures (MIXED procedures). Tukey's post-hoc tests for multiple comparisons were performed if main effects were significant ($P \leq 0.05$). The values are expressed as mean ± SEM. Proportional variables (ova/embryo ratio; proportion of total and different grades of morula on Day 4; proportion of early blastocyst, blastocyst or expanded blastocyst on Days 9 and 10; proportion of transferable embryos (≥ early blastocyst from Grades 1, 2, and 3) were compared using Chi-Square or Fisher's exact test. Cows that failed to ovulate were excluded from the analysis.

4.4 Results

4.4.1 Follicular dynamics

There were tendencies for differences among groups for the number of follicles present at the 8th FSH treatment; however, the only significant difference was more follicles ≥7 mm in diameter at CIDR removal in the long P_4 group compared to the FSH starvation group (Table 4.1). More ovulations occurred between CIDR removal and 12 hours after the second AI in the long P_4 group than in the FSH starvation group (P=0.02), whereas the short P_4 group did not differ from either (Table 4.1). Several cows in all three groups had at least 2 follicles ≥7 mm at 12 hours after second AI and they received a third insemination 48 hours after pLH administration (9/14, 13/13, and 4/14 in short P_4, FSH starvation, and long P_4 groups, respectively).

Table 4.1 The effect of duration of progesterone exposure and FSH starvation on the mean (± SEM) number of follicles during superstimulatory treatments in cows.

	Short P_4	FSH starvation	Long P_4	P value
No. follicles	n = 14	n = 13	n = 13	-
8th dose of FSH				
≥3 mm	18.6 ± 2.5	19.2 ± 1.8	25.4 ± 2.1	0.06
≥7 mm	10.5 ± 2.0	5.7 ± 1.2	9.6 ± 1.5	0.10
CIDR removal				
≥3 mm	18.6 ± 2.5	22.5 ± 2.1	25.3 ± 2.5	0.14
≥7 mm	10.5 ± 2.0[a]	9.1 ± 1.8[a]	18.1 ± 2.7[b]	0.02
12 hours after second AI				
≥3 mm	15.8 ± 4.2	15.8 ± 3.5	13.33 ± 2.3	0.88
≥7 mm	5.4 ± 1.6	9.0 ± 2.1	5.2 ± 1.5	0.20
Estimated number (%) of ovulations between CIDR removal and 12 hours after second AI	5.1 (71.3%)[ab]	0.1 (1.1%)[a]	12.9 (48.6%)[b]	0.02

[a,b]- Within a row, groups without a common superscript differed (P<0.05)

4.4.2 Plasma hormone concentrations

For plasma progesterone concentrations there was only a significant effect of time (Figure 4.2); concentrations were very low at the time of pLH treatment. However, for plasma LH concentrations, there was an effect of time and a group by time interaction. The long P_4 group had a significantly higher plasma LH concentration compared to the short P_4 group at the time of pLH treatment (Figure 4.3).

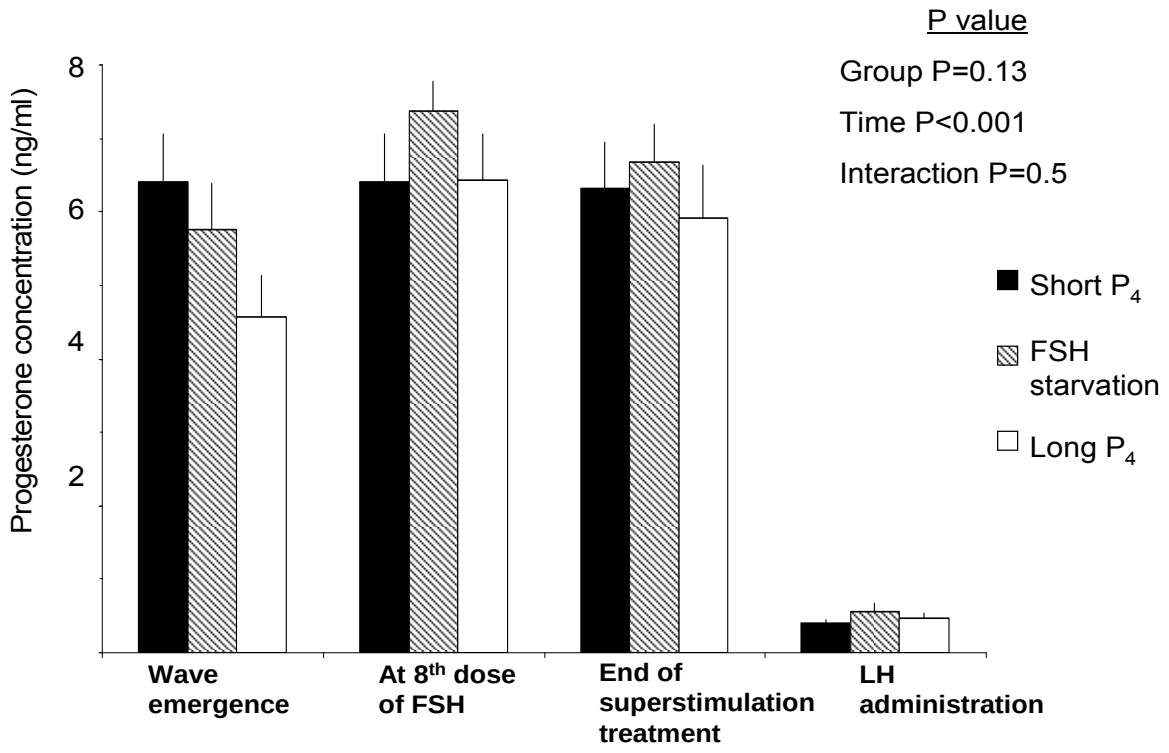

Figure 4.2 Mean (± SEM) plasma progesterone concentrations in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment. [a,b]- Values without a common superscript were different (P<0.05)

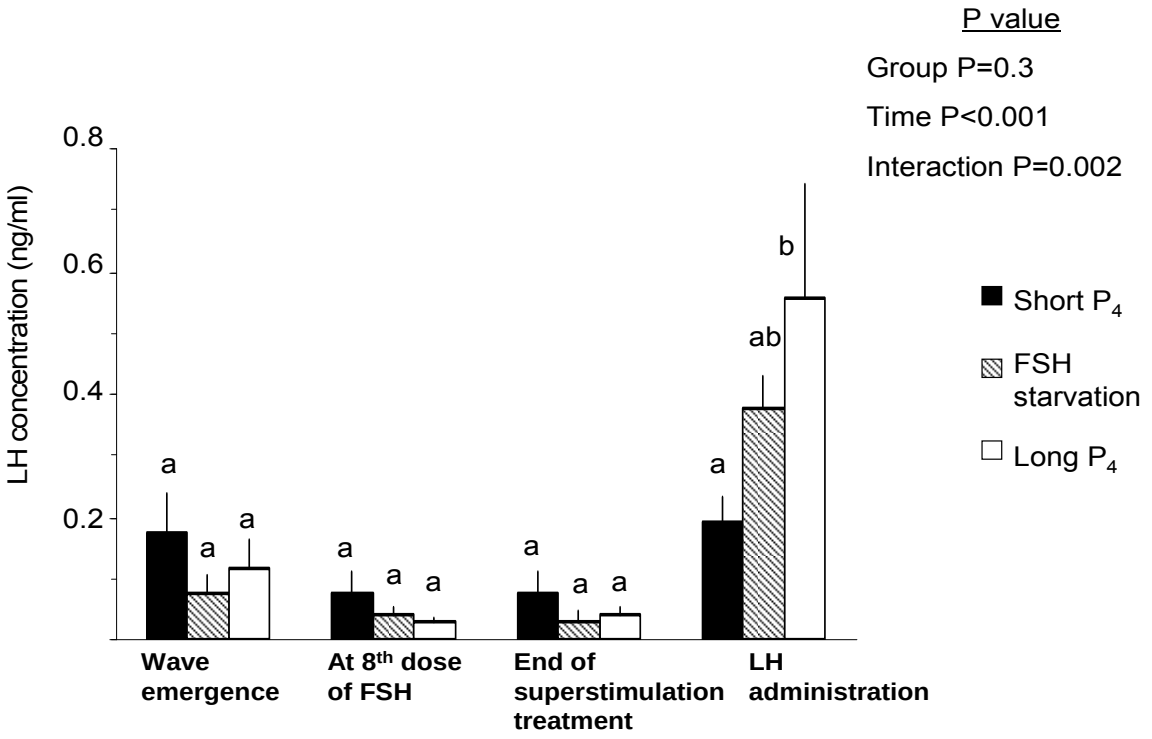

Figure 4.3 Mean (± SEM) plasma LH in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment. [a,b]- Values without a common superscript were different (P<0.05)

4.4.3 Ovulation and ova/embryo recovery

In the FSH starvation group, 6 of 13, and in the long P_4 group, 1 of 13 cows failed to ovulate (no follicles disappeared between the CIDR removal and 12 hours after the second AI and no new CL detected at slaughter) and were excluded from further analysis. Fewer ovulations were detected in the FSH starvation group than either the short P_4 or long P_4 groups (P=0.001; Table 4.2), whereas there was no significant difference between the latter two groups.

Most of the embryos were recovered from oviduct. Nine ova/embryos were collect from the uterus of one cow in the short P_4 group, whereas 23 ova/embryos were collected from the uteri of three cows in the long P_4 group. Total recovery rate varied among groups, with the lowest recovery rate in the FSH starvation group (Table 4.2). In the Short P_4 and FSH starvation groups, the majority of the embryos were Grades 1 or 2, whereas the majority of embryos in the Long P_4 group were Grades 3 or 4 (Table 4.2).

Table 4.2 Mean (±SEM) numbers of ovulations and ova/embryos recovered at slaughter 4 days after insemination in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment.

	Short P_4	**FSH starvation**	**Long P_4**	**P value**
No. cows	14	13	13	-
No. ovulations	11.6 ± 2.2^a	2.5 ± 1.2^b	15.4 ± 3.0^a	0.001
Total ova/embryos recovered	5.9 ± 1.3^a	0.9 ± 0.6^b	6.7 ± 1.5^a	0.048
Recovery rate (%)	50.9^a	36.0^b	43.5^a	0.048
No. uncleaved ova (%)	13/82 (15.9)	1/12 (8.3)	5/87 (5.7)	0.09
No. cleaved ova (%)	69/82 (84.1)	11/12 (91.7)	82/87 (94.2)	0.09
Grades 1 or 2 embryos (%)	87.0^a	81.8^a	37.8^b	0.0001
Grades 3 or 4 embryos (%)	13.0^a	18.2^a	62.2^b	0.0001

^{a,b}- Within a row, values without a common superscript differed ($P<0.05$)

There was no significant difference between Short P_4 and Long P_4 groups for embryo development at 7, 9, or 10 days after insemination (Table 4.3). Results from FSH starvation were excluded, due to a paucity of embryos.

Table 4.3 Embryo development in culture in cows subjected to a short or long progesterone exposure during superstimulatory treatment.

Interval after AI	**Short P_4**	**Long P_4**	**P value**
7 days			
≥ Morulas	17/69 (24.6%)	15/82 (18.3%)	0.39
Grades 1 or 2 morulas	0/69 (0%)	2/82 (2.4%)	0.19
Grades 3 or 4 morulas	17/69 (24.6%)	13/82 (15.8%)	0.19
9 days			
Early blastocyst	10/69 (14.5%)	16/82 (19.5%)	0.41
Blastocyst	9/69 (13.0%)	15/82 (18.3%)	0.38
≥ Expanded blastocyst	4/69 (5.8%)	7/82 (8.5%)	0.52
Transferable embryos	7/69 (10.1%)	14/82 (17.1%)	0.22
10 days			
≥ Early blastocyst	9/69 (13.0%)	15/82 (18.3%)	0.38
≥ Blastocyst	9/69 (13.0%)	16/82 (19.5%)	0.29
≥ Expanded blastocyst	8/69 (11.6%)	9/82 (11.0%)	0.90
Transferable embryos	7/69 (10.1%)	14/82 (17.1%)	0.22

4.5 Discussion

Oocytes in follicles that grew and developed under a prolonged period of progesterone exposure during superstimulation did not have compromised competence. In that regard, both Short P_4 and Long P_4 groups (analogous to 3-wave and 2-wave cycles, respectively) were similarly capable of producing equal numbers of healthy transferable embryos. Therefore, the hypothesis that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth was not supported. However, FSH starvation led to a substantial reduction in the in the capacity of

follicles to ovulate. Therefore, the hypothesis that oocyte competence is not affected by FSH starvation at the end of superstimulatory treatment was not adequately tested.

During the superstimulatory treatment, the long P_4 group had more follicles ≥7 mm at CIDR removal than the FSH starvation group; this was attributed to the difference in total dose of FSH (350 versus 200 mg, respectively). The effect of different total dose of FSH (from 100 to 900 mg) in a superstimulatory treatment has been tested and in many reports, increasing the total dose (up to ~400 mg) increased superovulatory response [26, 78, 126]. Furthermore, the greater number of follicles ≥7 mm at CIDR removal in the long P_4 group could also be attributed to a longer growing phase; a longer period of follicular development (e.g. 2-wave cycle) increased follicle diameter [5, 6, 127]. Most follicles ≥7 mm in diameter at CIDR removal were expected to ovulate within 48 hours after LH administration [3]; this accounted for the need for a third AI in most of the cows (i.e. 3 inseminations at 24, 36, and 48 hours, respectively, after LH treatment).

Based on follicle disappearance between CIDR removal and 12 hours after the second AI and the number of CL after slaughter, 72 hours of FSH starvation at the end of superstimulatory treatment decreased ovulatory response. This was in agreement with another study, in which 132 hours of FSH starvation decreased ovulation rates; however the FSH starvation was created under a prolonged low progesterone environment, inducing a large, persistent ovulatory follicle [2, 6]. It was not clear if the low ovulation rates in that study were caused by the development of persistent follicles.

Follicles in the FSH starvation and long P_4 groups were both under prolonged progesterone exposure, which could have resulted in an aged oocyte. In addition, the FSH starvation group had little FSH available to maintain follicle growth, which resulted in a reduced superstimulatory response, as demonstrated by fewer follicles ≥7 mm at CIDR removal when compared with long P_4 [128]. Follicle growth is dependent on gonadotrophin support (initially FSH and then LH); without this support, follicles undergo atresia [128]. Therefore, the long P_4 group did not have decreased ovulation rates, as FSH stimulation was continued. However, the long P_4 group had poor embryo quality, perhaps due to delayed LH (secondary to prolonged progesterone exposure) and suppressed preovulatory maturation (prior to the LH surge).

Progesterone concentrations were kept high during superstimulatory treatment to mimic mid-luteal progesterone concentration. However, $PGF_2\alpha$ treatment and CIDR removal resulted in low plasma progesterone concentrations at LH administration; these were needed to create a pre-ovulatory endogenous LH peak, since P_4 has a suppressive effect on pituitary release of LH [12, 20, 22, 80]. In the present study, plasma LH concentrations at the time of administration of exogenous LH were highest in the cows in the Long P_4 group, and tended to be higher in the FSH starvation group than in the Short P_4 group. Since LH is released by the pituitary in pulses [22], multiple samples at frequent intervals should be collected to ensure that the frequency and amplitude of LH is accurately characterized [22]. In the present study, LH concentrations were determined in only a single sample after plasma progesterone concentrations had declined. Therefore, the accuracy of determining LH release using a single blood sample is decreased; we may have detected only a portion of the LH surge, the peaks of a pulse, or increased basal concentrations of LH release in response to the feedback from follicles under prolonged development. Consequently, the lower LH concentration in the Short P_4 group could be an artifact due to a single blood sample, or it could represent a delayed LH surge.

Reproductive tracts were collected 4 days after the second AI; as expected, most of the embryos were retrieved from the oviducts, since bovine embryos enter the uterus 3 to 4 days after fertilization [129, 130]. Due to so few ovulations in the FSH starvation group, the recovery rate was lower than the other two groups, and there were insufficient numbers of embryos to adequately measure development competence.

At slaughter, the long P_4 group had proportionally more low-quality embryos than the short P_4 or FSH starvation groups. Although there were insufficient numbers of embryos to adequately measure development competence in the FSH starvation group, there was no obvious difference in the developmental competence of embryos recovered from cows in the Short or Long P_4 groups. Although this could reflect a latent effect, or an improvement in the competence of embryos in the Long P_4 group, development to expanded or hatched blastocyts was quite low in both the Short and Long P_4 groups, suggesting that effects may be more related to handling during collection (at slaughter), or culture conditions.

In conclusion, although there were differences in the response to superstimulation when the duration of the ovulatory wave was extended 3 days, there was no difference in oocyte competence. Long exposure of superstimulated follicles to progesterone (analogous to a 2-follicular wave cycle) decreased embryo quality 4 days after AI; however, superstimulated follicles exposured to progesterone for short and long intervals were similarly capable of producing healthy embryos that developed similarly in culture. Although FSH starvation treatment led to a loss of ovulatory capability, the hypothesis that FSH starvation at the end of superstimulatory treatment does not affect oocyte competence remains untested.

CHAPTER 5

GENERAL DISCUSSION

Several experiments were conducted to test the effects of progesterone on GnRH-mediated LH release, follicle growth, oocyte competence, and fertility. We examined 1) the effect of progesterone concentrations on GnRH-mediated LH release (Chapter 2); and 2) the effect of duration of progesterone exposure on oocyte competence and fertility (Chapters 3 and 4).

Based on present studies, we reached the following general conclusions: a) increased plasma progesterone concentrations during growth of the dominant follicle suppress LH release and tend to decrease ovulation rates following the administration of GnRH; although increasing the dose of GnRH not result in an increase LH release (and ovulation rate), administration of estradiol benzoate prior to GnRH promotes LH release in heifers with elevated plasma progesterone concentrations (Chapter 2); b) the dominant follicles of both short-progesterone exposure (analogous to the ovulatory wave of 3-wave cycles) and long-progesterone exposure (analogous to the ovulatory wave of 2-wave cycles) groups are capable of ovulating, achieving, and sustaining pregnancy (Chapter 3); c) although prolonged exposure to elevated plasma progesterone concentrations (analogous to 2-wave cycles) decreases the quality of early developmental-stage embryos, ovulatory follicles analogous to those resulting from both 2- and 3-follicular waves are similarly capable of producing healthy, developing embryos after superstimulation treatment (Chapter 4); d) a period of FSH starvation at the end of superstimulatory treatment led to a loss of ovulatory capability.

5.1 Effect of progesterone on follicular dynamics

As mentioned earlier, the bovine estrous cycle is composed of 2 or 3 follicular waves [12, 14, 110]. During an ovulatory wave, the dominant follicle grows under a high-

progesterone environment for 6 or 3 days in 2- and 3-wave cycles, respectively [20, 114]. Therefore, high progesterone exposure of developing dominant follicles for two different periods of time were tested in Chapters 3 and 4 during ovulatory wave (6 versus 3 days, analogous to 2 versus 3 waves cycle).

Follicle growth deviates (selection) at a largest follicle size of 8.5 mm diameter [35] and the dominant follicle acquires ovulatory capability at 10 mm; however it has been reported that ovulations from follicles >11 mm results in higher oocyte competence [99]. Larger follicles contain more granulosa cells, more LH receptors, and are more likely to ovulate [99]. Conversely, smaller follicles have fewer LH receptors and require a higher LH peak to ovulate [99]. In addition, it has been suggested that a larger follicle results in a larger CL, since more granulose cells are available for luteinization [99]. A larger CL is known to produce higher plasma progesterone concentrations, which is important to the maintenance of pregnancy [99]. Therefore, a pre-ovulatory follicle has to reach at least 10 mm in diameter to ovulate; however, follicles larger than 11 mm are more likely to contain a competent oocyte and develop into a CL that will produce more progesterone.

It has been postulated that progesterone affects follicular dynamics and consequently reproductive performance [17]. In study 1 (Chapter 2), the effect of different concentrations of progesterone on pre-ovulatory follicle diameter was clear; follicles which developed under high progesterone concentrations were smaller (~11 mm) and tended to be less able to ovulate. When durations of progesterone exposure during follicular development were tested (Study 2, Chapter 3), the means of follicular size in all groups were >11 mm and follicle size was larger in cows in the long progesterone group; however neither ovulation nor pregnancy rates were affected. Therefore, follicles might have a standard size prior to ovulation which will indicate the probability of ovulation, and it must be approximately 11 mm. In Study 3 (Chapter 4), there was a difference in number of large follicles (categorized as ≥7 mm) at CIDR removal after superstimulation treatment between a long and a short exposure to progesterone (long and short FSH). However, the main difference between Studies 2 and 3 is that cattle were superstimulated in Study 3 but the period of progesterone exposure during follicular growth in both studies was the same (3 vs 6 days). Therefore, differences in the duration of progesterone exposure during ovulatory

follicle development affected follicle dynamics, since long progesterone exposure resulted in larger follicles after synchronization for fixed-time AI and superstimulation programs.

5.2 Effect of progesterone on preovulatory LH release

It is well known that progesterone has a suppressive effect on the hypothalamic-pituitary axis [52, 131, 132]. In that regard, administration of exogenous progesterone to ovariectomized cows suppressed LH release [133]. Furthermore, elevated plasma progesterone concentrations suppressed LH pulse frequency [52, 131]. Similarly, in Chapter 2, high progesterone concentrations suppressed the release of LH in response to the administration of GnRH relative to a low-progesterone environment. However, pre-treatment with 0.25 mg of estradiol benzoate (8 hours before administration of 100 μg of GnRH) overcame the negative effect of progesterone on LH release. In cattle with high progesterone concentrations, maximum diameter of the dominant follicle was smaller and plasma estradiol concentrations were lower than in cattle with low plasma progesterone concentrations [52]. Estradiol increases GnRH receptors in the pituitary, thereby increasing the responsiveness to GnRH [31, 35, 131]. Therefore, in cattle with high progesterone concentrations, there are several consequences, including smaller follicles, reduced synthesis of estradiol, and decreased GnRH-mediated LH release [131] that must be considered.

In Chapters 3 and 4, only a single blood sample for measurement of plasma LH was collected; therefore, the LH profile could not be as well defined as in Chapter 2 [22] (LH was measured every hour for 3 or 4 hours after GnRH treatment). Although cattle in Chapters 3 and 4 were given exogenous pLH prior to AI (to maximize the probability of ovulation), the LH measured was endogenous LH, since blood samples were collected just before pLH treatment.

In Chapter 3, heifers that had long progesterone exposure during the ovulatory wave had higher peak LH concentrations compared with other groups (long-progesterone cows and short-progesterone heifers and cows). Furthermore, in Chapter 4, cows exposed to long-duration progesterone (concurrently long-duration FSH) had higher LH compared

with other groups. Therefore, cattle with the highest plasma LH concentrations were from long-duration progesterone treatments. However, it was noteworthy that measurements were not performed at the same time. In Study 2, LH concentrations were higher at the time of AI (48 hours after CIDR removal) and in Study 3, LH concentrations were higher at LH administration (24 hours after CIDR removal).

It has been reported that long-duration progestin exposure (ear implant for 8 days after wave emergence) resulted in estrus 39 hours after ear implant removal in beef heifers [16]. In cattle, the onset of estrus is approximately 2 hours after the LH peak [3]; therefore cattle in that study may have had an LH peak 37 hours after implant removal. It has also been reported that short progesterone exposure (use of a CIDR for 3 days after wave emergence) resulted in estrous behavior 52 hours after CIDR removal in heifers [41] and cows [37] (LH peak approximately 50 hours after CIDR removal). However, long-duration progesterone exposure during the growing phase of the ovulatory follicle could result in an early LH peak, presumably due to higher estradiol concentrations produced by larger follicles [2, 3]. Perhaps in a similar pathway, prolonged subluteal progesterone exposure results in larger sized, persistent follicles, with high estradiol concentrations, high LH pulse frequency, and reduced fertility.

5.3 Effect of progesterone on reproductive performance

It was confirmed in Chapter 2 that plasma progesterone concentrations during the ovulatory wave affected pre-ovulatory LH pulses and tended to affect ovulation. This was in agreement with other studies in which high progesterone concentrations suppressed LH release [132] and decreased ovulation rates [52]. As mentioned, high progesterone concentrations resulted in smaller follicles that produced less estradiol [52]. Small follicles require a higher pre-ovulatory LH peak [6]; however with less estradiol, the anterior pituitary is less sensitive to GnRH [3] and LH is released in low frequency and high amplitude, which does not promote ovulation. Preovulatory LH peak is characterized by high frequency and low amplitude of pulses [22].

Reproductive performance was also evaluated based on the duration of progesterone exposure during the growing phase of the preovulatory follicle. Long and short progesterone exposures were tested using fixed-time AI, where pregnancy rates were recorded, and superstimulation treatment where superovulatory response, embryo recovery rates, and evaluations were recorded.

Duration of progesterone exposure during the ovulatory wave did not affect pregnancy rates or blastocyst rates (Chapters 3 and 4, respectively). Furthermore, ovulation rates were not affected by duration of progesterone exposure. Perhaps treatment with exogenous pLH induced ovulation, thereby masking the effects of the duration of progesterone treatment. However, long-duration progesterone (analogous to 2-wave cycles) decreased oocyte quality 4 days after insemination, but did not affect further development.

The effect of the duration of the growing phase of the ovulatory follicle (i.e. 2- vs 3-wave cycles) on reproductive performance has been an intriguing topic [6, 15, 20]. Some reports suggested that 2-wave cycles were associated with lower fertility; this was attributed to an ovulatory follicle that grows 3 days longer relative to the wave emergence, perhaps resulting in an aged oocyte [115]. In contrast, other reports detected no differences in fertility between the wave patterns [17]. In the present studies, long or short growing phases of ovulatory follicle (similar to 2- or 3-wave cycles) under the influence of high progesterone concentrations did not affect blastocyst rates (Chapter 4) or pregnancy rates (Chapter 3).

It is well known that prolonged exposure of growing dominant follicles to sub-luteal progesterone concentrations results in oversized, estrogen-activated persistent follicles [21], with reduced fertility [21]. Furthermore, when oocytes that originated from a persistent follicles are fertilized, the incidence of embryonic death is increased [33]. However, 3 and 6 days of mid-luteal progesterone concentrations were compared in our experimental design. Although long-duration exposure to relatively high progesterone concentrations compromised embryo quality 4 days after insemination, further development was not compromised, while those in persistent follicles resulted in abnormal embryonic development [33]. Presumably, progesterone concentrations in the present studies were higher than those that produced the changes associated with persistent follicles [2].

A period of 72 hours of FSH starvation at the end of superstimulatory treatment decreased ovulatory response (Chapter 4). Follicles subjected to FSH starvation were under prolonged midluteal progesterone exposure, which could have resulted in an aged oocyte. This was in agreement with another study, in which 132 hours of FSH starvation decreased ovulation rates [2]; however, in that study, FSH starvation was created under a prolonged subluteal progesterone environment [2]. Exogenous LH was given prior to AI, in both studies but anovulation still occurred. Perhaps follicles which were subjected to FSH starvation did not acquire sufficient LH receptors on granulosa cells to respond to a preovulatory LH peak. Therefore, regardless progesterone concentrations, FSH starvation at the end of superstimulatory treatment led to a loss of ovulatory capability, even though exogenous LH was given.

For both timed-AI and superstimulation protocols, it is noteworthy that progesterone concentrations are decreased at the end of the protocol, thereby enabling plasma LH concentrations to reach a pre-ovulatory peak, optimizing both ovulation and reproductive performance. To improve superstimulation protocols, FSH starvation should be avoided. Although different durations (3 or 6 days) of progesterone exposure during the growing phase of the ovulatory follicle can be used in estrus synchronization and superstimulation protocols, exogenous LH should be given prior artificial insemination. If progesterone concentrations remain elevated or are unknown, giving estradiol 8 hours before administration of GnRH could overcome the negative effect of progesterone on LH release in GnRH-based synchronization protocols.

CHAPTER 6

GENERAL CONCLUSION

Based on the work conducted in this thesis, we concluded that:

- When systemic progesterone concentrations were high, increasing the dose of GnRH (from 100 to 200 μg) does not overcome the suppressive effect of progesterone on LH release. However, this should be reconfirmed, since handling stress may have confounded the results in this study. Although increased plasma progesterone concentrations during the growth of the dominant follicle suppress the release of LH and tend to decrease ovulation rates in heifers, the administration of estradiol benzoate prior to GnRH promote LH release.
- To maximize the LH response, GnRH treatment should be given when P_4 concentrations are low, or alternatively, following pretreatment with estradiol benzoate.
- A long duration of the growing phase of the ovulatory follicle (analogous to 2-wave cycles) increases follicle size.
- A long duration of the growing phase of the ovulatory follicle (analogous to 2-wave cycles) results in higher LH concentrations, perhaps due to an early LH peak.
- The duration of the growing phase of the ovulatory follicle (under mid-luteal progesterone concentrations) does not affect reproductive performance after fixed-time AI or superstimulation in cattle.
- Two-wave reproductive cycle is likely to be as fertile as a 3-wave cycle.
- A period of FSH starvation during superstimulatory treatment led to a loss of ovulatory capability.

CHAPTER 7

FUTURE DIRECTIONS

New questions were raised from the results and conclusions presented in this thesis. Further research should be conducted to answer the following questions.

- Can a higher dose of GnRH (200 μg) or multiple injections overcome the negative effect of high progesterone concentrations on ovulation?
- Does different duration of progesterone exposure during the growing phase of the ovulatory wave (analogous to 2- or 3-wave cycles) affect fertility when no exogenous LH is administered?
- Does a short progesterone exposure (analogous to the ovulatory wave of a 3-wave cycle) affect oocyte competence after superstimulatory treatment when the same total dose of FSH as that given to long progesterone is administered?
- What are the effects of duration of progesterone exposure during the ovulatory wave in *Bos indicus* cattle?
- Is there any difference between animals which exhibit a 4-wave pattern compared with 2- or 3-wave cycles on oocyte competence and fertility?
- Is the effect on LH by the long progesterone exposure caused by high estradiol from larger follicles?
- Are follicles from the long progesterone exposure groups following the same pattern of follicle dynamics, LH release, and fertility as persistent follicles?

CHAPTER 8

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