OVARIAN RESPONSE, FOLLICULAR FUNCTION AND OOCYTE DEVELOPMENTAL COMPETENCE IN GONADOTROPIN TREATED PREPUBERTAL CALVES

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ABSTRACT

Sexual immaturity during the prepubertal period in cattle is characterized by low pulse frequency of LH, anovulatory waves of follicle development, absence of corpora lutea (i.e., progesterone) and oocytes of reduced developmental capacity *in vitro* when compared to oocytes of sexually mature animals. However, calf ovaries are responsive to exogenous gonadotropin treatment early in life, and the use of prepubertal animals as a source of oocytes for *in vitro* embryo production may have significant potential to decrease generation interval and accelerate the rate of genetic gain. The overall objective of this thesis was to investigate the effect of exogenous FSH treatment on the ovarian response, hormonal profiles, ovulation and oocyte developmental competence in prepubertal calves.

In the first study (Chapter 3) the effect of cumulative dose (200 mg vs. 350 mg) and duration (4 vs. 7 days) of FSH treatment on the ovarian response and the number of spontaneous and induced ovulations in 5-month-old calves were compared. Calves (n=24) were selected for gonadotropin treatment from a group of spring-born calves (n=51) based on the antral follicle counts (AFC) at the time of wave emergence. Calves were classified in low, medium and high AFC, and the ones in the medium classification (25±0.8, range of 20 to 32 follicles) were used in this study. At the end of the FSH treatment and 24 hours after treatment with pLH, the number of follicles ≥ 9 mm was greater in the 7-day than in the 4-day treatment group and in calves given a cumulative dose of 350 mg of FSH compared to those given 200 mg. Spontaneous ovulations were observed in 14 calves between Day 4 of FSH treatment and 12-hours post-LH treatment. The number of total and spontaneous ovulations was higher in the 7-day treatment groups than in the 4-day groups, and the number of spontaneous ovulations was higher in calves given a cumulative dose of 200 mg FSH than 350 mg. Numbers of ovulations in response to exogenous LH did not differ among groups.

In the second study (Chapter 4), data showed in prepubertal calves (n=46) that the number of follicles at the beginning of a wave was predictive of the number recruited into subsequent waves and that after FSH treatment, the number of medium and large sized follicles available for follicular aspiration was positively associated with the number of follicles ≥ 1 mm at the time of wave emergence. In calves with low (n=12) and high (n=10) AFC at wave emergence, 7 days of FSH treatment resulted in a higher number of large than small size follicles than the 4 days of FSH

treatment. High AFC at wave emergence resulted in a greater number of follicles ≥ 6 mm available for aspiration and a greater number of cumulus oocyte complexes (COC) collected than low AFC.

The third study (Chapter 5) was designed to investigate the relationship between the antral follicular counts and plasma concentrations of AMH and FSH at the time of wave emergence in prepubertal calves and to compare the effects of age and duration of gonadotropin treatment on ovarian response of prepubertal and pubertal cattle. The AFC and the plasma concentrations of AMH at the time of wave emergence were positively correlated and both were positively correlated with the number of follicles ≥ 6 mm at the time of oocyte collection. Ovarian response was greater in calves at 4 months of age than at 7 months and was characterized by a higher number of medium to large (i.e., ≥ 6mm) sized follicles and higher number of recovered oocytes following transvaginal ultrasound-guided aspiration. Prolonged follicular growth by the 7-day treatment resulted in a greater number of large follicles at the end of the treatment and a greater degree of follicular maturation, characterized by lower intrafollicular E2:P4 ratio and higher proportion of fully expanded COC after LH treatment.

In the fourth study (Chapter 6), different methods to control endogenous LH release and prevent ovulations during 7 days of exogenous FSH treatment were investigated. A long-acting progesterone (Long-acting P4) treatment at the time of follicular ablation and the GnRH antagonist Cetrorelix given at 48 hours intervals during FSH treatment were effective in preventing endogenous LH release and spontaneous ovulations during 7 days of exogenous FSH treatment. Ovulations were observed after FSH treatment in the Long-acting P4 treatment groups, while ovulations were prevented in calves of the Cetrorelix group. Luteal structures formed due to spontaneous ovulations were functional and a positive correlation was observed between the number of structures and the plasma concentrations of progesterone.

In Chapter 7, oocyte developmental competence following *in vitro* vs. *in vivo* maturation of oocytes from prepubertal calves and *in vivo* maturation of oocytes from calves and pubertal heifers given 4 or 7 days of exogenous FSH treatment were evaluated in Study I. Spontaneous ovulations before oocyte collection were observed in calves and prevented meaningful comparisons among groups, resulting in lower rates of cleavage and blastocyst in the 7-day *in vivo* group. Blastocyst rates did not differ between 4-day *in vivo* vs. 4- and 7-day *in vitro*. In Study II, 4, 6 and 7 days of exogenous FSH treatment under controlled endogenous LH release were

compared and six days of exogenous FSH support was associated with the greatest developmental competence of oocytes collected from 5-month-old calves.

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DEDICATION

I dedicate this thesis to my brother, Luiz Gustavo, and my father, Luiz Felipe Krause. They were my best friends, my true heroes, my inspiration, and the best part of me.

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AFC	antral follicle counts
AMH	anti-Müllerian hormone
ART	assisted reproductive techniques
ATP	adenosine triphosphate
AUC	area under the curve
C	Celsius
cAMP	cyclic adenosine monophosphate
CIDR	controlled internal drug release device
CO2	carbon dioxide
COC	cumulus-oocyte complex
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
E2	estradiol
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
g	gram
G	gauge
GnRH	gonadotropin-releasing hormone
GV	germinal vesicle
hCG	human chorionic gonadotropin
IGF-I	insulin-like growth factor
im	intramuscular
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
Kg	kilogram
LH	luteinizing hormone
mcg	microgram
μm	micrometer
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mm	millimeter
ng	nanogram
N2	nitrogen
NIH	National Institute of Health
O2	oxygen
P4	progesterone
PBS	phosphate buffered saline
pg	picogram

PGC	primordial germ cell
rBST	recombinant bovine somatotropin
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
vs.	versus

CHAPTER 1

1. GENERAL INTRODUCTION

The female mammal is born with a limited number of germ cells; this reserve of primordial follicles provides the gametes for their entire reproductive life. Each primordial follicle contains an oocyte surrounded by a flat layer of granulosa cells. Activation and growth of these follicles are required for the development of a fertilizable oocyte through a complex and very well coordinated process, regulated by the primordial follicle itself and by the hypothalamus-pituitary-gonadal axis.

In female cattle, sexual maturation is expressed by the occurrence of regular estrous cycles and the ability to carry a gestation to term. The average age at puberty has been reported to be around 15 months of age [1-5], and although the prepubertal period is characterized for sexual immaturity, the growth of primordial follicles to antral stages is initiated during fetal life. Therefore, calf ovaries offer a source of potentially fertilizable oocytes from birth that can be used in assisted reproductive technologies (ART) with the potential to decrease generation interval and accelerate the rate of genetic gain by *in vitro* embryo production and embryo transfer. However, the developmental capacity of oocytes from prepubertal calves has been reported to be lower when compared to oocytes from sexually mature animals [6-11]. As oocyte developmental competence has been considered the critical limiting factor in female fertility [12], methods to improve oocyte competence and the establishment of successful *in vitro* embryo production systems are required.

Exogenous FSH treatments have been used widely in order to increase the number of recovered oocytes for *in vitro* embryo production or embryos for transfer. Our laboratory has provided support for the hypothesis that duration of FSH support can influence follicular growth, granulosa gene expression, and oocyte developmental competence in sexually mature animals. Seven days of follicular growth under FSH support resulted in a higher number of large sized follicles available for aspiration, activation of ovulation and oocyte competence markers in granulosa cells and higher number of transferable embryos when compared to four days of FSH treatment [13, 14]. Accordingly, it has been shown that oocytes recovered from follicles > 6 mm in diameter have higher developmental competence than oocytes from smaller follicles [15].

A wave pattern characterizes follicular development in calves, as in sexually mature animals [1, 2, 16, 17] and ovarian ultrasound examinations of calves from 2 to 36 weeks of age revealed that growing, static and regression phases of follicular development did not change within this age period. Moreover, ultrasound examinations starting 12 weeks before puberty revealed no changes in the maximum diameter of the dominant and largest subordinate follicle as puberty approached, and no differences in follicle growth or regression rates, but an increase in interwave interval [1]. Therefore, exogenous FSH treatment may be a strategy to influence follicular growth and produce oocytes of higher developmental capabilities in prepubertal calves.

A review of the literature regarding current knowledge about ovarian and follicular development in cattle and the onset of puberty is presented in the following sections. Meiotic and developmental competence of oocytes have also been reviewed, with an emphasis on studies in prepubertal calves. Finally, the effects of exogenous gonadotropin treatment on the ovarian response and oocyte developmental competence in prepubertal calves is presented from previous studies.

1.1. Ovarian and Follicular Development in Cattle

In cattle, oocytes are formed during fetal development [18]. It has been assumed that primordial germ cells (PGC), the embryonic precursors of the gametes, develop in the yolk sac and start migration, while proliferating, along the wall of the hindgut and dorsal mesentery to the developing gonads around days 18 to 31 of gestation in cattle [19]. However, in 18-day old bovine embryos the first potential PGC were identified in the caudal wall of the proximal yolk sac and it was demonstrated that the folding process from the flat trilaminar disc into a cylindrical embryonic body incorporates the PGC into the embryo. Therefore, PGC are located predominantly in the axial body region, at the level of the mesonephros in 23-25 days old bovine embryos [20]. The PGC are unevenly distributed in the gonadal ridge which starts to form around day 27 in a bovine embryo, and the same distribution has been observed in the undifferentiated gonads by day 32 to 39 [20]. The invasion of epithelial proliferations into the mesenchyme of the genital ridges stimulates local connective tissue to proliferate, forming the primitive sex cords [21].

Primordial germ cells differentiation into oogonia or spermatogonia is chromosome dependent, and in cattle, sexual differentiation of the gonads occurs in 40-day old embryos [19]. Oogonia and

spermatogonia show a high frequency of mitotic and transcriptional activity [22], and the maximum number of primordial germ cells is reached between the transition from mitosis to meiosis. In cattle, the maximum number of PGC has been estimated to be around 2 100 000 during fetal development, and this number is reduced through apoptosis to around 130 000 by birth [21]. Between days 130 and 170 of gestation, the number of germ cells present in the ovaries decreases by 80% [23]. In the ovary, groups of oogonia are formed by mitotic divisions of PGC, and they connect via intercellular cytoplasmic bridges [24]. Germ cell clusters or ovigerous cords are formed when these oogonia become surrounded by somatic cells of mesonephros stroma origin, and oogonia are still mitotically active diploid germ cells. It has been shown recently that granulosa and ovarian surface epithelial cells shares a common precursor, named as the gonadal ridge epithelial-like (GREL) cell [25]. The DNA in oogonia is then replicated, and the first meiotic division is entered and arrested at the diplotene stage, developing the primary oocytes [21]. These oocytes are larger and contain more cytoplasmic organelles than oogonia [24]. During ovarian development, the ovarian stroma increases and large ovigerous cords are segmented into smaller clusters of cells, resulting in the formation of primordial follicles containing an oocyte surrounded by one layer of flattened granulosa cells and a basal lamina [25]. Primordial follicles constitute the ovarian reserve of non-growing follicles, from which follicles are recruited for development throughout the female reproductive life.

Activation of primordial follicles to leave the resting pool is characterized by transition of granulosa cells from flattened to cuboidal, characterizing the primary follicle stage, but oocytes surrounded by both cell types have been observed *in vivo*. The transition to a secondary follicle stage is followed by additional layers of granulosa cells, from 2 to 6 or 7 layers and is characterized by the initial appearance of the zona pellucida around the oocyte, the cortical granules within the oocyte cytoplasm [26] and oocyte RNA synthesis [27]. The appearance of gap junctions between the oocyte and surrounding granulosa cells also occurs at the secondary stage [26]. The gradual development of an antral cavity at a follicular diameter of 200 μm is also observed during the secondary stage [28], which characterizes follicular development from the secondary to the preantral stages, while oocyte growth is initiated when follicles have at least 40 granulosa cells observed in a cross-section [29]. The secondary follicle stage to the tertiary stage transition is marked by the development of internal and external theca cell layers [30]. Oocytes enclosed in

early tertiary follicles are transcriptionally active, and transcription is observed until the oocyte reaches around 110 μm inside an antral follicle of 2 to 3 mm in diameter [27, 31].

In cattle, it was established that primordial, primary and secondary follicles first appear around 90, 140, and 210 days of gestation, suggesting that at least a 50-day gap exists between the first appearance of primordial versus primary follicles [32]. It was also shown that the majority of oocytes are at pre-diplotene stages between 91 and 140 days of gestation and that after day 140, the majority of the oocytes are resting at the diplotene stage. Studies *in vitro* have shown that gonadotropins are not essential for the initiation of follicle growth in bovine ovaries supporting the hypotheses that gonadotropins are not essential for follicular activation, nor granulosa cell proliferation. Although it has been shown that FSH does not affect the proportion of follicles that enter the growth phase [30], FSH increased proliferation of granulosa cells and follicle survival [33, 34]. Expression of FSH receptors has been detected in follicles with one and two layers of granulosa cells in cattle [35, 36], and FSH and activin have been shown to stimulate steroidogenesis in isolated bovine preantral follicles [37]. It seems that responsiveness of bovine follicles to FSH increased as the follicle develops since preantral follicles of around 150 to 220 μm produced more progesterone in response to FSH than follicles of 60 to 179 μm *in vitro* [38]. Interestingly, steroidogenesis in bovine fetal ovaries decreases around the time of follicle formation, remaining low until the first growing follicles appear [32]. It has been proposed that the mechanisms that regulate steroid production are established during fetal life since the effects of gonadotropins on steroid production in bovine fetal ovarian tissue are similar to the effects in theca and granulosa cells from bovine preovulatory follicles [39]. The latter study supported the hypothesis that FSH and LH target different cell types in the fetal ovaries, as in the adult ovary, resulting in the production of androgens and estrogens by different cell types (two-cell, two gonadotropin model; [40]).

1.2. Antral follicular development in prepubertal and pubertal cattle

In cattle, and most of the domestic species and in humans, antral follicular development can be divided in two distinct phases, starting at antrum formation, when the follicle is around 200 μm [28] to around 3 mm, followed by the second phase that encompasses cohort growth, dominant follicle selection and dominant follicle growth [41].

It has been established in cattle, that follicle growth occurs in a wave-like pattern [42]. A folliular wave is characterized by the synchronous development and growth of a group of follicles [43] and that a surge in circulating FSH concentrations precedes the emergence of a follicular wave [44]. Interestingly, it has been shown that the developmental pattern of follicles < 1 mm in diameter, observed through histological analysis of bovine ovaries on different days following ovulation, was also in a wave-like manner and that changes in the number of 0.5 to 1 mm follicles and 1 to 3 mm follicles indicates a synchronized system of recruitment for the successive waves [45]. Ovarian ultrasound examinations of prepubertal calves from 2 to 36 weeks of age showed that follicular development occurred in a wave-like pattern at all ages [2] and that an apparent increase in plasma FSH concentrations preceded each wave of follicular development, as was observed in sexually mature animals. Synthesis and release of FSH and LH from the anterior pituitary gland are controlled by a pulsatile pattern of GnRH synthesis and release. Lower GnRH pulse frequencies stimulate FSH synthesis and release, while higher pulse frequencies stimulate LH synthesis and release [46-48].

The number of follicular waves in each estrous cycle varies from 2 to 4 in cattle, resulting in different estrous cycle and interovulatory interval (i.e., the interval between ovulatory dominant follicle appearance) lengths. The follicular growth, static, and regression phases also will vary among animals, according to the number of waves in the individual estrous cycle. Follicular waves are characterized by the simultaneous growth of a group of small follicles of 1 to 3 mm in diameter (wave emergence, recruitment) [45], selection and growth of a dominant follicle, while subordinated follicles become atretic and regress, with either atresia or ovulation of the dominant follicle. Follicle-stimulating-hormone not only precedes wave emergence but also maintains follicular growth, cell proliferation, and steroidogenesis until dominant follicle selection [45, 49-51]. Dominant follicle selection in cattle is characterized by deviation in the growth of the largest follicle in the wave, while subordinate follicles undergo atresia, enhanced intrafollicular estradiol production and increased number of LH receptors in granulosa cells. These features characterize the follicle that is developmentally prepared to continue growing under declining FSH concentrations and able to respond to a transient increase in LH to become the dominant follicle [52]. Indeed, an increase in LH receptors in theca cells and the acquisition of LH receptors in granulosa cells are coincident with dominant follicle selection [35, 53], and a higher estradiol production was observed by the selected dominant follicle [54, 55].

Estradiol is produced in granulosa cells by the aromatization of androgens produced by theca cells from cholesterol [40]. Dominant ovulatory and anovulatory follicles produce high concentrations of estradiol, elevating the circulating concentrations of estradiol, and during the majority of the estrous cycle, estradiol acts via negative feedback to reduce GnRH pulse amplitude and inhibit LH release from the pituitary [48, 56, 57]. In contrast to the prepubertal period that is characterized by anovulatory waves of follicular growth, absence of ovulation and corpus luteum formation, progesterone produced during regular luteal phases in sexually mature cattle, controls LH pulse frequency and consequently follicular wave development [43, 56]. During the late luteal phase, progesterone concentrations decline after luteolysis and the high circulating estradiol concentrations produced by the dominant follicle cause a positive feedback in the hypothalamus and the pituitary to induce a GnRH and LH surge of sufficient amplitude to stimulate final follicular and oocyte maturation and ovulation [58].

In cattle, sexual maturity is characterized by the development of regular estrous cycle. The estrus cycle can be divided into four phases that are characterized by ovarian and hormonal changes leading up to the estrus phase. The estrus phase is characterized by basal circulating levels of progesterone in the absence of a corpus luteum, elevated circulating levels of estradiol produced by the pre-ovulatory dominant follicle and sexual receptivity. Its duration varies among individuals and breeds but has been reported to be less than 24 hours. Spontaneous ovulation occurs between 20 to 30 hours after the onset of estrus, followed by differentiation of granulosa and theca cells of the ovulated follicle into the corpus luteum, gradually increasing circulating progesterone concentrations during the metestrus phase. The metestrus is followed by the diestrus phase, in which a functional corpus luteum is present until luteolysis is triggered in the absence of pregnancy during the proestrus phase [59].

The prepubertal period, as well as seasonal anestrus which is observed in some species and the anestrus post-partum, is characterized by regular and periodic surges in plasma FSH secretion and the emergence of anovulatory follicle waves [43]. It has been reported that the maximum diameter of the dominant follicle increased between 2 to 34 weeks of age, in prepubertal calves while the maximum diameter of the largest subordinate follicle increased from 2 to 14 weeks of age, remaining steady thereafter. The total number of follicles in the wave also increased with age, but no differences were observed in the duration of the growing, static and regression phases of dominant follicle growth between 2 and 34 weeks of age; however, the time interval in which the

dominant follicle could be detected increased with age [2, 4]. Repeated low doses of purified bovine LH for 48 hours has been shown to induce an LH surge, ovulation, and corpus luteum formation in prepubertal heifers [60]. Indeed, episodic pattern of LH secretion following repeated injections of low doses of GnRH in 4-month-old calves confirmed the existence of a fully functional anterior pituitary [61, 62] and continuous infusion of GnRH consistently induced ovulation in 9-month-old prepubertal heifers [63], providing evidence that the prepubertal period is characterized by a lack of pituitary stimulation, rather than an inability to respond to GnRH since low frequency episodes characterize LH secretion in prepubertal calves. Therefore, anovulatory cycles in prepubertal calves are due solely to inadequate stimulation of follicle development. In ewes, simultaneous measurements of GnRH in the pituitary portal blood and of LH in the peripheral blood demonstrated that the preovulatory GnRH and LH surge occurs as a response to the endogenous increases in estradiol concentrations during the late follicular phase [64, 65].

No significant changes in circulating concentrations of LH and progesterone were detected in association with follicular waves in calves between 2 and 36 weeks of age, and at 34 weeks of age, respectively, although there was an increase in the basal concentrations of LH over time [2]. In fact, there is an observed marked increase in the total number of follicles and maximum follicular diameter in the first 20 weeks of age in calves that was coincident with a transient increase in the levels of gonadotropin secretion [2, 3, 66], suggesting that an initial stimulation of the hypothalamus-pituitary axis may be a critical step for maturation of the reproductive tract. Ultrasonographic examinations in calves have shown that there is a substantial growth of the reproductive tract during the first few months of life. Ovarian dimensions were shown to increase rapidly from 2 to 14 weeks of age, followed by a second increase at 34 weeks of age [4].

1.3. Puberty

The age of puberty in beef heifers, based in first ovulation and concentrations of progesterone ≥ 1 ng/mL has been observed to be between 52 and 67 weeks of age [1-4] and in cattle, puberty can be defined as the age at first ovulation, that is followed by regular estrous cycles [59]. No differences in the mean age and weight at puberty were found between spring and autumn-born heifers, but a broader range in age and weight at puberty was observed in autumn-born heifers [3]. A short ovulatory cycle of 7.7±0.2 days was observed in heifers at the onset of puberty, that resulted

in the formation of a short-lived corpus luteum and shortened interovulatory interval that was followed by a normal ovulatory cycle of 20.3±0.5 days [1].

In the prepubertal heifer, LH secretion is more sensitive to the negative feedback to estradiol than in the sexually mature heifer. It has been demonstrated that the onset of puberty is a result of decreased estradiol negative feedback on LH secretion due to a decrease in the number of receptors for estradiol in the hypothalamus and pituitary as puberty approaches [67, 68]. There is an increase in basal, mean, and pulse frequency of LH in the months preceding puberty, and this is accompanied by a decline in LH pulse amplitude [67, 68]. When these endocrine changes were associated with the pattern of follicular growth and development in heifers during the twelve weeks preceding first ovulation, no increase in the maximum diameter of the dominant, or largest subordinate follicle was observed. However, the interwave interval increased as heifers approached first ovulation, and the mean serum concentrations of estradiol and LH, and LH pulse frequency increased [1].

The treatment of prepubertal calves with a GnRH agonist to reduce gonadotropin secretion, at 8 and 12 weeks of age, the period in which a transient rise in gonadotropin secretion has been observed, did not affect the age and body weight at puberty. However, there was a suppression of ovarian follicular growth, a decrease in basal and mean concentration of FSH, and FSH and LH pulse amplitude. Basal LH concentrations increased, and a rebound in LH and FSH secretion was observed at 25 and 35 weeks of age, respectively, which may have prevented long-term effects on sexual development [69]. On the other hand, the treatment of prepubertal calves with gonadotropins (4 mg bFSH or 3 mg bLH; subcutaneously) every other day between 8 and 12 weeks of age, resulted in a reduction in maximum antral follicle size and the number of large sized follicles during the first 17 days of treatment. Treatment with FSH, but not with LH, caused a delay in the age of onset of puberty of almost 8 weeks when compared to non-treated control calves. Lower estradiol concentrations were observed at 25 and 35 weeks of age in FSH-treated calves and at 35 weeks of age in LH treated calves [70]. Limiting dietary energy during the prepubertal period also delayed the onset of puberty in heifers in comparison to control heifers fed a growing diet. A less pronounced increase in mean LH concentrations and no increase in LH pulse frequency was also observed in heifers given an energy deficient diet [71].

1.4. Ovarian Response to Gonadotropin Treatment in Calves

The gonadotropins FSH and LH are part of a family of glycoprotein hormones that are produced and secreted by the gonadotrophs in pituitary cells, to stimulate steroidogenesis and gametogenesis in the gonads. Follicular growth is regulated by circulating concentrations of FSH that precedes each wave of follicular development. By maintaining circulating concentrations of FSH elevated, dominant follicle selection is prevented, and follicles supposed to undergo regression and atresia are rescued, allowing the growth of multiple follicles to ovulatory size [72]. Follicle-stimulating hormone (FSH) acts through specific G-protein coupled receptors (GPCRs) on target cells surfaces. In females, FSH receptor is expressed in granulosa cells of ovarian follicles [73]. Glycoprotein hormones are polypeptide units (heterodimers) consisting of non-covalently associated α and β subunits. The α-chain is common among the glycoprotein hormones, while the β-chain provides functional specificity within a specie [74, 75].

It was demonstrated several decades ago that calf ovaries were responsive to the administration of gonadotropins almost from birth, and since then attempts to superovulate calves have shown that although ovulation was induced and fertilization was achieved, both were very low when compared to sexually mature animals [76-78]. It was shown that circulating LH concentrations after superstimulation in the prepubertal calf exhibited a rate of LH release that was similar to that of sexually mature animals [79] and that a positive relationship was observed between plasma estradiol concentrations during treatments and the number of follicles that ovulated after treatment [80]. A great individual variation in the ability of the ovary to respond to the treatment, which could not only be attributed to the animal age was detected [76-78, 81, 82]. Steroid hormone levels in the superovulated calves were observed to be much higher than observed in sexually mature animals suggesting adverse consequences for oocyte maturation, fertilization, and implantation. Treatment of prepubertal calves with LH after superstimulation with exogenous FSH increased ovulation, fertilization, and embryo recovery rates in a different study [83].

Currently, purified pituitary extracts have been used to increase the number and size of follicles available for aspiration, and consequently the number of recovered oocytes to be used for *in vitro* embryo production. It has been demonstrated that oocytes recovered from medium to large sized follicles have higher developmental competence *in vitro* than oocytes from smaller follicles [15, 84, 85], therefore, manipulation of the follicular growth and development has been used as a

strategy to improve *in vitro* embryo production [13, 86]; although differences in the gonadotropin regimens used in calves have made comparisons between studies very difficult.

The treatment with FSH increased the number of ≥ 6 mm follicles by more than 10 times in 16-week-old calves when compared to 12-week-old non-treated calves, resulting in 12 times more follicles that were aspirated and 18 times more oocytes recovered. Similar results were also obtained in 6-week-old calves [17]. In prepubertal calves, 3 days of FSH treatment resulted in a more significant proportion of follicles > 5 mm than no treatment [87] and no additional effects on the number and size of follicles were observed when FSH was given at higher frequency intervals (every 8 hours, instead of 12 hours) in 2 to 6-month-old calves submitted to repeated laparoscopy for oocyte collection. However, replacing FSH by eCG towards the end of the treatment (i.e., 36 hours before oocyte collection) resulted in higher rates of development to the blastocyst stage [85]. The numbers of visualized follicles immediately before oocyte collection by laparoscopy and the numbers of recovered oocytes were greater in 3 to 4-month-old calves that were given two days of FSH treatment than in calves and sexually mature heifers that were not given FSH treatment [88]. The use of a GnRH antagonist for 13 days in 3-month-old calves resulted in significant increase in the mean number of > 5.4 mm follicles compared to control calves and when FSH was given for the last 3 days of GnRH antagonist treatment, a greater number of follicles ≥ 3 mm and reduced rate of atresia of follicles between 3 and 5.4 mm was observed [89]. By investigating the effect of GnRH given after gonadotropin treatment in 5-month-old calves, it was observed a higher number of > 2 mm follicles in GnRH treated animals; although, there were no differences in the number of oocytes recovered by ultrasound-guided transvaginal aspiration and GnRH treatment was associated with reduced recovery rate [90].

It has been demonstrated in calves between 6 and 7 months of age that the characteristics of an induced wave were not different from those of a spontaneous wave, and that gonadotropin treatment initiated at the time of wave emergence resulted in the growth of more follicles than treatment initiated later in the wave. The total number of aspirated follicles and recovered oocytes were greater in calves that were given exogenous FSH treatment after induced wave emergence than in calves treated at random stages of follicular growth [91]. Accordingly, the use of estradiol and progesterone to synchronize follicle wave emergence 4 days before FSH treatment resulted in a more homogeneous group of 3 to 7 mm follicles available for aspiration, an increased proportion of usable oocytes and numerically higher rates of cleavage and blastocyst production [92]. In 2-

and 4-week-old calves, the number of follicles available for aspiration doubled after FSH treatment, and there was a high correlation between the number of follicles pre and post-FSH treatment [93]. It was suggested that both age and hormonal treatment influenced the acquisition of developmental competence in prepubertal calf oocytes since a higher proportion of high quality oocytes were recovered from gonadotropin-treated calves at 5 and 7 months of age, but at 9 and 11 months no such improvement was observed [94].

It has been shown in a commercial setting that, in terms of number of transferable embryos recovered per donor, the highest success rate was obtained in heifers at or more than 10 months of age; the number of viable embryos was lower in heifers between 7 to 10 months of age, and the number of degenerated embryos was higher in heifers < 10 months of age than in heifers ≥ 14 months of age [95]. It was shown that morula and blastocyst formation was significantly lower with oocytes obtained from 5 to 10 months old calves than from sexually mature heifers, and that different periods of coasting (i.e., FSH withdrawal; 19, 30 or 43 hours) after exogenous FSH treatment did not affect embryo development. Coasting periods after FSH treatment have been proposed as a means of collecting oocytes at the optimal time of follicular differentiation, and its use has been associated with improved oocyte competence [86, 96, 97]. The authors concluded that a larger number of oocytes per cycle could be recovered from animals younger than 9 months of age, but that the optimal response to superstimulation and oocyte recovery was observed at the peripubertal period, between 11 and 12 months of age [11]. On the other hand, similar rates of blastocyst production were observed in heifers between 7 and 12 months of age and sexually mature animals that were submitted to same gonadotropin treatment over successive cycles of oocyte collection was reported in another study [98]; however, lower pregnancy rates occurred after the transfer of cryopreserved *in vitro*-produced embryos from calves. A correlation between the number of aspirated follicles before and after puberty was also observed, and it was suggested that this individual characteristic was established before puberty.

1.5. Meiotic and Developmental Competence of Prepubertal Calf Oocytes

Meiotic competence of an oocyte is related to its ability to resume and complete meiosis. It has been shown that the competence of the bovine oocyte to undergo meiotic maturation is gradually achieved at a diameter of 100 to 110 μm [31], corresponding to a follicle of approximately 3 mm;

however, further ultrastructural modifications are still needed for the oocyte to acquire full developmental competence during follicular growth within the dominant follicle. During the final stages of oocyte maturation, these ultrastructural modifications are characterized by the continued development of the lipid store in the cytoplasm, reduction in the Golgi compartment and the alignment of the cortical granules in the periphery of the ooplasm, forming the structural background against polyspermy [99, 100]. In agreement with these observations, lack of embryonic development was observed in oocytes recovered from 1 to 2 mm follicles [101], and lower rates of blastocyst formation were observed in oocytes from 2 to 6 mm than from large-sized follicles [15, 84]. The reduced developmental capacity of prepubertal calf oocytes has been attributed to several independent factors, which reflects the sexual immaturity and related endocrine profile of the animal [6]. In one study, calf oocytes did not reach the morula stage *in vitro*, and their protein pattern was similar to that of low-quality cow oocytes before *in vitro* maturation [102]. However, in another study protein patterns between calf and cow oocytes did not differ, but blastocyst rates from calf oocytes were half those observed from cow oocytes [8]. The authors attributed these inconsistencies to differences in the oocyte population and suggested that developmental competence of oocytes cannot be attributed uniquely to the protein profile or protein synthesis since the microenvironment in which the oocyte develops may play a role. Indeed, differences in the protein profile of the follicular fluid of calf and cow follicles have been reported [9]. However, neither cow nor calf follicular fluid supplementation during oocyte maturation improved embryo development of calf oocytes, despite being beneficial to adult oocytes. The authors concluded that even if follicular fluid did provide factors that stimulate the acquisition of competence, calf oocytes were not able to respond [9].

It has also been suggested that oocytes recovered from untreated prepubertal calves have lower developmental competence than cow oocytes and are therefore less suitable for commercial *in vitro* embryo production [103]. Although a greater rate of embryo development was observed with oocytes recovered from follicles > 8 mm than from follicles between 3 to 8 mm in calves treated with FSH, a relationship between oocyte competence to form a blastocyst and the size of the follicle from which it originates probably also exists [6, 96]. Although calf oocytes were shown to resume meiosis and arrest at metaphase II stage at rates similar to that of adult oocytes [7], a delay in the kinetics of nuclear maturation of calf oocytes when compared to cow oocytes has been observed [10]. A comparison of oocytes from 3 to 4-month-old calves and cow revealed no differences in

the *in vitro* fertilization and cleavage rates, but a reduced proportion of cleaved calf embryos reached the blastocyst stage. It is possible that calf oocytes arrest at the time of embryonic genome activation, but even after blastocyst formation, a limited competence to develop further following transfer to recipients was observed with calf-derived embryos, which reasons required further investigation (i.e., lack of embryo signalling molecules for implantation).

Nuclear transfer has been used to compare developmental capabilities between calf and cow oocytes, and no differences were observed in fusion and cleavage rates, although, blastocyst rate was significantly lower in calf oocytes after the nuclear transfer (9 vs. 21%) as well as after *in vitro* fertilization (12 vs. 32%; [104]. The authors suggested that a deficiency at the cytoplasmic level could explain the low developmental competence of calf oocytes. Indeed, after *in vitro* maturation, the activity of MPF (maturation-promoting factor), MAPK (mitogen-activated protein kinase) and the relative amount of IP3R (inositol 1,4,5-triphosphate receptor), key biochemical components of fertilization, cleavage, and development, were substantially lower in the calf than cow oocytes [105]. Calf oocytes also had a reduced metabolism of glucose, pyruvate, and glutamine during the first few hours of oocyte maturation when compared to cow oocytes [106, 107]. A higher mitochondrial population was observed in cow oocytes, as an indicator of higher oxidative metabolism, while calf oocytes showed a greater number of lipid droplets in cumulus cells, an indication of greater metabolism exchange between these two cell types, likely due to oocyte immaturity [108].

Incomplete or impaired cytoplasmic maturation of calf oocytes has also been suggested by the observations of a delay in organelle migration and redistribution following *in vitro* maturation, and a higher percentage of abnormal chromatin and microtubule configurations following *in vitro* fertilization than in cow oocytes [7]. Gene expression during oocyte maturation, early embryo development, and embryonic genome activation depend upon mainly on the RNA that was accumulated during oocyte growth. Differences in the maternal RNA store and its regulation machinery during oocyte maturation was observed between cow and calf oocytes [109, 110], suggesting that calf oocyte transcriptome may be deficient and responsible for the low developmental competence that has been observed. DNA methylation of three developmentally important, nonimprinted genes (SLC2A1 – solute carrier family 2 member 1, PRDX1 – peroxiredoxin 1 and ZAR1 – zygotic arrest gene 1) and two satellite sequences (BTS - bovine testis satellite I and BTαS – Bos Taurus alpha satellite I) were analyzed in cow and calf oocytes after

treatment with FSH and/or IGF-I before and after *in vitro* maturation. Results indicated that neither gene-specific expression nor methylation levels were affected in the different treatment or age groups. Results appeared to suggest that the treatment of calves with exogenous FSH and intraovarian application of IGF-I before oocyte collection yielded oocytes with an epigenetic status similar to untreated adult oocytes [111].

Results of comparisons between cow and calf embryos revealed no differences in the total number of cells or the inner cell mass and trophectoderm cell distribution [10, 112] but a higher rate of pregnancy loss after the transfer of *in vitro* produced embryos from calf oocytes was observed [10]. Calf embryos were characterized by a higher rate of developmental arrest before the 9-cell stage and a longer duration of the fourth cell cycle, that precedes embryonic genome activation [112]. Nutrient uptake at the blastocyst stage was equivalent between cow and calf embryos, despite differences in glucose and pyruvate uptake at earlier stages of embryo development [113]. Cow and calf 8 to 16 cell and blastocyst stage embryos showed different gene expression profiles, suggesting that small differences at the molecular level may have a profound impact on the developmental capacity of the calf oocyte and subsequent embryo [114]. Another study showed different expression profiles of three metabolic pathways (mTOR and PPAR signaling pathway and NRF2-mediated oxidative stress response pathway) between cow and calf embryos, suggesting that current culture conditions might not be optimal for the development of calf oocytes and embryos [115]. Indeed, maturation medium enrichment with hormones (human chorionic gonadotropin - hCG, equine chorionic gonadotropin - eCG and insulin), growth factors (fibroblast growth factor - FGF, insulin-like growth factor I - IGF-I and epidermal growth factor - EGF), energy substrates (glutamine and pyruvate) and precursors of glutathione synthesis (cysteine and β-mercaptoethanol) dramatically improved the developmental competence of calf oocytes, with a substantial increase of 38% in the blastocyst rate when compared to basal media, with only EGF supplementation [116]. Supplementation of *in vitro* maturation medium of calf oocytes for the first 12 hours of maturation with insulin-transferrin-selenium plus L-ascorbic acid resulted in oocytes with a higher proportion of peripherally distributed cortical granules than control oocytes, and higher blastocyst rates than control medium without supplementation [117]. The addition of leptin in the *in vitro* maturation medium was also investigated, without an increase in the developmental competence of 9-month-old calf oocytes [118]. It was also shown that the addition

of L-carnitine and/or resveratrol to the *in vitro* maturation medium did not increase the embryo developmental potential of either fresh or vitrified calf oocytes [119].

In order to provide oocytes sufficient time to complete cytoplasmic maturation *in vitro*, calf oocytes were maintained in germinal vesicle (GV) stage for 24 hours, by supplementing the *in vitro* maturation medium with roscovitine, an inhibitor of maturation promoting factor (MPF) kinase activity. Although embryo production was not increased with this pre-treatment when compared to controls, maintaining calf oocytes in artificial meiotic arrest did not compromise subsequent developmental competence [120]. However, in a different study pre-treatment of calf oocytes with roscovitine had a negative impact on their development potential (12 vs. 3% of blastocyst rate after 7 days of culture for control and treatment, respectively; [116]. The increase of intra-oocyte cAMP (cyclic adenosine monophosphate) levels during maturation delayed meiotic progression but did not increase developmental rates of either cow or calf oocytes. Interestingly, it was shown that oocytes from 6 to 9-month-old prepubertal animals, appeared to have a functional cAMP system, similar to adult oocytes [121]. Cyclic adenosine monophosphate is a second messenger involved in various cellular functions, and in mammalian oocytes, high levels of cAMP maintain meiotic arrest by the inactivation of MPF.

Intraovarian injection of IGF-I 48 hours prior to oocyte collection, followed by FSH treatment in prepubertal calves resulted in blastocyst rates similar to control cows (28% vs. 25%), but higher than control calves (11%) and rbST- (recombinant bovine somatotropin) treated calves (16%) [122]. Although a positive effect of intraovarian injection of IGF-I on the developmental competence of calf oocytes was not observed in a different study, blastocyst rates were low for IGF-I treated and untreated calves, as well as for treated heifers when compared to untreated heifers and cows [123]. The authors suggested that differences in the medium in which the IGF-I was delivered might be the cause of such discrepancies between studies. It was also shown by the latter, that the proportion of apoptotic cells in blastocysts decrease with age, while the developmental capacity of oocytes increased. The follicular fluid of prepubertal calves had lower levels of glucose, and fatty acids than the follicular fluid of sexually mature cows and calf oocytes were characterized by lower diameter and lipid droplet numbers, which apparently determined their reduced quality and limited maturation potential [124].

It can be assumed from the presented studies that the low developmental competence of prepubertal calf oocytes seems to be a consequence of the sexual immaturity of the animal and that

artificial manipulation of follicular growth may provide a suitable hormonal environment for the prepubertal oocyte to acquire full meiotic and developmental competence.

CHAPTER 2

2. GENERAL OBJECTIVES AND HYPOTHESES

The overall objective was to determine the effects of different durations of exogenous FSH treatment on the ovarian response, follicular maturation, ovulation and oocyte developmental competence in prepubertal calves. The overall hypothesis was that a longer duration of FSH treatment, that matches the interwave interval in 6-month-old calves would be optimal for follicular growth and oocyte developmental competence.

2.1. Specific Objectives

1. To evaluate the ovarian response to gonadotropin treatment based on cumulative dose (200 mg vs. 350 mg FSH) and duration (4 versus 7 days) of treatment.
2. To determine:
    a. The repeatability of AFC at wave emergence;
    b. The relationship between AFC and ovarian response.
3. To determine the effect of duration of gonadotropin treatment in calves with high and low AFC.
4. To investigate the relationship between AFC and plasma concentrations of AMH and FSH at wave emergence.
5. To compare the effect of age and duration of exogenous FSH treatment on the ovarian response of prepubertal calves at 4 and at 7 months of age and sexually mature heifers.
6. To evaluate three methods of controlling endogenous LH release and the prevention of ovulations during 7 days of exogenous FSH treatment.
7. To compare the developmental competence of *in vitro* versus *in vivo* matured oocytes following 4 versus 7 days of exogenous FSH treatment.
8. To compare 4, 6 and 7 days of exogenous FSH treatment on ovarian response and oocyte developmental competence.

2.2. Specific Hypotheses

1. Both a higher cumulative dose and a longer duration of FSH treatment will result in a greater number of large follicles by the end of treatment and a greater number of ovulations in response to exogenous LH treatment;
2. The AFC at wave emergence is repeatable within individuals and is predictive of the ovarian response following exogenous FSH treatment;
3. A longer duration of FSH treatment (4 versus 7 days) will result in a greater number of follicles available for aspiration and higher COC collection efficiency;
4. The total number of follicles ≥ 1 mm at wave emergence will be correlated positively with plasma AMH concentrations and negatively with plasma FSH concentrations;
5. Regardless of animal age, a greater ovarian response will be observed after 7 than after 4 days of gonadotropin treatment;
6. Controlling plasma concentrations of LH during FSH treatment by maintaining higher circulating levels of progesterone or by GnRH antagonist treatment will prevent ovulations during treatment;
7. Oocyte developmental competence will be increased by 7 days of follicular growth under FSH support and *in vivo* oocyte maturation;
8. Ovarian follicular response and oocyte developmental competence will be increased by 7 days of follicular growth under exogenous FSH compared to 4 or 6 days.

CHAPTER 3

3. EFFECT OF DOSE AND DURATION OF FSH TREATMENT ON OVARIAN RESPONSE IN PREPUBERTAL CALVES

Krause ART, Dias FCF, Adams GP, Mapletoft RJ, Singh J

Relationship of this study to the dissertation

Bovine ovarian follicles are responsive to exogenous gonadotropins since the first few days of life. The treatment of animals of such a young age with exogenous FSH may help to decrease the generation interval and accelerate the rate of genetic gain in the bovine specie, by the production of embryos *in vitro* and transfer to healthy recipients. Luteinizing hormone (LH) secretion in prepubertal calves is more sensitive to the estradiol negative feedback than in sexually mature animals, therefore, exogenous gonadotropin treatment of sexually immature animals also provides a model to study the interrelationship of the hypothalamus-pituitary-gonadal axis during the prepubertal period. The literature provides no consensus regarding dose or duration of FSH treatment needed to sustain follicular growth during superstimulation in prepubertal cattle. Therefore, the first study aimed to evaluate the ovarian response in prepubertal calves resulting from differences in the dose and duration of FSH treatment. The optimal ovarian response among the doses tested in this study was used for all future studies.

Authors' contribution: ARTK, FCFD, GPA, RJM and JS conceived and designed the experiment. ARTK conducted and coordinated the experiment, collected and analyzed the data. ARTK wrote the draft of the manuscript. FCFD, GPA, RJM and JS reviewed the manuscript.

3.1. Abstract

The objective of the study was to evaluate the ovarian response in prepubertal calves resulting from differences in the dose and duration of FSH treatment. Twenty-four calves (173.2±1.5 days of age) were selected from a larger group (n=51) based on antral follicle counts at wave emergence to represent the medium AFC group. Follicular ablation was performed to induce wave emergence and calves were assigned to treatment with a cumulative dose of 200 vs. 350 mg FSH over a 4-day vs. 7-day period (2x2 factorial). Calves were given 8 im treatments (at 12-hour intervals) of 25 mg (n=6) or 44 mg (n=6) of pFSH in the 4-day groups and 14 im treatments of 14 mg (n=6) or 25 mg (n=6) of pFSH in the 7-day groups starting 36 hours after follicular ablation (i.e., day of wave emergence, Day 0). Calves were given 12.5 mg of pLH im 12 hours after the last FSH treatment. The ovarian response was evaluated by ultrasound examinations performed every 48 hours starting at 24 hours after follicular ablation until LH treatment, and every 12 hours thereafter for 3 days. On Day 4, calves given 14 mg of FSH per treatment had fewer follicles ≥ 3 mm (15.1±1.9; P=0.04) than those given 25 mg (27.9±3.3). Spontaneous ovulations were observed in all groups after Day 4. At the end of treatment (24 hours post-LH), the number of follicles ≥ 9 mm was greater in the 350 mg vs. 200 mg FSH groups including (13.5±1.8 vs. 8.8±1.3; P=0.02) or excluding (8.9±2.0 vs. 2.5±0.8; P=0.01) the number of ovulated follicles. Including the follicles that ovulated, the number of follicles ≥ 9 mm was greater in the 7-day vs. 4-day treatment groups (13.3±1.8 vs. 9.0±1.3; P=0.03). The number of spontaneous ovulations was greater in the 7-day vs. 4-day groups (5.6±1.3 vs. 1.7±1.0; P=0.02) as was the total number of ovulations (9.7±0.9 vs. 6.9±1.0; P=0.05). More spontaneous ovulations were detected in the 200 mg vs. 350 mg FSH groups (5.1±1.5 vs. 1.9±0.6; P=0.05). The number of ovulations in response to exogenous LH did not differ among treatments (overall 5.7±0.8). In summary, a dose of 25 mg of FSH per treatment given twice daily for 7 days resulted in a greater ovarian response than other treatments in prepubertal calves.

Keywords: calves, gonadotropins, ovarian stimulation, follicular growth, ovulation.

3.2. Introduction

More than 80 years ago, antral follicle development was discovered in calves as young as one week of age [125]. In a series of studies done over 25 years ago, antral follicle development was characterized as wave-like in prepubertal calves from 2 weeks of age to puberty [1, 2, 126]. An increase in the number and size of antral follicles with calf age was also noticed in the original study [125], and was confirmed in a later study in which ovarian weight was found to increase four times more rapidly than body weight from birth to 5 months of age, remain static from 5 to 8 months, and increase commensurate with body weight from 8 to 12 months of age [126]. The number of antral follicles detected by ultrasonography reached a peak at 4 months of age, decreased from 5 to 8 months, and remained constant thereafter [2, 43, 126]. Since the discovery of gonadotropin responsiveness in calves [76], attempts to superovulate prepubertal calves have demonstrated wide individual variation in ovarian response regardless of animal age, and low ovulation and fertilization rates [77, 78, 81, 82].

Given the variability in the number of antral follicles among calves at any given time (i.e., stage of the follicular wave, calf age), determining the ovarian status of each animal before treatment may be predictive of the ovarian response [127]. In adult cattle, the number of antral follicles growing in each follicular wave and the wave pattern is variable among individuals but repeatable within an individual [128-130], and in two-month-old calves, the number of follicles before and after gonadotropin treatment was highly correlated [93]. While the number of follicles inherent at wave emergence accounts for most of the variability in ovarian response to gonadotropin treatment [129], the superstimulatory response is also influenced by the dose and duration of exogenous FSH support [13, 87, 131-133].

The effects of dose and duration of gonadotropin treatment have not been critically evaluated in prepubertal animals. Calves have lower body mass and blood volume than adults, but it is not known whether a lower dose of FSH will elicit a similar follicular response in calves. Three days of FSH treatment given to 6-week-old calves increased the number of follicles ≥ 6 mm almost ten times [17], and increased the proportion of follicles > 5 mm compared to 1.5 days of treatment or no treatment in 2- to 6-month old calves [87]. Histological analysis of the ovaries showed that 3 days of FSH treatment increased the number of follicles between 3 to 5.4 mm and > 5.4 mm in 3-month-old calves [89]. Even 2 days of FSH treatment resulted in a doubling of the number of

follicles visualized laparoscopically in 3 to 4-month-old calves [88]. Gonadotropin treatment in 7-month old calves initiated at the time of wave emergence resulted in the growth of more follicles than treatment initiated later in the wave [91]. The interwave interval in 6-month-old calves has been reported to be an average of 7 days, with a growing, static, and regression phase of 3.5, 4.4, and 4.4 days, respectively [2]. We propose that a longer duration of FSH treatment that matches the interwave interval in prepubertal calves, will be optimal for follicular growth and ovulatory potential in calves.

The objective of this study was to evaluate the ovarian response to gonadotropin treatment based on cumulative dose (200 mg versus 350 mg FSH) and duration (4 days versus 7 days) of treatment. We hypothesize that 1) a higher FSH dose per treatment will result in a greater number of follicles and a greater mean follicle diameter than a lower dose and 2) both a higher cumulative dose and a longer duration of FSH treatment will result in a greater number of large follicles by the end of treatment and a greater number of ovulations in response to exogenous LH treatment.

3.3. Material and Methods

3.3.1. Animals

Animal procedures were approved by the University of Saskatchewan's Animal Care Committee in accordance with the Canadian Council on Animal Care. The experiment was conducted between September and October at the Goodale Research Farm (52° North and 106° West), University of Saskatchewan.

Calves (n=24) were selected in September from a group of spring-born Hereford crossbred female calves (n=51; born between March 31 and May 3). The selection was based on the number of follicles ≥ 1 mm detected at the time of wave emergence (antral follicle count) to minimize the variation among animals assigned to treatment groups. Calves were 5 months old and not yet weaned; cow-calf pairs were temporarily separated for each handling. Follicular wave emergence was synchronized among calves by transvaginal ultrasound-guided ablation of follicles ≥ 5 mm in both ovaries, as described previously [91]. Briefly, caudal epidural anesthesia was induced with 2 to 3 mL of lidocaine (2% lidocaine HCl and epinephrine USP; Bimeda-MTC Animal Health Inc., Lavaltrie, Québec, Canada) and the perineal region was washed with surgical scrub. Sterile lubricant was applied to the custom-modified needle guide with the transducer before it was guided into the vagina. The other hand of the operator was placed into the rectum to manipulate the ovary

and position it against the vaginal wall. A disposable 18-gauge needle connected to an aspiration line (WTA, Cravinhos, SP, Brazil) was placed in the needle guide and advanced through the vaginal fornix and into the follicular antrum. Follicular fluid was aspirated by syringe and curettage of the follicle wall was performed by slowly rotating the needle in the follicle during aspiration. Follicle ablation was defined as evacuation and collapse of all follicles ≥ 5 mm in diameter in both ovaries. Transrectal ultrasonography was performed 1.5 days later (i.e., expected day of wave emergence) [134] with a 7.5-MHz linear-array transducer (Esaote MyLab Five, Canadian Veterinary Imaging, Georgetown, Ontario, Canada). The total number of follicles ≥ 1 mm per calf was determined from video recordings (i.e., follicle numbers from both ovaries were summed). The calves (n=51) were categorized according to the number of follicles (range: 12 to 53 follicles), and 24 of those in the center quartiles (25±0.7 follicles, range 20 to 32 follicles) were selected for inclusion in the study. Ovulation was defined as the number of follicles ≥ 8 mm that disappeared between successive ultrasound examinations.

3.3.2. Experimental Design

At least 7 days after the first ablation, a second transvaginal ultrasound-guided follicle ablation (Day -1) was performed on the selected calves (n=24; 200.6±5.2 Kg body weight, 173.2±1.5 days of age) to induce the emergence of a new follicular wave (Day 0). Using a 2x2 factorial design, calves were assigned randomly to one of four groups (n=6 per group) to determine the effects of duration (4 vs. 7 days) and cumulative dose of FSH (200 mg vs. 350 mg). Superstimulatory gonadotropin treatment consisted of 200 mg or 350 mg of pFSH (Foltropin-V, Vetoquinol, Lavaltrie, Quebec, Canada) divided and given intramuscularly at 12-hour intervals for 4 days (8 equal doses) or 7 days (14 equal doses; Fig. 3.1). Treatments were initiated 36 hours after follicle ablation. FSH doses were based in similar studies in sexually mature animals [13, 133].

Calves were given 12.5 mg pLH (Lutropin-V, Vetoquinol, Lavaltrie, Canada) im 12 hours after the last FSH treatment to induce follicle and oocyte maturation, and ovulation. Transrectal ovarian ultrasonography was performed 24 hours after follicular ablation and then every-other-day until LH treatment when ultrasound examinations were performed twice daily for the next 72 hours. Cine-loops of ovarian images were recorded at each examination and the number of small (3 to 5 mm), medium (6 to 8 mm) and large (≥ 9 mm) follicles and the number of corpora lutea were counted. In order to compare follicular size among treatments, the mean diameter of the five largest follicles per pair of ovaries was used to calculate the mean follicular diameter.

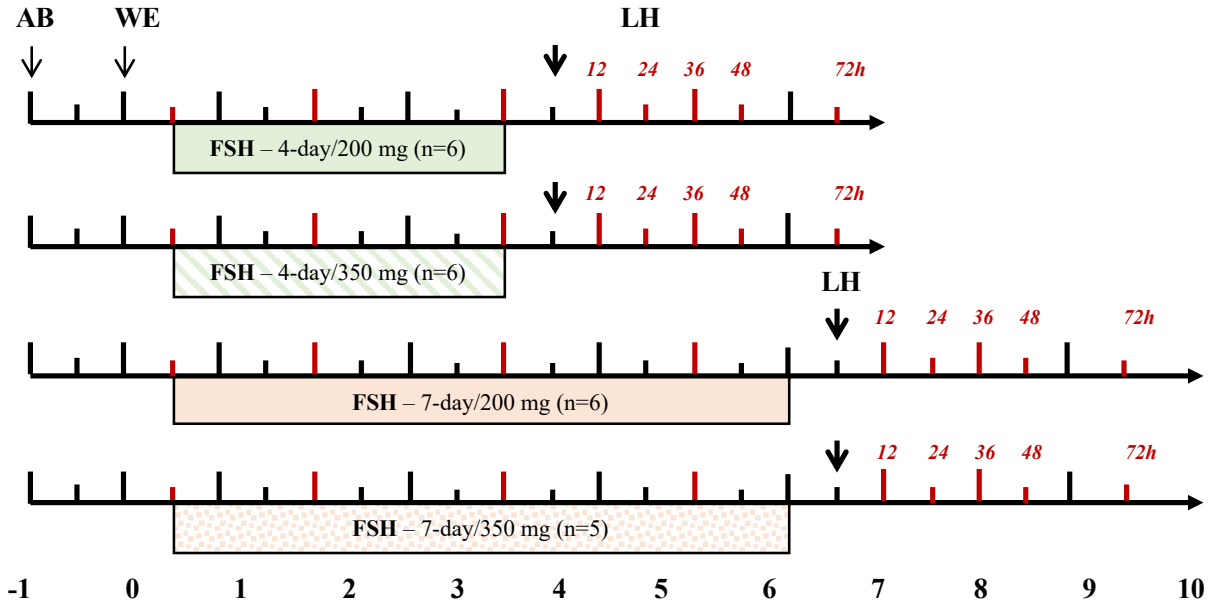

Fig. 3.1. Experimental Design. Transvaginal ultrasound-guided follicular ablation (AB) of follicles ≥ 5 mm was performed (n=24 calves) to synchronize the emergence of a new follicular wave (WE). Exogenous FSH treatment were initiated 1.5 days after ablation and consisted of pFSH given intramuscularly at 12-hour intervals over 4 days (4-day groups) or 7 days (7-day groups) with a cumulative dose of 200 mg or 350 mg. Calves were given 12.5 mg of pLH im 12 hours after the last FSH treatment. Ovarian ultrasound examinations were performed at 24 hours after follicular ablation (Day 0), on Day 4, Day 6 (7-day groups) and at 12, 24, 36, 48 and 72 hours after LH treatment (red labels).

3.3.3. Statistical Analysis

Statistical analyses were performed using SAS Enterprise Guide 6.1 (SAS 9.4; SAS Institute Inc., Cary, NY, USA). Changes in the number of follicles at the end of treatment were compared at each time point for the treatment effect (Dose and Duration), follicle size (small, medium and large) and their interactions using a general linear model (GLM) factorial design analysis. Factorial analysis was also used to evaluate the effect of treatments on the number of ovulations.

To assess the ovarian response based on the FSH dose-rate, data on Day 4 were arranged into 3 groups to compare rates of 14 mg per treatment (7-day group/200 mg), 25 mg per treatment

(4-day group/200 mg + 7-day group/350 mg), and 44 mg per treatment (4-day group/350 mg) using one-way analysis of variance (number of follicles ≥ 3 mm, mean diameter of 5 largest follicles) or by 3x3 factorial analysis of variance (number of follicles in 3 size categories versus treatments). Similarly, data on Day 6 were arranged into 2 groups to compare rates of 14 mg per treatment (7-day group/200 mg) and 25 mg per treatment (7-day group/350 mg) using T-tests or 2x3 factorial analysis of variance.

If the main effects (dose, duration, follicle size) or their interactions were significant ($P \leq 0.05$), multiple comparisons were performed by Tukey's post-hoc test. Log transformation was used for data that did not have equal variances and normal distribution of the residuals. The mean and standard error of the mean (mean±SEM) of raw data is reported in tables and figures to describe all response variables.

3.4. Results

By design, calves (n=51) were classified based on antral follicle count after the first follicular ablation, and 24 calves in the center quartiles were selected for gonadotropin treatment. The age at the first follicular ablation and antral follicle count was 161±1.7 days (n=24), and at the second ablation (pre-gonadotropin treatment) it was 173.2±1.5 days. The number of ≥ 1 mm diameter follicles at 24 hours after the second ablation was 26.4±1.6 follicles (range 14 to 46) and there was no difference between treatment groups. One calf in the 7-day group/350 mg treatment became sick on the first day of the experiment and was removed from the study.

3.4.1. Effect of cumulative dose and duration of the FSH treatment on follicular response

The number of follicles in small (3 – 5 mm), medium (6 – 8 mm) and large (≥ 9 mm) size categories were compared among groups at 24 hours and at 48 hours after pLH treatment (i.e., 36 and 60 hours after the last FSH treatment, respectively; Fig. 3.2). At 24 hours after pLH treatment, the number of small follicles was greater in the 4-day group/200 mg (18.7±4) than in the other three groups (combined data 6.6±0.9; Duration*Dose interaction P=0.04). At 48 hours after pLH treatment, the 4-day groups (combined data 9.7±1.9) had a greater number of small follicles (Duration P=0.05) than the 7-day groups (5.1±0.9).

At 24 hours after pLH treatment, the number of medium size follicles was higher in the 7-day group given 350 mg than in the 7-day group given 200 mg (11.8±2.5 vs. 5.3±1.2;

Duration*Dose interaction P=0.05). At 48 hours, groups given a cumulative dose of 350 mg FSH tended to have a greater number of medium size follicles than the 200 mg groups (9.4±1.2 versus 6.8±1.9, respectively; P=0.06).

At 24 hours after pLH treatment, the number of large sized follicles (≥ 9 mm; excluding or including the number of ovulated follicles) was greater in the 350 mg FSH groups than in the 200 mg FSH groups (8.9±2.0 vs. 2.5±0.8 and 13.5±1.8 vs. 8.8±1.3 respectively, $P \leq 0.02$) and at 48 hours (8.5±2.1 vs. 2.7±0.6 and 16.5±2.5 vs. 11.2±1.0; $P \leq 0.04$). The number of large follicles 24 hours after pLH treatment, including ovulated follicles, was greater (P=0.03) in the 7-day groups (13.3±1.8) than the 4-day groups (9.0±1.3).

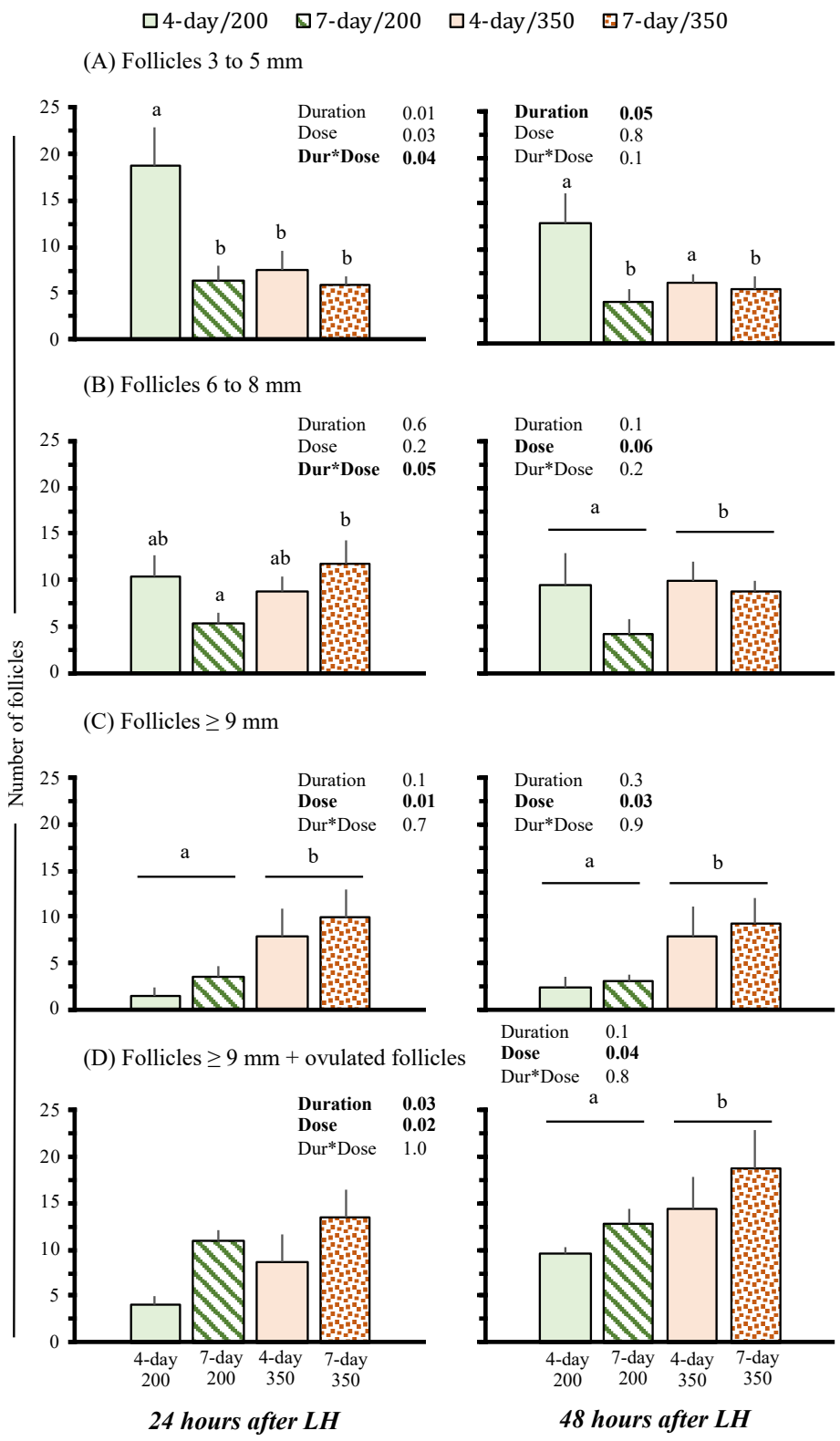
4-day/200
7-day/200
4-day/350
7-day/350
(A) Follicles 3 to 5 mm
Duration 0.01
Dose 0.03
Dur*Dose 0.04
Duration 0.05
Dose 0.8
Dur*Dose 0.1
(B) Follicles 6 to 8 mm
Duration 0.6
Dose 0.2
Dur*Dose 0.05
Duration 0.1
Dose 0.06
Dur*Dose 0.2
(C) Follicles ≥ 9 mm
Duration 0.1
Dose 0.01
Dur*Dose 0.7
Duration 0.3
Dose 0.03
Dur*Dose 0.9
(D) Follicles ≥ 9 mm + ovulated follicles
Duration 0.03
Dose 0.02
Dur*Dose 1.0
Duration 0.1
Dose 0.04
Dur*Dose 0.8
Number of follicles
4-day 200
7-day 200
4-day 350
7-day 350
24 hours after LH
48 hours after LH

Fig. 3.2. The ovarian response (mean±SEM) in prepubertal calves to a 4-day vs. 7-day regimen of gonadotropin treatment using a cumulative dose of 200 mg vs. 350 mg FSH. The ovarian response was assessed at 24 hours (left panel) and 48 hours (right panel) after LH treatment (i.e., 36 and 60 hours after the last FSH treatment, respectively).

[ab] Values with no common superscript are different ($P<0.05$).

3.4.2. Effect of FSH dose-rate on ovarian response

Comparisons among groups based on dose-rate (mg FSH per treatment) are presented in Figure 3.3. On Day 4, fewer follicles ≥ 3 mm were detected in calves in the 14 mg dose-rate group than the 25 mg dose-rate group (15.1±1.9 vs. 27.9±3.3; $P=0.04$); the 44 mg dose-rate group was intermediate (26.8±4.5; Fig. 3.3A). Combined for all groups, there was a greater ($P=0.001$) number of small (9.0±1.2) and medium sized (11.7±1.3) follicles than large follicles (3.5±0.6; Fig. 3.3A) on Day 4. The mean diameter of the five largest follicles did not differ among groups (Fig. 3.3A).

On Day 6 (comparison of 7 days duration groups only), the total number of follicles ≥ 3 mm (19.6±2.5 vs. 27.4±5.5) and the numbers of small, medium and large follicles did not differ ($P > 0.2$) between calves given 14 vs. 25 mg FSH per treatment (Fig. 3.3B) whether ovulated follicles were included or excluded (data shows following inclusion of ovulated follicle counts). Similarly, the mean diameter of the five largest follicles also did not differ between dose-rate groups (Fig. 3B).

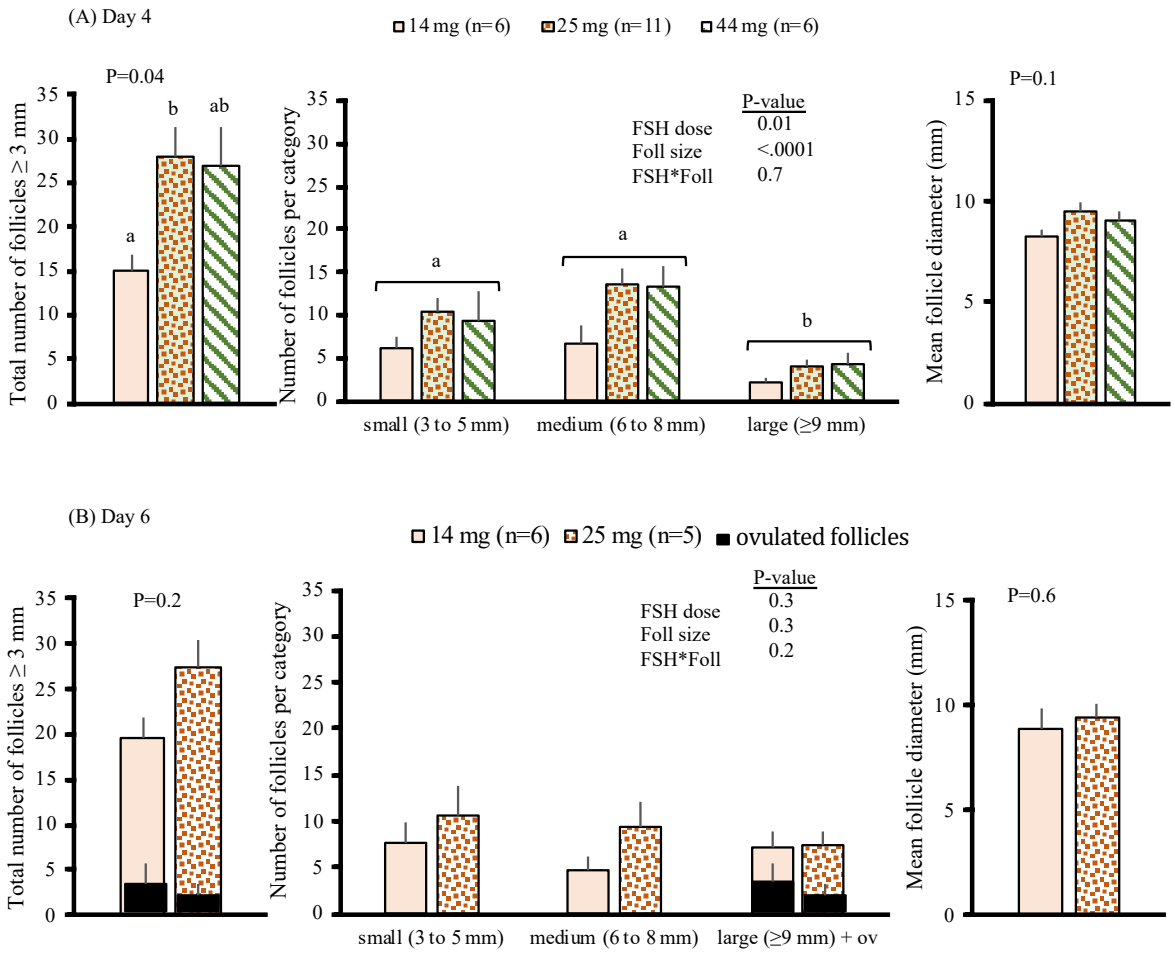

Fig. 3.3. The effect of FSH dose-rate on the number of follicles and the mean diameter of the 5 largest follicles on Day 4 (Day 0 = day of follicular wave emergence; A) and Day 6 (B) in prepubertal calves given a 4-day vs. 7-day regimen of gonadotropin treatment using a cumulative dose of 200 mg vs. 350 mg of FSH. Day 4 data from the respective treatment groups were arranged into 3 groups to compare rates of 14 mg per treatment (7-day group/200 mg), 25 mg per treatment (4-day group/200 mg + 7-day group/350 mg), and 44 mg per treatment (4-day group/350 mg). Similarly, Day 6 data were arranged into 2 groups to compare rates of 14 mg per treatment (7-day group/200 mg) and 25 mg per treatment (7-day group/350 mg). The number of follicles that ovulated (based on the number of follicles ≥ 8 mm that disappeared between ultrasound examinations on Days 4 and 6) were added (black bars) to the total number of follicles ≥ 3 mm and the number of follicles ≥ 9 mm.

3.4.2.1. Effect of cumulative dose and duration of FSH treatment on the number of ovulations

No ovulations were detected before Day 4 (0/23; all groups combined). In the 4-day groups, ovulations were detected in 4 of the 12 calves (2/6 from the 200 mg group and 2/6 from the 350 mg group; 1.7±1.0 ovulations) between Day 4 (i.e., last FSH treatment) and Day 5 (i.e., 12 hours post-LH). In the 7-day groups, ovulations were detected in 5 of 11 calves between Day 4 and Day 6 of FSH treatment (2/6 from the 200 mg group; 3/5 from the 350 mg; 2.7±1.3 ovulations) and in 5 of the remaining 6 calves between Days 6 and Day 8 (i.e., 12 hours post-LH; 5.3±1.8 ovulations). The number of spontaneous ovulations was higher in the 7-day than in the 4-day groups (5.6±1.3 vs 1.7±1.0; P=0.02) and was also higher in the 200 mg than in the 350 mg groups (5.1±1.5 vs. 1.9±0.6; P=0.05).

Between 24- and 48-hours post pLH treatment, ovulations were observed in all but 4 calves (one calf each from the 4-day - 200 mg and 350 mg groups, 2 calves from the 7-day/200 mg group). Excluding the 4 calves that failed to ovulate by 48 hours post pLH treatment, the number ovulations did not differ among groups (5.7±0.8 ovulations per animal).

The total number of ovulations (i.e., spontaneous and pLH induced) did not differ between 200 mg and 350 mg groups (P=0.7), but the 7-day groups had a greater number of ovulations than the 4-day groups (9.7±0.9 vs. 6.9±1.0; P=0.05). The proportion of follicles ≥ 3 mm present in the ovaries at Day 4 that ovulated by 48 hours post pLH was higher in the 7-day than in the 4-day groups (55.7±5.8 vs. 28.1±5.2%; P=0.001), and tended to be higher in the 200 mg than in the 350 mg groups (48.0±7.7 vs. 34.0±5.0%; P=0.07; Table 3.1 and Fig. 3.4).

The number of CL at 72 hours post-LH treatment did not differ (Dose P=0.7, Duration P=0.1) among treatment groups (overall, 6.2±0.6 per animal).

Table 3.1. Total number of ovulations (spontaneous and induced by pLH treatment), total number of follicles ≥ 3 mm on Day 4 of FSH treatment (mean ± SEM), and the proportion of follicles that ovulated in prepubertal calves given a 4-day vs. 7-day regimen of gonadotropin treatment using a cumulative dose of 200 mg vs. 350 mg pFSH.

Endpoint	4-day/200	7-day/200	4-day/350	7-day/350	P-value
Number of ovulations (Day 4 to 48 hours post-LH)	7.2±0.9[a]	9.8±1.2[b]	6.7±1.8[a]	9.6±1.4[b]	**Dur 0.05** Dose 0.7 Dur*Dose 0.9
Number of ≥ 3 mm follicles on Day 4	30.5±4.4[a]	15.0±1.9[b]	26.8±4.5[a]	24.8±5.1[b]	**Dur 0.04** Dose 0.4 Dur*Dose 0.1
Proportion of ovulated follicles (ovulated/follicle count)	28.9±8.2[a]	67.1±6.8[b]	27.3±7.2[a]	42.0±5.7[b]	**Dur 0.001** Dose 0.07 Dur*Dose 0.1

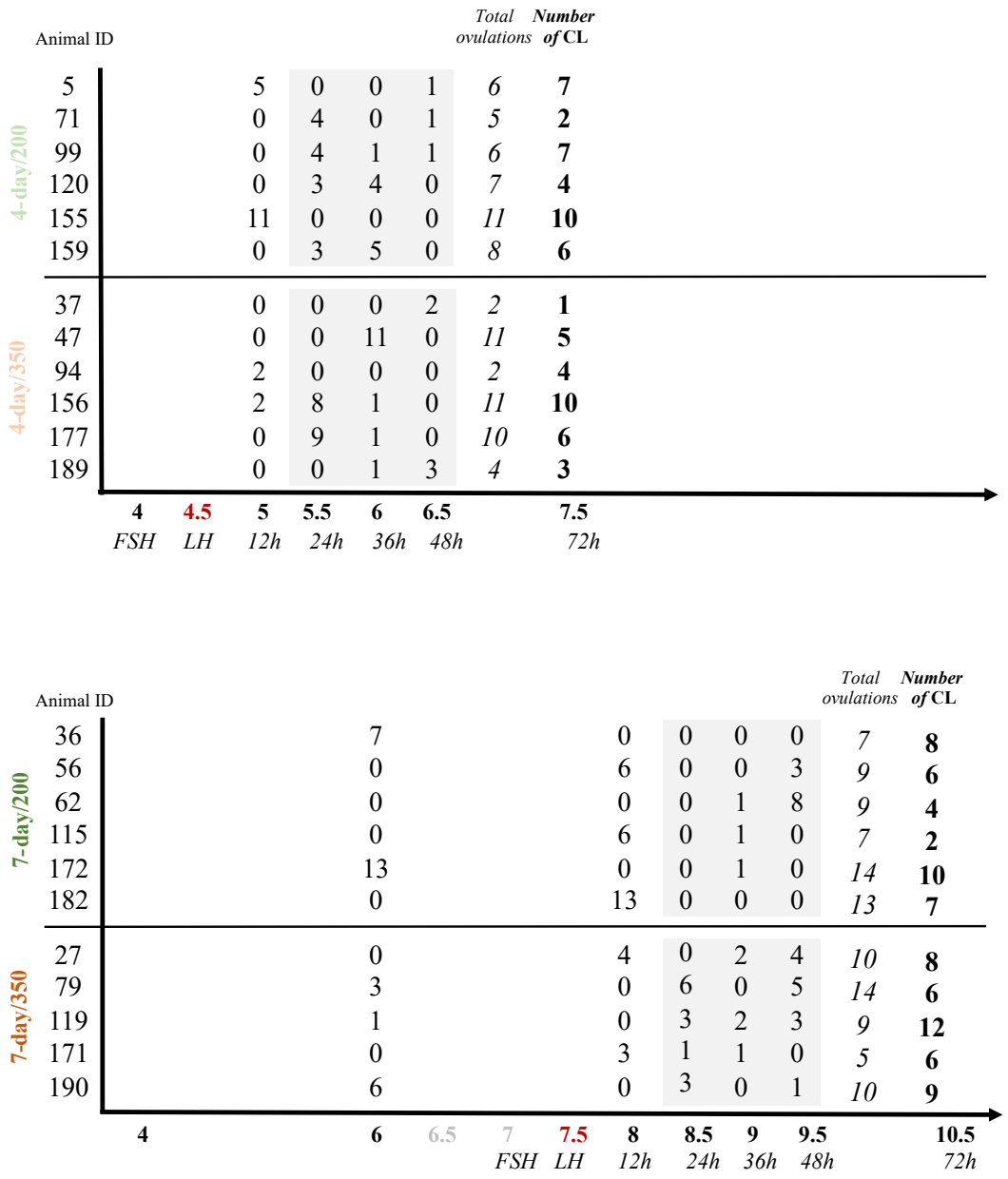

Fig. 3.4. Diagram showing the time distribution of spontaneous and induced (pLH treatment – grey area) ovulations in individual calves to a 4-day vs. 7-day regimen of gonadotropin treatment using a cumulative dose of 200 mg vs. 350 mg FSH. Calves were given pLH 12 hours after the last FSH treatment. Ultrasound examinations were performed Days 4 (4-day and 7-day groups) and 6 (7-day groups) and at 12, 24, 36, 48 and 72 hours after LH treatment (Day 0 = day of follicular wave emergence). Ovulation counts were based on the disappearance of follicles ≥ 8 mm between successive examinations. The number of corpora lutea (CL) was assessed at 72 hours after LH treatment. Total ovulations = total number of spontaneous and induced ovulations; Number of CL = number of corpora lutea detected at 72 hours after pLH treatment.

3.5. Discussion

This study aimed to determine the effects of FSH dose, dose-rate, and duration of treatment on ovarian response in prepubertal calves. After 4 days of FSH treatment (8 doses), 44 mg of pFSH per dose resulted in a similar number of follicles ≥ 3 mm as 25 mg per dose, while 25 mg per dose produced more follicles than 14 mg per dose. More than 80% of follicles in all groups were in the small and medium size categories (3 to 8 mm) following 4 days of FSH treatment. After 6 days of pFSH treatment, the number of follicles ≥ 3 mm was not different between calves given 14 mg or 25 mg of pFSH per dose; therefore, data were not consistent with the hypothesis that a higher FSH dose rate will result in a greater number of follicles than a lower dose rate. The growth rate of the largest follicles appeared to be similar among groups since the mean diameter of the 5 largest follicles was not different on Day 4 or Day 6. However, the number of follicles ≥ 9 mm at the end of the treatment (24 hours post-LH) was greater in groups given a cumulative dose of 350 mg FSH than 200 mg, and in the 7-day than the 4-day groups, supporting the hypothesis that a higher cumulative FSH dose and a longer duration of FSH treatment results in a greater follicular response in calves.

The follicular response to exogenous FSH in calves has been reported to increase progressively [135] from 3 to 9 weeks of age, and twice-daily administration of FSH over 3 days induced a greater response than a single bolus of FSH, which might be expected as the half-life of FSH in cattle has been reported to be approximately 5 hours [136, 137]. Results of the present study provide evidence that the ovarian response in prepubertal calves is affected by FSH dose per treatment since a greater number of follicles $\geq$ 3 mm was recorded with a dose of 25 mg per treatment than 14 mg per treatment. By design, the number of follicles ≥ 1 mm at the time of wave emergence was not different among calves in our study; hence, a higher FSH dose-rate rescued more follicles within the wave to grow during gonadotropin treatment. Surprisingly, the administration of either 25 or 44 mg of FSH resulted in a similar number of follicles $\geq$ 3 mm, suggesting that exogenous FSH support is dose-dependent until an upper plateau is reached. In sexually mature cows, the superstimulatory effect of exogenous FSH treatment was attributed to the rescue of small antral follicles present at the time of wave emergence; i.e., there was no evidence for continuous recruitment of new follicles during 4 or 7 days of treatment [132]. Contrary to expectations, a similar number of follicles was observed in calves given 14 mg vs. 25 mg of FSH

per treatment after 6 days of treatment in the present study, suggesting that a dose rate of 25 mg may not be sufficient to maintain the growth of multiple follicles for an extended duration. Although there was no difference in the mean diameter of the five largest follicles, the disappearance of follicles due to spontaneous ovulations interfered with the accuracy of this endpoint and since the 44 mg dose-rate was not given for more than 4 days, the hypothesis that a higher FSH dose per treatment will result in a greater number of follicles than a lower dose will require further evaluation with more than 4 days of treatment.

Four days of exogenous FSH treatment was not sufficient for the recruited follicles to grow to larger sizes with a dose rate of 25 mg since the number of small-sized follicles was greater in calves given a cumulative dose of 200 mg of pFSH than in the other groups. Perhaps the higher dose-rate of FSH enhanced the responsiveness of follicles to endogenous and exogenous LH, thus supporting continued follicle growth. Furthermore, extending follicular growth by 3 days in the 7-day groups resulted in a greater number of large-sized follicles than in the 4-day groups resulting in a greater number of ovulations, likely due to increased responsiveness to the LH surge. Similar results were observed in sexually mature animals where a greater number of large-sized follicles at the end of the treatment was associated with a greater number of ovulations after a pLH stimulus with the 7-day than with the 4-day treatment [13, 133].

Differences in the number of follicles that ovulated (spontaneous or induced) among groups in the present study may be explained by differences in follicular size and the expression of LH receptors in granulosa cells at the time of the endogenous LH surge or pLH treatment. Despite considerable variation in the time at which ovulations occurred, the number of spontaneous (presumably due to endogenous LH) and the total number of ovulations (spontaneous and induced) per calf were greater in the 7-day groups than in the 4-day groups. Surprisingly, the 200 mg FSH groups resulted in a greater number of spontaneous ovulations than the 350 mg groups, while the number of ovulations in response to exogenous LH treatment did not differ among treatments. Secretion of LH is critical to maintain dominant follicle growth and viability, and the number of LH receptors on granulosa cells has been shown to increase at the time of dominant follicle selection [138]. Exogenous FSH treatment prevents follicle selection and allows the growth of multiple follicles that otherwise would undergo regression and atresia [72, 139]. Ovulatory capacity in adult cattle is acquired when a follicle reaches a diameter of about 10 mm, but a 10 mm follicle requires a higher LH dose to ovulate than a larger follicle [140]. Plasma concentrations of LH after

gonadotropin treatment in calves were similar to that observed in mature cows [79, 80], but LH concentration in pituitary tissue was much higher in calves than in sexually mature heifers (5.0 versus 1.1 mg LH/g of pituitary), and pituitary LH concentrations decreased markedly after superstimulation in calves, reaching values found in mature heifers [79].

The number of corpora lutea detected at 72 hours after LH treatment was not different among groups and was lower than the number of ovulations detected, perhaps because not all CL were sufficiently developed at the time of examination. The developing CL was detectable by ultrasonography, on average, 3 days after ovulation [141, 142].

In summary, 4 days of exogenous FSH treatment was not sufficient for follicles to reach the large size category (≥ 9 mm), regardless of the dose-rate given. A dose rate of 25 mg of FSH resulted in a greater number of follicles ≥ 3 mm than a dose-rate of 14 mg after 4 days of treatment, but not after 6 days, therefore not fully supporting our hypothesis that a higher FSH dose rate would result in greater number of follicles than a lower dose rate. Prolonged follicular growth in the 7-day groups resulted in a greater number of follicles reaching an ovulatory size and ovulating from an endogenous LH stimulus than in the 4-day groups. The number of ovulations in response to the pLH treatment was not different among groups, likely due to a higher number of follicles ovulating from endogenous LH surge. A cumulative dose of 350 mg of FSH resulted in a greater number of large-sized follicles at the end of the treatment than a dose of 200 mg. The greatest ovarian response was observed in calves given a 7-day treatment regimen with a cumulative dose of 350 mg of FSH. The effect of prolonged follicular growth on the oocyte developmental competence in prepubertal calves under controlled endogenous LH release awaits future evaluation.

CHAPTER 4

4. ANTRAL FOLLICLE COUNTS IN PREPUBERTAL CALVES AND ITS RELATIONSHIP TO OVARIAN SUPERSTIMULATORY RESPONSE

Krause ART, Dias FCF, Adams GP, Mapletoft RJ, Singh J

Relationship of this study to the dissertation

In the previous study, we reported that ovarian response was greater with a cumulative FSH dose of 350 mg over 7 days of treatment, which is equivalent to a dose-rate of 25 mg of pFSH per treatment. In this study the ovarian response in prepubertal calves with low and high AFC resulting from different durations of exogenous FSH treatment with same dose-rate per injection of 25 mg of pFSH was studied. In adult cattle, the number of follicles growing in each wave were shown to be highly variable among individuals, but repeatable within individual, therefore a single ovarian ultrasound examination can predict the ovarian response to superstimulation. However, the repeatability of the AFC at wave emergence and its relationship with the ovarian response have not been critically evaluated in prepubertal calves. Since the dynamics of follicle development have been shown to be similar between pubertal and prepubertal cattle, the repeatability of AFC and its relationship with ovarian response in prepubertal calves was evaluated.

Authors' contribution: ARTK, FCFD, GPA, RJM and JS conceived and designed the experiment. ARTK conducted and coordinated the experiment, collected and analyzed the data. ARTK wrote the draft of the manuscript. FCFD, GPA, RJM and JS reviewed the manuscript.

4.1. Abstract

Antral follicle counts (AFC) assessment provides an estimation of the ovarian reserve and is a useful method to predict the ovarian response to exogenous gonadotropin treatment in assisted female reproduction. The objectives were to determine the repeatability of the antral follicle counts (AFC) at follicular wave emergence (FWE) and the relationship between the ovarian response following superstimulation (Study I) and the effect of duration of gonadotropin treatment in prepubertal calves (low and high AFC; Study II). In Study I, follicular ablation of follicles ≥ 5 mm was performed in calves at 5.5 months of age (n=46; 166.3±2.1 days) to synchronize FEW and the number of follicles ≥ 1 mm was counted. Calves were classified according to the AFC as low (15.5±0.7; range 12 to 20 follicles), medium (25.0±0.7; 20 to 32 follicles) and high (38.9±2.7; 29 to 53 follicles) AFC groups. Follicular wave emergence was induced a second time before exogenous FSH treatment when calves were at 6.4 months of age (192.6±3.3 days; 195.5±4.3 Kg). The FSH treatment and the ovarian response for calves in the medium AFC group were reported previously (*Unpublished, Chapter 3*). In Study II, using a 2x2 factorial design, high- and low-AFC calves were assigned randomly to two treatment groups and given 25 mg of pFSH at 12-hour intervals for either 4 days (8 doses) or 7 days (14 doses). Treatments were initiated 36 hours after transvaginal ultrasound-guided follicle ablation to induce FEW, and 12.5 mg of pLH was given 20 hours after the last dose of FSH. Transvaginal ultrasound-guided collection of cumulus-oocyte complexes (COC) was done 16 hours after LH treatment. In Study I, there was a positive correlation between the number of follicles ≥ 1 mm at wave emergence at 5.5 months and 6.4 months of age (r=0.4; P=0.003). The number of follicles at FWE was also positively correlated with the number of follicles at the end of superstimulatory treatment (i.e., 36 hours after last FSH treatment) in both the medium (≥ 6 mm; r=0.5, P=0.0005) and large (≥ 9 mm; r=0.3, P=0.01) follicle size categories. At the time of COC collection, corpora lutea were detected in calves in the 7-day treatment groups due to spontaneous ovulations; therefore, the number of corpora lutea was included in the counts of large follicles for treatment group comparisons in Study II. A greater number of large vs. small follicles was detected at the end of treatment in the 7-day treatment groups (13.6±2.7 vs. 2.9±0.9) than the in the 4-day treatment groups (6.1±1.7 vs. 7.2±1.7; Duration*Follicle P=0.01). The number of follicles ≥ 6 mm was greater in high than low AFC groups (25.3±5.4 vs. 10.3±1.8; p=0.01) as was the number of COC collected (7.8±0.7 vs. 3.7±0.7; p=0.001), but both endpoints were not

affected by the duration of FSH treatment. In summary, the number of follicles at the beginning of a wave was predictive of the number of follicles in subsequent waves and the ovarian response following superstimulation in prepubertal calves. Gonadotropin treatment for 7 days resulted in a greater number of large follicles at the end of treatment than 4 days but did not result in a greater number of COC collected since recovery rate was adversely affected by spontaneous ovulations. Prolonged follicular growth under exogenous FSH support requires suppression of endogenous LH release to prevent spontaneous ovulations.

Keywords: wave emergence, superstimulation, follicular growth, follicular aspiration, ovulation.

4.2. Introduction

In cattle, follicular development is characterized by a wave-like pattern of follicular growth [42] and the emergence of each wave is characterized by simultaneous growth of a group of small antral follicles (1 to 3 mm in diameter; [45]) followed by selective growth of the dominant follicle and regression of subordinates [reviewed in [143]]. This pattern has been observed in calves as young as two weeks of age, but while in sexually mature animals the dominant follicle that is present at the time of luteolysis ovulates, in prepubertal calves follicular development is characterized by anovulatory waves and the absence of corpus luteum [43]. In sexually mature cattle, the number of antral follicles growing in each wave were shown to be very variable among individuals but highly repeatable within individual [128, 129], therefore, a single ovarian ultrasound examination was shown to reliably identify the animals that have low or high follicle numbers per wave and thus predict the ovarian response to gonadotropin treatment [129]. Since the mechanisms involved in follicular dynamics (recruitment, selection, and regression) were shown to be similar between prepubertal and adult animals [16, 91], antral follicle counts (AFC) assessed by ultrasonography may be useful in calves, as in adult cattle, to investigate individual variation and predictive response to gonadotropin treatments.

Estimations of the ovarian reserve have been used as a marker of fertility in cattle [144], in sexually mature animals the AFC has been positively correlated with the ovarian response to gonadotropin treatments [129], *in vitro* and *in vivo* embryo production [145, 146], pregnancy rates [147] and fecundity [148]. In prepubertal calves, little is known about the relationship between

AFC and the ovarian superstimulatory response, but in 2 and 4-month-old calves, the number of follicles before FSH treatment was highly correlated with the number of oocytes collected post-treatment [93]. In a study of calves involving serial ultrasonography ovarian examinations from 2 to 36 weeks of age, the total number of small and medium antral follicles increased from 2 to 14 weeks, remained constant until 34 weeks; while the number of large follicles increased steadily between 2 and 34 weeks of age [2]. In agreement with these observations, when exogenous FSH treatment was given to heifers between 5 to 18 months of age, a greater number of follicles was observed post-treatment in heifers between 5 and 8 months of age than in older heifers [11]. Therefore, we infer that a transitional phase of the hypothalamic-pituitary-gonadal axis occurs between 4 and 8 months of age, affecting the overall number of follicles within an animal, but perhaps maintaining individual AFC pattern.

Prevention of single follicle dominance and the rescuing of subordinate follicles from regression and atresia are the principles of exogenous FSH treatment, which stimulates follicular growth by maintaining the circulating concentrations of FSH elevated [72, 127]. Ovarian response to exogenous FSH was enhanced when treatment was initiated at the time of wave emergence in adult [149] and in prepubertal cattle [91]; and FSH treatment was shown to rescue small antral follicles at the time of wave emergence, since there was no evidence for continued recruitment of small follicles during treatment [132]. In sexually mature cattle, prolonged follicular growth under exogenous FSH support for 7 days resulted in a greater number of large follicles at the end of the treatment, a greater number of fully expanded COC collected after LH treatment and 2.5 more transferable embryos produced *in vitro* than 4 days of treatment [13]. In prepubertal cattle, 3 days of FSH treatment resulted in a higher proportion of > 5 mm follicles than 1.5 days or no treatment [87]. Our previous study showed that a greater number of large follicles and ovulations were observed following the 7 than the 4 days treatment in prepubertal calves classified at 5 months of age as having an intermediate number of follicles at wave emergence (*Unpublished, Chapter 3*). In this study, we are interested in the effects of the prolonged follicular growth in the 7 days treatment in animals with previous low and high AFC at the time of wave emergence.

Two studies were performed with the objectives of to determine 1) the repeatability of AFC at wave emergence and 2) the relationship between AFC and ovarian response following exogenous FSH treatment in Study I and 3) the effect of duration of gonadotropin treatment in calves with high vs. low AFC in Study II. We hypothesized that 1) the AFC at wave emergence is

repeatable within individuals and is predictive of the ovarian response to gonadotropin treatment in Study I; and 2) that a longer duration of FSH treatment (7 vs. 4 days) will result in a greater number of follicles available for aspiration and a higher COC collection efficiency in Study II.

4.3.Material and Methods

4.3.1. Animals and treatments

Animal procedures were approved by the University of Saskatchewan's Animal Care Committee in accordance with the Canadian Council on Animal Care. The experiment was conducted at the University of Saskatchewan's Goodale Research Farm, Saskatoon, SK, Canada (52° North and 106° West).

Calves were selected in September at 5.5 months of age (n=46; 166.3±2.1 days) from a group of Spring-born Hereford crossbreed calves (n=51; born between March 31 and May 3) according to antral follicle counts (AFC) at wave emergence. To synchronize the emergence of a new follicular wave among calves, transvaginal ablation of follicles ≥ 5 mm in diameter was performed under caudal epidural anesthesia (2% lidocaine HCl and epinephrine USP; Bimeda-MTC Animal Health Inc., Lavaltrie, Québec, Canada) using a 7.5 MHz convex-array transducer (Esaote MyLab Five, Canadian Veterinary Imaging, Georgetown, ON, Canada) using a custom-designed handle as described previously [91]. Briefly, the perineal region was washed with surgical scrub, and sterile lubricant was applied to the transducer before guiding it into the vagina. The ovaries were manipulated transrectally and positioned against the vaginal wall. A disposable 18-gauge needle connected to an aspiration line was placed in the needle guide and advanced through the vaginal fornix and into the follicular antrum. Follicular fluid was aspirated by syringe; follicle ablation was defined as evacuation and collapse of all follicles ≥ 5 mm in diameter in both ovaries. Follicles were measured and counted 24 to 36 hours after ablation (i.e., expected time of wave emergence, Day 0; [91]) using a transrectal 7.5-MHz linear-array transducer (Esaote MyLab Five). The total number of follicles ≥ 1 mm was used to classify calves with low (15.5±0.7 follicles, range: 12 to 20 follicles, n=12), medium (25±0.8 follicles, range 20 to 32 follicles, n=24), and high AFC (38.9±2.7 follicles, range: 29 to 53 follicles, n=10). All the 46 calves classified were included in Study I, while only calves in the low and high AFC groups were included in Study II.

Second wave emergence synchronization, exogenous FSH treatment, and data collection for calves in the medium AFC classification group were described previously (*Unpublished, Chapter 3*). For calves in the low and high AFC classification groups, approximately 1.5 months after classification (7.1±0.1 months of age, 213.7±2.2 days; 187.8±7.2 Kg) calves were submitted to a second follicular ablation procedure to synchronize emergence of a new follicular wave, and gonadotropin treatments were initiated 36 hours later. Using a 2x2 factorial design (Fig. 4.1), high- and low-AFC calves were assigned randomly to two treatment groups and given 25 mg of pFSH (Folltropin-V; Vetoquinol, Lavaltrie, QB, Canada) at 12-hour intervals for either 4 days (8 doses) or 7 days (14 doses). Calves were given 12.5 mg of pLH (Lutropin-V; Vetoquinol, Lavaltrie, QB, Canada) 20 hours after the last FSH treatment and cumulus-oocyte complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration 16 hours after pLH treatment. During the trial, calves were already weaned and were maintained in outdoors in pens with hay and water *ad libitum*.

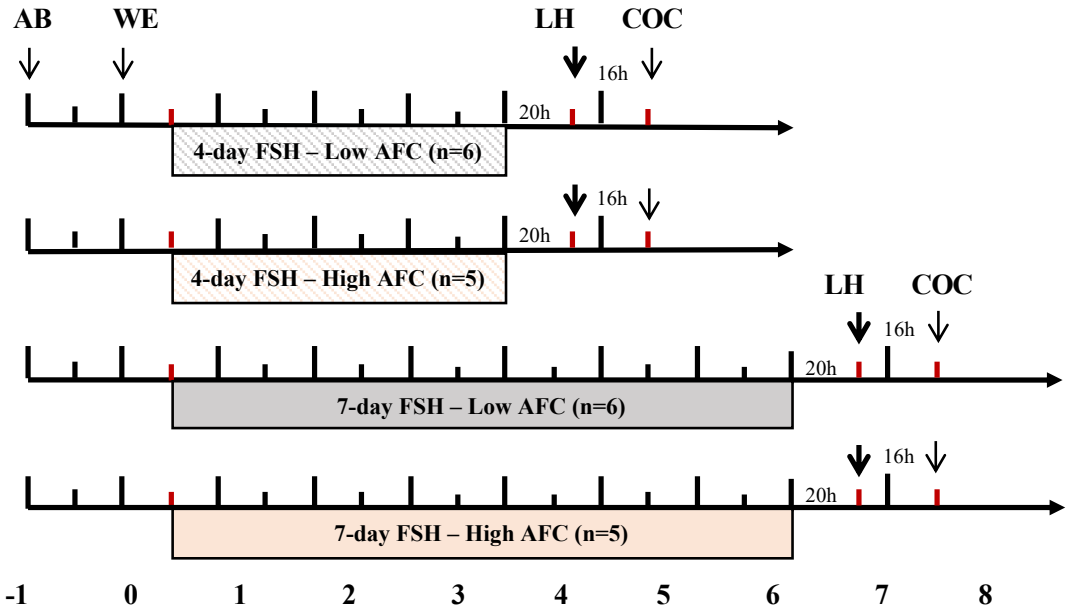

Fig. 4.1. Experimental Design and treatments. Transvaginal ultrasound-guided ablation (AB) of follicles ≥ 5 mm was done to synchronize emergence of a new follicular wave (WE, Day 0) among calves (n=22). Using a 2x2 factorial design, calves previously classified with high- and low-AFC at the time of wave emergence were assigned randomly to two treatment groups and given 25 mg of pFSH at 12-hour intervals for either 4 days (8 doses) or 7 days (14 doses) beginning at 36 hours

after follicle ablation. Calves were given 12.5 mg of pLH (LH) 20 hours after the last dose of FSH. Cumulus-oocyte complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration 16 hours after LH treatment. Ovarian ultrasound examinations were performed at the time of the first FSH treatment, at the time of LH treatment, and immediately before COC collection (red hash marks).

4.3.2. Ovarian ultrasound examinations and COC collection

Ovarian examinations were done by transrectal ultrasonography at the time of the first FSH treatment (i.e., 36 hours after follicular ablation), immediately before pLH treatment, immediately before COC collection, and 80 hours COC collection. Cine-loops of each ovary were recorded during each examination and the number of small (3 to 5 mm), medium (6 to 8 mm) and large (≥ 9 mm) follicles, as well as the number of corpora lutea (CL).

COC were collected under caudal epidural anesthesia (2% lidocaine HCl and epinephrine USP; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) by transvaginal ultrasound-guided follicle aspiration using a disposable 18-gauge needle and a line system connected to a vacuum pump set at a flow rate of 22 mL/min. Follicular contents were aspirated in a 50 mL conical tube containing 5 mL of collection medium at 36°C. Collection medium consisted of Dulbecco's phosphate-buffered saline (DPBS 1X containing calcium and magnesium; Gibco, Carlsbad, CA, USA) with ET surfactant (0.3% Pluronate; Bioniche Animal Health, Belleville, ON, Canada) and sodium heparin (2 IU/ml; Heparin Sodium Injection USP; Sandoz Canada Inc.). The aspirate was immediately filtered (EmCon Filter, Immuno Systems, Inc., Spring Valley, WI, USA), and the COC were located and classified according to cumulus cell characteristics as expanded, partially expanded, compact or denuded. All collections were performed in a standing position by the same operator.

4.3.3. Blood sampling and progesterone assay

Blood samples were taken by jugular venipuncture using an 18G x 1.5" vacutainer needle in a 10 mL heparinized tubes (Becton Dickinson Vacutainer Systems, Franklyn Lakes, NJ, USA) at the time of the last FSH treatment, at the time of pLH treatment, and immediately after COC collection. Samples were centrifuged within 3 hours of collection at 3000 x g for 15 minutes, and plasma was stored at -20°C until analyses. Plasma progesterone concentrations were measured in a single assay using a commercial radioimmunoassay kit (ImmunoChem Progesterone^{125} kit, MP

Biomedicals, Costa Mesa, CA, USA). The range of the standard curve was 0.15 to 80 ng/mL and the intraassay coefficients of variation were 8.9, 6.0 and 13.5% for low (0.57 ng/mL), medium (4.06 ng/mL) and high (11.52 ng/mL) reference sera, respectively.

4.3.4. Statistical Analyses

Pearson and Spearman correlation coefficients and regression analysis were used to evaluate the repeatability of AFC at wave emergence at 5.5 and 6.4 months of age, and the association between AFC and ovarian response. Analysis of variance was used to compare follicle counts at 5.5 and 6.4 months of age using repeated measures and ovarian response at 6.4 months of age among AFC groups (one-way). Factorial analyses of variance were used to compare the ovarian response (number of follicles and COC collected) and plasma progesterone concentrations between treatment groups (4-day vs. 7-day), and/or follicle size category (small – 3 to 5 mm, medium – 6 to 8 mm and large - ≥9 mm), and/or AFC groups (low vs. high). The proportion of large follicles and the number of follicles that ovulated were compared by t-test. Log transformation for numerical data and arcsine transformation for proportion data were performed when data did not present equality of variances and normality of the residuals. Multiple comparisons were performed by Tukey's test. Data are presented as mean±SEM, and statistical significance was set at a P-value of ≤ 0.05, while tendencies were considered for P-values from ≥ 0.06 to ≤ 0.1.

4.4.Results

One calf in the 7-day/High AFC group became sick during the study and was removed from the experiment. However, data on follicle counts at the time of wave emergence for this calf were included in statistical analyses.

4.4.1. Study I

4.4.1.1. Repeatability of AFC at wave emergence

The AFC at wave emergence at 5.5 months of age (first counts; 166.3±2.1 days; n=46) was correlated with the AFC at 6.4 months of age (second counts; 192.5±4.3 days) (Pearson correlation coefficient = 0.4; P=0.03). Animal ranks based on AFC were also correlated (Spearman correlation

coefficient = 0.5; P<.0001). Regression analysis of follicle counts (P=0.003) and animal ranks (P<.0001) are shown in Fig. 4.2A and B, respectively. An interaction between main effects (AFC and age) was attributed to a drop in AFC from 5.5 months of age to 6.4 months of age in the High AFC group only (38.9±2.7 [range: 29 to 53 follicles] vs. 24.3±1.3 [range: 20 to 31 follicles], respectively; AFC group*Time, P=0.0001; Fig. 4.2C). At both ages, AFC in the High AFC group was higher than in the low AFC group (15.5±0.7 vs. 38.9±2.7 at 5.5 months and 15.4±1.0 vs. 24.3±1.3 at 6.4 months).

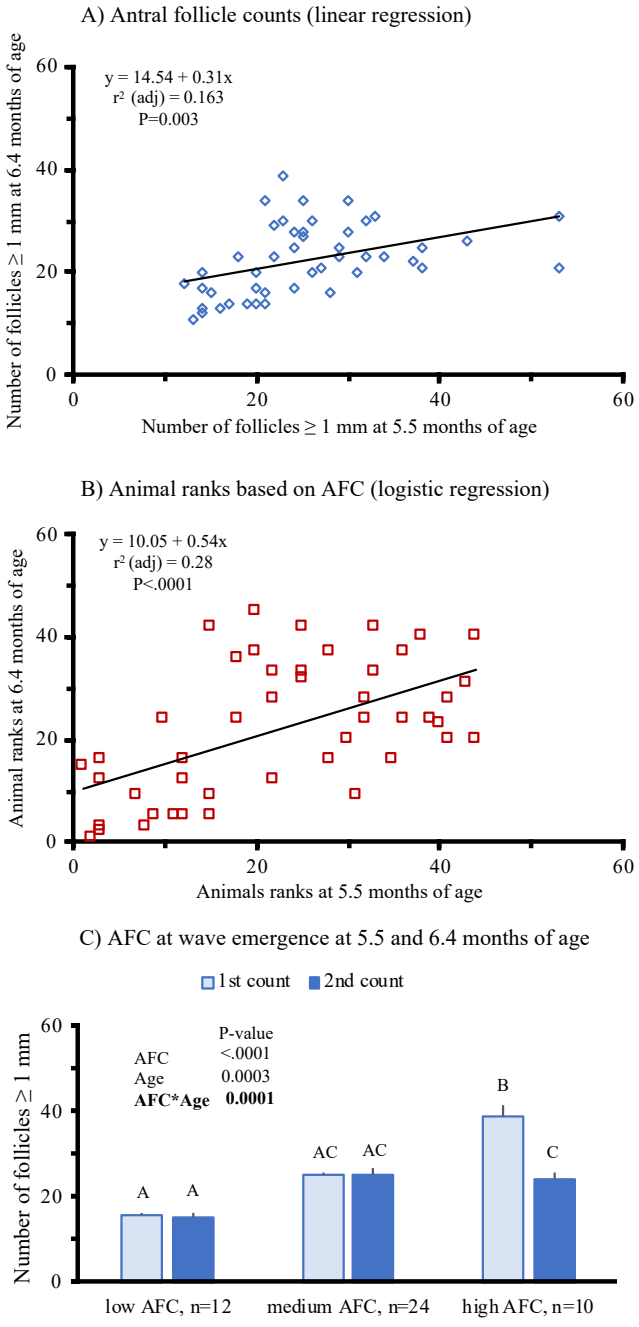

Fig. 4.2. Repeatability of antral follicle counts (AFC) at wave emergence; A) Linear regression of the AFC at 5.5 and 6.4 months of age; B) Logistic regression of animal ranks based on AFC at 5.5 and 6.4 months of age; C) Number of follicles ≥ 1 mm at wave emergence (mean±SEM) for the first count at 5.5 months of age (AFC classification; light blue bar) and second count at 6.4 months of age (pre-stimulation; dark blue bar).

Values with no common superscript are different (P<0.05).

4.4.1.2. AFC at wave emergence and ovarian response to gonadotropin treatment

Spontaneous ovulations were observed during FSH treatment; therefore, the number of ovulated follicles (follicle disappearance or CL counts) were included in the number of follicles ≥ 9 mm for statistical analysis.

The number of follicles ≥ 1 mm at wave emergence pre-stimulation (second counts; n=44) was correlated with the number of follicles ≥ 6 mm (r=0.5; P=0.0005) and the number of follicles ≥ 9 mm (r=0.3; P=0.01) at the end of FSH treatment (i.e., 36 hours after the last FSH treatment). Linear regression analysis between follicle counts at wave emergence and the ovarian response are presented in Figures 4.3A and B. Overall among calves (Fig. 4.3C), the number of follicles ≥ 6 mm at the end of FSH treatment was greater in the high than in the low AFC group (26.9±5.2 vs. 11.6±2.1; P=0.005), while in the medium AFC group was intermediate (18.1±1.8). The number of follicles ≥ 9 mm was not different among groups (P=0.3).

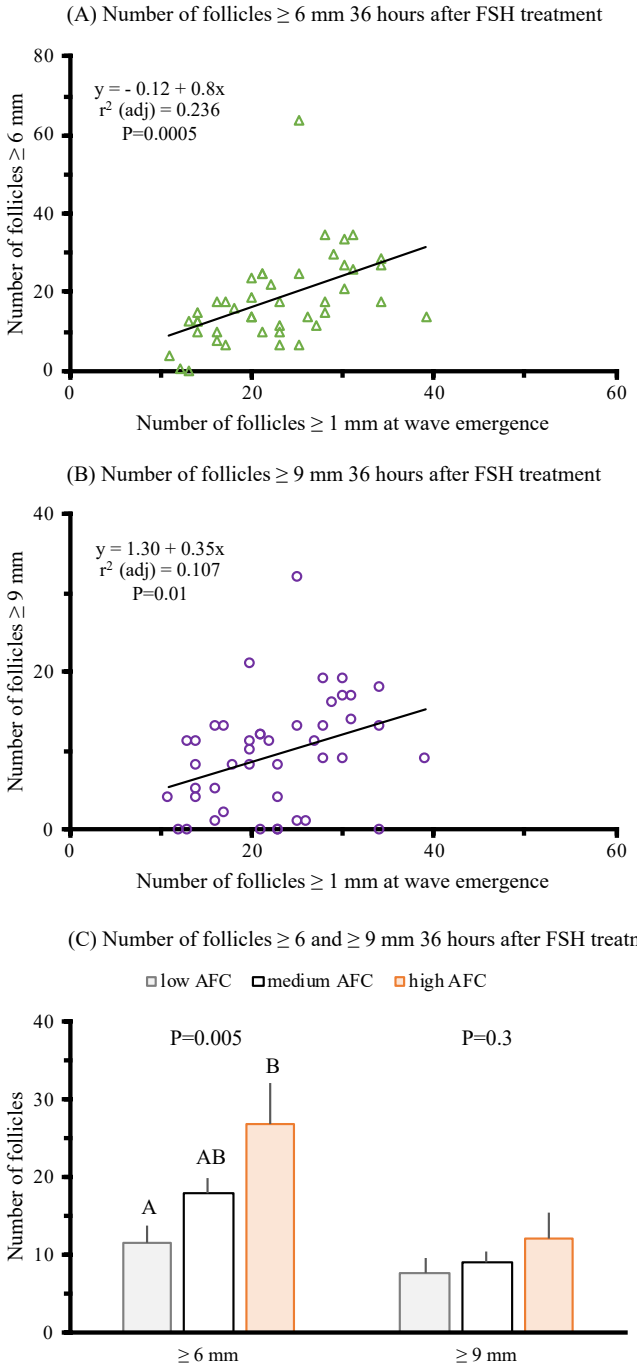

Fig. 4.3. Relationship between antral follicle count (AFC) at the time of wave emergence and the ovarian response at the end of FSH treatment in calves at 6.4 months of age. Linear regression of the number of follicles ≥ 1 mm at wave emergence and (A) the number of follicles ≥ 6 mm or (B) ≥ 9 mm at the end of the FSH treatment (i.e., 36 hours after last FSH treatment); and (C) Number of follicles ≥ 6 mm and ≥ 9 mm (mean±SEM) after FSH treatment at 6.4 months of age according

to classification at 5.5 months in low (n=12; light grey bar), medium (n=23; white bar) and high (n=9; light orange bar) AFC.

Values with no common superscript are different (P<0.05).

4.4.2. Study II

4.4.2.1. Effect of duration of gonadotropin treatment in calves with high vs. low AFC

To compare the ovarian response between 4- and 7-day gonadotropin treatment groups, follicular number data were analyzed both with and without the inclusion of follicles that ovulated. The number of ovulations was estimated from CL counts at the time of COC collection. Using both counting methods, there was a Duration*Follicle category interaction (P<0.04) resulting from a greater number of large- than small-sized follicles in the 7-day groups (13.6±2.7 and 2.9±0.9, respectively; Fig. 4.4) but not in the 4-day groups (6.1±1.7 and 7.2±1.7). Further analyses included the number of follicles that ovulated, as estimated by CL counts. A Duration*Follicle category interaction (P=0.02) was attributed to a greater number of large- than medium- and small-size follicles in the Low AFC group given 7-days of treatment (10.7±2.8 vs. 2.3±0.7 and 1.7±0.7) but not in those given 4-days of treatment (4.8±2.0 vs. 5.3±1.7 vs. 5.3±2.1, respectively). No effect of duration of gonadotropin treatment (P=0.3) was observed in the High AFC groups; overall, the number of medium-size follicles tended to be higher than the number of small-size follicles (14.7±2.5 vs. 7.3±1.7; P=0.07; Fig. 4.4C).

The proportion of large-size follicles (i.e., number of follicles ≥ 9 mm/ number of follicles ≥ 3 mm) was higher in the 7-day than in the 4-day treatment groups (56.4±8.3 vs. 27.8±7.5%; P=0.01) for all calves combined, while in the Low AFC groups, the proportion of large-sized follicles tended to be higher in the 7-day than in the 4-day groups (63.8±13 vs. 30.4±12%; P=0.08; Figure 4.4 right panels).

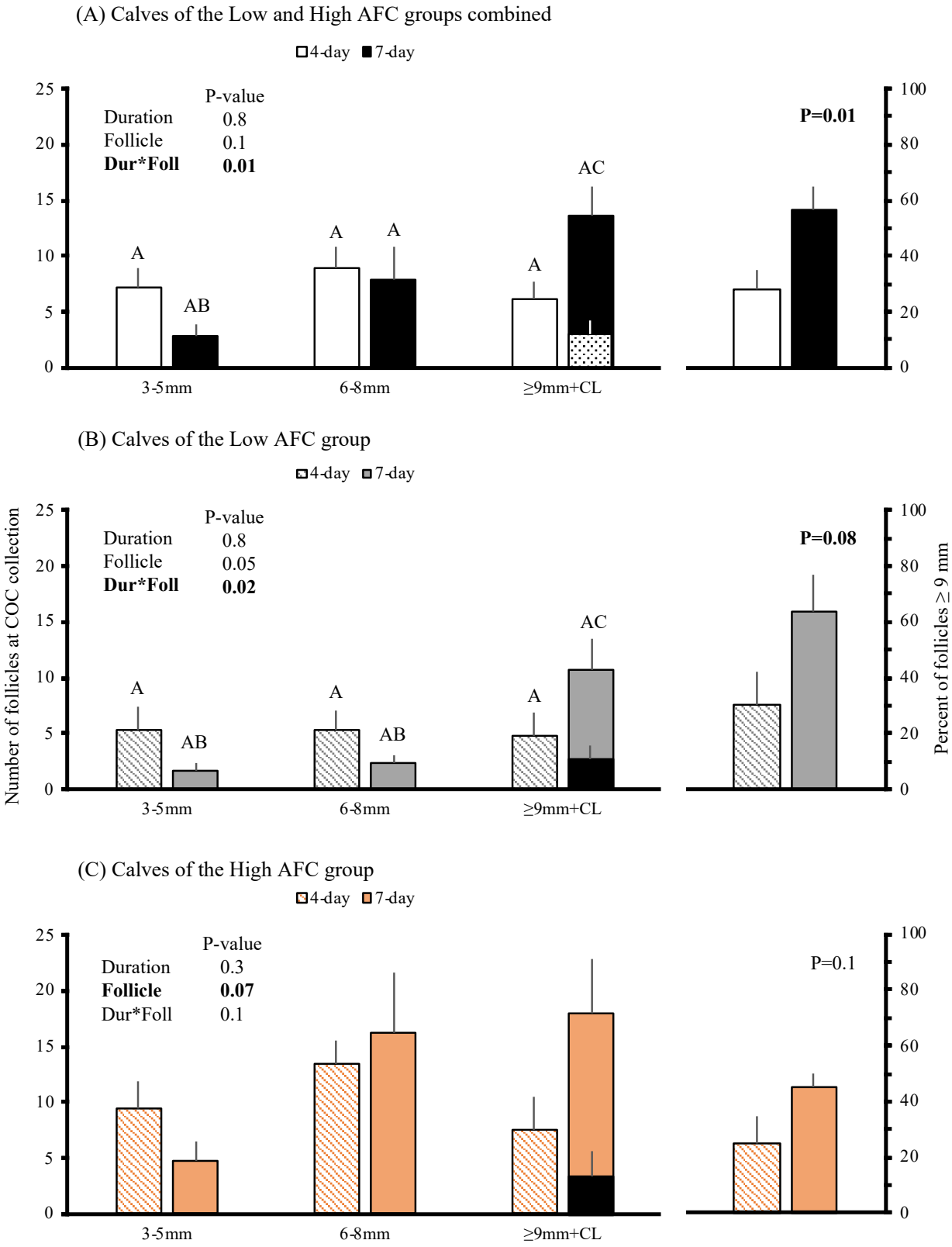
(A) Calves of the Low and High AFC groups combined
4-day
7-day
P-value
Duration 0.8
Follicle 0.1
Dur*Foll 0.01
A
AB
A
A
A
AC
3-5mm
6-8mm
≥9mm+CL
P=0.01
(B) Calves of the Low AFC group
4-day
7-day
Number of follicles at COC collection
P-value
Duration 0.8
Follicle 0.05
Dur*Foll 0.02
A
AB
A
AB
A
AC
3-5mm
6-8mm
≥9mm+CL
P=0.08
Percent of follicles ≥ 9 mm
(C) Calves of the High AFC group
4-day
7-day
P-value
Duration 0.3
Follicle 0.07
Dur*Foll 0.1
3-5mm
6-8mm
≥9mm+CL
P=0.1

Fig. 4.4. Ovarian response at the time of cumulus-oocyte complex (COC) collection in prepubertal calves given exogenous FSH treatment for 4 days vs. 7 days. Number of follicles (mean±SEM) in each size category (small, 3 to 5 mm; medium, 6 to 8 mm; large, ≥ 9 mm) for calves in both high and low AFC groups combined (A), and for the low and high AFC groups separately (B and C, respectively). The dotted bar and the black bars represent the number of CL added to the number of large sized follicles. The percent of follicles ≥ 9 mm (to the right side of each figure) = (number of follicles ≥ 9 mm/number of follicles ≥ 3 mm) x 100.
Values with no common superscript are different (P<0.05).

4.4.2.2. Ovulations and COC collection

Corpora lutea were detected in 5 calves of the 7-day groups (3/6 in the 7-day/Low AFC group and 2/4 in 7-day/High AFC group) (2.7±1.3 vs. 3.5±2.4; P=0.7; Fig. 4.5D), while no CL was detected in calves in the 4-day groups. Overall, plasma progesterone concentrations at the time of COC collection were higher in the 7-day than in the 4-day groups (9.9±3.4 vs. 0.7±0.2; P=0.002; Fig. 4.5E), confirming the formation of functional corpora lutea. At 80 hours after COC collection, CL was detected in 19 calves (19/21; one calf in each of the Low AFC groups did not have CL detected in any examination). More CL were detected in calves of the 7-day groups than in the 4-day groups (9.0±1.4 vs. 5.5±1.1; P=0.03), and calves in the High AFC groups tended to have a greater number of CL than in the Low AFC groups (8.9±1.4 vs. 5.9±1.2; P=0.07).

High AFC groups had a greater number of follicles available for COC collection (≥ 6 mm; 25.3±5.4 vs. 10.3±1.8; P=0.01; Fig. 4.5A) and greater number of COC collected than Low AFC groups (7.8±0.7 vs. 3.7±0.7; P=0.001; Fig. 4.5B), but the duration of gonadotropin treatment had no effect on either endpoint. The COC collection efficiency (i.e., the number of COC collected/the number of follicles ≥ 6 mm) was greater in the 4-day than in the 7-day groups (56.0±9.5 vs. 25.8±6.3%; P=0.02; Fig. 4.5C).

The majority of COC collected from calves in all groups were denuded (57.9%, 66/114) and not suitable for *in vitro* embryo production (25.4% - 29/114 expanded + partially expanded; 16.7% - 19/114 compact).

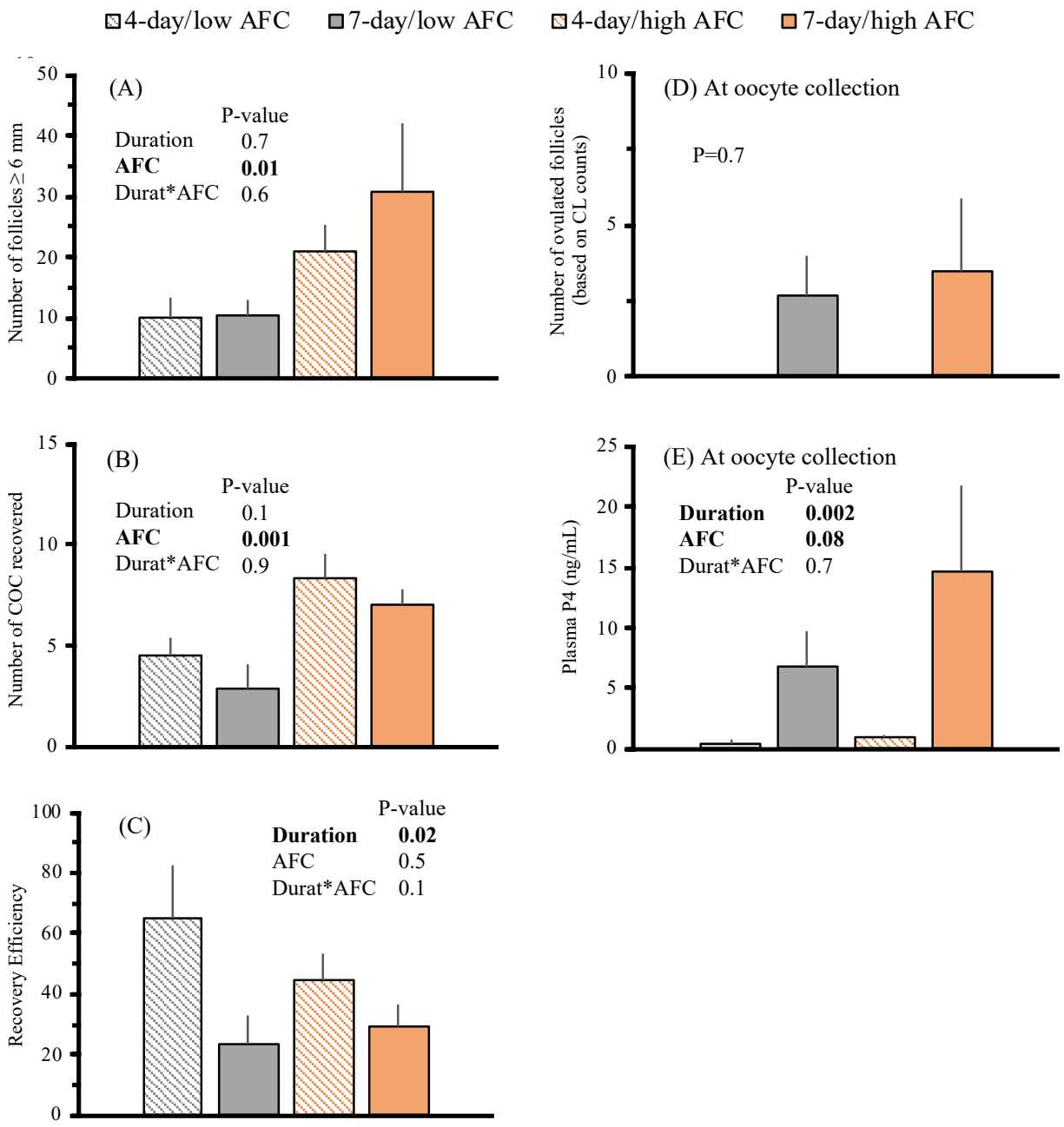

Fig. 4.5. Follicles available for aspiration and collection of cumulus-oocyte complexes (COC) (mean±SEM) in prepubertal calves with low vs. high antral follicle counts (AFC) given exogenous FSH treatment for 4 days vs. 7 days. (A) Number of follicles ≥ 6 mm; (B) Number of COC collected; (C) COC collection efficiency (i.e., number of COC collected/number of follicles ≥ 6 mm); (D) Number of ovulations at the time of COC collection (based on counts of corpora lutea); and (E) Plasma progesterone concentrations.

4.5.Discussion

The data from Study I demonstrates in prepubertal calves that the number of follicles at the beginning of a wave was predictive of the number recruited into subsequent waves and that AFC can be used to predict the ovarian response to gonadotropin treatment, therefore supporting our hypotheses. The AFC pattern at the time of wave emergence was maintained from 5.5 to 6.4 months of age, despite a decrease in the number of follicles ≥ 1 mm in calves of the high AFC group. This observation is perhaps a representation of the marked transition in follicle counts that have been observed in calves between 4 and 8 months of age [2, 126]. Furthermore, the number of follicles ≥ 1 mm at wave emergence was correlated with the number of follicles ≥ 6 and ≥ 9 mm post-FSH treatment, confirming a positive relationship between AFC and ovarian response in prepubertal calves. Spontaneous ovulations during gonadotropin treatment were confirmed by the presence of visible corpora lutea at the time of COC collection in calves of the 7-day groups, which were included into the number of large follicles for statistical comparisons. Study II showed that calves in the high AFC groups had a higher number of follicles ≥ 6 mm and COC collected than calves in the low AFC groups. Seven days of gonadotropin treatment resulted in a greater number of large than small-sized follicles and greater proportion of large-sized follicles at the end of the treatment than 4 days, but since the number of follicles ≥ 6 mm available for aspiration at COC collection and the number of COC collected was not different between treatment groups, our Study II hypothesis was not supported. Although it was not the objective of these studies, it is important to note that the majority of COC collected from all calves were denuded and not suitable for *in vitro* embryo production, which requires further investigation.

In the first study, we measured and counted antral follicles by transrectal ovarian ultrasonography after an induced wave of follicular development in calves at 5.5 and at 6.4 months of age and we concluded that the number of follicles ≥ 1 mm at the beginning of a wave was predictive of the number of follicles ≥ 1 mm recruited into subsequent waves as has been described in sexually mature cattle [129]. Transrectal ovarian ultrasonography in prepubertal calves poses a challenge in the field because it requires physical restraint of the animal, suitable personnel and ultrasound equipment; however, counting and measuring antral follicles after an induced wave of follicular growth in calves at 5 months of age was accomplished. The AFC pattern based on the number of follicles at the time of wave emergence remained consistent for all calves when a wave

of follicular development was induced a month later, even after an observed decrease in the number of follicles in the high AFC group. Major changes in the number of antral follicles and the diameter of the dominant follicle were observed to occur between birth and 8 months of age in prepubertal calves, characterizing a transitional phase of the hypothalamus-pituitary-gonadal axis. It was reported that after birth there is an increase in the mean number of antral follicles that is followed by a decrease between 4 and 8 months of age [2, 126], and possibly this decrease in the number of follicles is more pronounced in animals with high AFC, as we observed.

A strong relationship between the number of follicles ≥ 2 mm at the time of wave emergence of consecutive waves has been shown in cows and the number of follicles at the time of wave emergence can reliably predict the ovarian response to gonadotropin treatment [129]. In our study, ovarian response to superstimulation was also positively associated with the number of follicles ≥ 1 mm at the time of wave emergence, suggesting that donors can be selected at the prepubertal stages based on the number of follicles at the time of wave emergence. It has been suggested that AFC phenotype is established during the prepubertal period and sustained over time in cattle [150]. Higher AFC at the time of wave emergence resulted in a greater number of follicles available for aspiration and in a greater number of oocytes collected than lower AFC. In sexually mature animals, phenotypic classification based on AFC resulted in a greater number of transferable embryos recovered following superovulation in cows with high AFC [145] and high AFC was positively correlated with the number of morphologically healthy follicles and oocytes in the ovaries [151]. *In vitro* studies showed that granulosa cells recovered from follicles of animals with low AFC responded minimally to an FSH stimulus, resulting in less estradiol and AMH production when compared to granulosa cells of high AFC follicles [152]. Interestingly, the 7 days treatment resulted in greater ovarian response than the 4 days treatment in calves of the low AFC group in our study, suggesting that prolonged follicular growth may rescue more follicles, perhaps benefiting animals that have low AFC.

Gonadotropin treatment has been shown to increase not only the diameter but also the number of follicles of a suitable diameter available for aspiration when compared to non-stimulated animals. While the majority of follicles observed in non-stimulated prepubertal calf ovaries were under 3 mm in diameter, gonadotropin treatment given for 3 days increased the proportion of follicles > 5 mm and resulted in higher rates of embryo production than treatment given for 1.5 days or no treatment [87]. An increase in oocyte developmental competence has been observed

with increased follicle size [153] at least until follicles reach 8 mm in diameter [97] and COC collected from follicles > 6 mm in diameter showed superior developmental capacity than those from smaller follicles [15]. In Study II, calves with low and high AFC given 7 days of FSH treatment had a greater number of large than small-sized follicles that calves given 4 days of treatment, similar to the observations of our previous study in calves with medium AFC (*Unpublished, Chapter 3*). Our results are in agreement with previous results from our lab in sexually mature heifers [13] in which prolonged follicular growth under exogenous FSH support for 7 days resulted in a higher number of medium and large-sized follicles available for aspiration at the end of the treatment than 4 days; moreover, the 7 days treatment resulted in higher proportion of fully expanded COC collected and 2.5 more transferable embryos produced *in vitro*. Therefore, increasing follicular size by prolonged follicular growth may be a strategy to improve oocyte competence in prepubertal calves, since calf oocytes showed reduced developmental capacity when compared to oocytes from sexually mature animals [6, 7, 105].

Corpora lutea (CL) were detected in some calves at the time of COC collection confirming that ovulations had occurred during gonadotropin treatment. Because ultrasound examinations were not performed daily, it was not possible to determine the precise timing of ovulations based on the disappearance of large follicles between successive examinations; therefore, the number of ovulations was estimated from CL counts at the time of oocyte collection. In an earlier study, spontaneous ovulations were also reported in gonadotropin-treated calves by the visualization of corpora lutea during laparoscopy to recover COC [154]. It is plausible to assume that spontaneous ovulations reduced the number of follicles available for aspiration; moreover, higher recovery rates have been reported in follicles between 7 and 10 mm in diameter than from larger follicles [155]. Therefore, recovery efficiency in 7 days treatment groups might have been impaired not only by the reduced number of follicles available for aspiration but also by the proportionally higher number of large-sized follicles. Lower recovery rates have also been reported after *in vivo* oocyte maturation, due to increased viscosity of the follicular fluid and expansion of cumulus cells [154]. Cumulus-cell expansion is a morphological marker of oocyte maturation [156], but the majority of the COC collected from all calves in this study were denuded, regardless of the treatment group and the reasons remain to be addressed.

Based on plasma progesterone levels at the time of COC collection (≥ 1 ng/mL), calves that spontaneously ovulated during gonadotropin treatment produced functional corpus luteum. Eighty

hours after COC collection, a greater number of corpora lutea was observed in calves of the high AFC group that were given the 7 days treatment, than in the other calves. It is important to note that during this ovarian examination, differentiation of corpora lutea formed from spontaneous ovulations from those possibly resulting from follicular aspiration was impracticable.

In conclusion, the number of follicles ≥ 1 mm at wave emergence was predictive of the number of follicles recruited into subsequent waves, and the ovarian response to exogenous FSH treatment was positively associated with the number of follicles ≥ 1 mm at the time of wave emergence in the prepubertal calves of our study. Seven days of exogenous FSH treatment resulted in a higher number of large than small-sized follicles than 4 days of treatment, but spontaneous ovulations during FSH treatment resulted in a lower number of COC collected in the 7 days than in the 4 days groups. A greater number of follicles ≥ 6 mm were available for aspiration, and a greater number of COC was collected from calves of the high AFC than from calves of the low AFC. The majority of COC collected from all calves was not suitable for *in vitro* embryo production due to the absence of cumulus cells. Prolonged follicular growth under exogenous FSH support requires control of endogenous LH release to prevent spontaneous ovulations before oocyte collection.

CHAPTER 5

5. RELATIONSHIP BETWEEN ANTRAL FOLLICLE COUNTS, ENDOGENOUS AMH AND FSH LEVELS, AGE AND DURATION OF FSH TREATMENT ON THE OVARIAN RESPONSE IN CATTLE

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Relationship of this study to the dissertation

Previous studies showed that exogenous FSH treatment for more than 4 days in prepubertal calves requires the control of endogenous LH release to prevent spontaneous ovulations during treatment. In this study, the ovarian response of prepubertal calves at 4 and 7 months of age and sexually mature heifers to different durations of FSH treatments under controlled endogenous LH release was investigated. In adult cattle and other female mammals such as women, AFC has been shown to correlate with plasma concentrations of AMH and both are considered to be reliable markers of the ovarian reserve and the ovarian response to exogenous gonadotropin treatments. Circulating concentrations of AMH were reported to be stable during the estrous cycle of pubertal animals but were shown to have a characteristic pattern during the prepubertal period. Therefore, the relationship between AFC and plasma AMH and FSH concentrations at wave emergence in prepubertal calves were evaluated.

Authors' contribution: ARTK, FCFD and JS conceived and designed the experiment. ARTK conducted and coordinated the experiment. ARTK and SLC collected the data. ARTK analyzed and interpreted the data. ARTK wrote the draft of the manuscript. FCFD, SLC, GPA, RJM and JS reviewed the manuscript.

5.1. Abstract

The objectives were to investigate the relationship between the antral follicle counts (AFC) and plasma concentrations of AMH and FSH at the time of wave emergence, as well as the relationship between the AFC and plasma concentrations of AMH at wave emergence and the ovarian response following superstimulation in prepubertal calves. The effects of age and duration of FSH treatment on the ovarian response of prepubertal and pubertal cattle were also compared. Crossbreed Hereford calves (n=20) were assigned randomly to 4 days (4-day, 8 treatments; 25 mg i.m. B.i.d.) or 7 days (7-day, 14 treatments; 25 mg i.m. B.i.d.) of pFSH treatment at 4-months and again at 7-months of age. Sexually mature heifers were also assigned randomly to the two gonadotropin treatments (n=8 at first and 10 second replicates) along with the calves. Transvaginal ultrasound-guided follicle ablation was done in all animals to synchronize follicular wave emergence followed by insertion of progestin ear implants (Crestar) in calves and progesterone intravaginal device (CIDR) in heifers of the first trial, while heifers in the second trial had a mid-cycle functional corpus luteum. Gonadotropin treatments were initiated 36 hours after follicle ablation and followed by 12.5 mg of pLH treatment 12 hours after the last FSH treatment. Blood samples were collected at the time of first FSH treatment, and follicular fluid and oocytes were collected by transvaginal ultrasound-guided follicular aspiration 24 hours after pLH. Data were analyzed using correlation analysis, paired t-tests, and factorial ANOVA for repeated measures. At the time of wave emergence, the counts of antral follicle ≥ 1 mm (AFC, 31.1±4.0 vs. 16.2±1.8; $P<0.001$) and plasma anti-Müllerian hormone concentrations (AMH, 606.4±90.5 vs. 279.6±28.3 pg/mL; $P=0.001$) were higher at 4-months than at 7-months of age, while plasma FSH concentrations did not differ (0.54±0.05 vs. 0.51±0.03 ng/mL, respectively; $P=0.4$). Plasma AMH was correlated with AFC at 4-months ($r=0.8$; $P<0.001$) and 7-months ($r=0.7$; $P=0.0001$) of age but FSH was not significantly correlated with either AFC or AMH. AFC and AMH concentrations were correlated with the number of follicles ≥ 6 mm at the end of the gonadotropin treatment at both 4 ($r=0.7$ and 0.6, respectively; $P<0.01$) and 7 months ($r=0.7$ and 0.4; $P\leq0.04$) of age. After gonadotropin treatment, a higher number of follicles ≥ 6 mm were observed at 4 than at 7 months of age (32.4±5.4 vs. 14.9±2.0; $P=0.003$), and in pubertal heifers (22.0±2.3) than in 7-month-old calves. The number of large (≥ 9 mm) follicles was greater in calves given the 7-day than the 4-day FSH treatment at 4 months of age (36.2±6.9 vs. 10.4±2.9; $P=0.006$), but not at 7 months

(10.5±2.4 vs. 6.9±1.9; P=0.9). In follicles aspirated at the time of oocyte collection, the 7-day FSH treatment resulted in lower intrafollicular estradiol concentrations (23.7±4.5 vs. 144.0±29.5 ng/mL; P<0.0001), while intrafollicular progesterone tended to be higher (217.5±29.3 vs. 157.0±33.9 ng/mL; P=0.07) than in the 4-day treatment. The number of recovered cumulus-oocyte-complexes (COC) was greater in calves at 4 months than at 7 months of age and heifers (13.4±2.6 vs. 5.8±1.1 vs. 6.0±1.0; P=0.008). The proportion of fully expanded recovered COC was greater in calves at 7-months of age than in heifers (57.7±9.4 vs. 30.8±8.4%; P=0.05), but not different from calves at 4 months of age (33.2±8.2), while the 7-day treatment tended to result in greater proportion of expanded COC than the 4-day (50.1±7.7 vs. 31.9±6.8%; P=0.07). In summary, plasma AMH concentrations were useful as a marker of the AFC in prepubertal calves. Both AMH and AFC were predictive of ovarian response to exogenous FSH treatment in calves. Higher follicular responses to FSH treatment was recorded in calves at 4 than at 7 months of age, and prolonged follicular growth under exogenous FSH support resulted in evidence of follicular maturation as characterized by estrogen-inactive follicles and a higher proportion of fully expanded COC recovered.

Keywords: prepubertal, pubertal, follicular growth, superstimulation, oocytes, intrafollicular steroids.

5.2. Introduction

Gonadotropin treatment has been used widely to increase the number of recovered oocytes and bovine embryos for *in vitro*/*vivo* procedures, cryopreservation and embryo transfer. Prepubertal calf oocytes have been reported to have low developmental competence [7, 8, 10, 105, 106, 112, 113, 124, 157], and although *in vitro* embryo production from calf oocytes have been achieved, embryo production rates are lower even after superstimulation when compared with sexually mature animals [6, 11, 107, 112]. Since the follicular environment has direct effects on oocyte competence, special attention has been given to the length of follicular growth under exogenous FSH support. Extension of the follicular growth phase with exogenous FSH resulted in a higher number of follicles reaching an ovulatory size in prepubertal (*Unpublished, Chapter 3 and 4*) and adult cattle and a greater number of competent oocytes [13, 133]. In sexually mature heifers,

extending follicular growth by three days with exogenous FSH resulted in 2.5 times more embryos produced than conventional treatment [13], while in prepubertal calves, three days of exogenous FSH support resulted in higher rates of embryo production than 1.5 days or no FSH treatment [87].

As oocytes from follicles > 6 mm have been shown to have greater developmental capacity than oocytes from smaller follicles [15], increasing the length of follicular growth may enable oocytes to complete the synthesis of molecular products that are required for further development; which could be of great importance for prepubertal calf oocytes since their low developmental capacity has been presumed to be related to poor cytoplasmic maturation [7, 105]. According to a study that examined calves from 2 to 36 weeks of age [2], the interwave interval in 6-month old calves was approximately 7 days, in which growing, static and regression phases lasted 3.5, 4.4 and 4.4 days, respectively. Furthermore, it has been shown that the developmental competence of oocytes is acquired late in the follicular phase, in follicles that present first signs of regression [158, 159] or atresia [84]. Therefore, in order to recover oocytes of high developmental capacity in gonadotropin-treated animals, an ideal follicular environment must be achieved, and a greater understanding of follicular dynamics is essential for the control of follicular development during hormonal treatments. FSH dose regulates follicular growth, and an optimal FSH dose is required in order to sustain follicular development. Reduced FSH support following few days of exogenous FSH treatment, known as FSH 'coasting' or 'starvation', aiming to improve oocyte developmental competence by simulating preovulatory changes experienced by the dominant follicle, has resulted in consistent embryo production in sexually mature cattle that are dependent on a precise coasting period [13, 86, 97, 133, 160], but with no significant improvements in embryo production in prepubertal calves [11].

Follicular dynamics in cattle is characterized by a wave-like pattern that is observed in 2-week old calves [2, 44]. In sexually immature animals, there is an increase in follicle number and size with age, while the number of large follicles increases from 2 to 34 weeks of age, remaining steady after that [2, 126]. The increase in the number of follicles from birth to 4 months of age likely represents a major phase of postnatal follicular development [2] and was associated with increased response to gonadotropin treatment (superstimulation [82]). Indeed, a strong effect of age on the number of follicles and recovered oocytes was observed in donor calves in a commercial setting [11], in which a higher number of follicles were aspirated after gonadotropin treatment in calves between 5 and 7 months of age than in sexually mature donors.

Recent data have shown that the number of follicles at the beginning of a wave is predictive of the number of follicles in subsequent waves in prepubertal calves (*Unpublished, Chapter 4*). One study showed that there was an apparent increase in serum FSH concentrations before wave emergence, but significant peaks were observed only in two-week-old calves [2]. Estradiol concentrations tended to increase with age when associated with the end of the growing phase of the dominant follicle in 2- to 36-week-old calves, while serum concentrations of LH and progesterone did not change during follicular development [2]. In another study plasma concentrations of AMH at the time of oocyte collection were correlated positively with the antral follicle population, the number of recovered oocytes and the number of embryos produced *in vitro* in gonadotropin-treated calves [88]. AMH is a glycoprotein expressed by granulosa cells of small growing antral follicles and has been used as an endocrine marker of the size of the ovarian follicular pool and predictive of ovarian response to gonadotropin treatment [161-163]. Although an understanding of ovarian physiology and endocrine profiles at the time of wave emergence in prepubertal calves is increasing, knowledge of these events during exogenous gonadotropin treatment is incomplete.

The objectives of this study were to 1) investigate the relationship between the antral follicular counts (AFC) and plasma concentrations of AMH and FSH at the time of wave emergence at 4 and 7 months of age in prepubertal calves, and 2) to compare the effect of age and duration of gonadotropin treatment on a) number and size of follicles at the end of the treatment, b) intrafollicular concentrations of estradiol and progesterone and c) number and quality of recovered oocytes after *in vivo* maturation. We hypothesized that the total number of follicles ≥ 1 mm at the time of wave emergence would be correlated positively with plasma AMH concentrations and negatively with plasma FSH concentrations in calves at 4 and 7 months of age. Also, regardless of age, extending the follicular growth phase by three days with exogenous FSH support will result in 1) a higher number of follicles ≥ 3, ≥ 6 and ≥ 9mm at the end of treatment (i.e., 24 hours after pLH treatment), 2) lower intrafollicular estradiol to progesterone ratio; and 3) a greater number of recovered (and expanded) cumulus-oocyte complexes (COC). By design, gonadotropin treatments were administered under exogenous progesterone to prevent spontaneous ovulation.

5.3.Material and Methods

5.3.1. Animals and Experimental Design

The experiments were conducted at the Goodale Research Farm, University of Saskatchewan, Saskatoon, SK, Canada. All animal procedures were approved by the University of Saskatchewan's Protocol Review Committee and in accordance with the Canadian Council on Animal Care. The studies were performed in two replicates, Replicate one was done during July and August, while Replicate two was done during November and December.

Twenty crossbreed Hereford calves were selected from a group of spring-born calves to form a homogeneous group, based on the date of birth and body weight at birth (the heaviest calves were selected) for gonadotropin treatment. The study was initiated when calves were at 4.1 months of age (124±1.8 days; 177.2±4.2 kg; Replicate 1) and repeated when the calves reached 7.6 months of age (228.3±1.1 days; 279.7±5.7 kg; Replicate 2). During each replicate, sexually mature heifers (n=8, 2.6±0.3 years old; 422.1±8.7 kg during the first replicate and n=10, 2.8±0.2 years old; 557.5±23.9 kg during the second replicate) were included for gonadotropin treatment.

On Day -1, transvaginal ultrasound-guided follicular ablation of all follicles ≥ 5 mm was done under caudal epidural anesthesia (2% lidocaine HCl and epinephrine USP; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) in all animals to synchronize the emergence of a new follicular wave (Day 0). At the time of follicular ablations, calves received an auricular progestin ear implant containing 3.0 mg of Norgestomet (Crestar®; MSD Animal Health, Cruzeiro, SP, Brazil), while heifers in Replicate 1 received an intravaginal device containing 1.38 g of progesterone (Eazi-Breed™ CIDR®; Zoetis Canada Inc., Kirkland, QB, Canada). In Replicate 2, follicular ablation was done in heifers between Days 5 and 7 of the estrous cycle in the presence of a functional corpus luteum (therefore, a progesterone device was not used). Animals were randomly assigned to gonadotropin treatments that were initiated 36 hours after follicular ablation (Day 0.5) in all animals and consisted of 8 (4-day group, n = 10 calves/4 and 5 heifers) or 14 (7-day group, n = 10 calves/4 and 5 heifers) i.m. treatments of 25 mg of pFSH (Folltropin-V®; Vetoquinol, Lavaltrie, QB, Canada) administered at 12-hour intervals. All animals received 12.5 mg of pLH (Lutropin-V®; Vetoquinol, Lavaltrie, QB, Canada) i.m. 12 hours after the last FSH treatment. Heifers were given two doses of a prostaglandin F2α analog i.m. (500 μg of cloprostenol; Estrumate®; Intervet/Merck Animal Health, Madison, NJ, USA) concomitant with the last two FSH

treatments and progestin implants/devices were removed at the time of the last FSH treatment in all animals. Transvaginal ultrasound-guided follicle aspiration for oocyte collection was done 24 hours after the pLH treatment.

5.3.2. Ovarian Ultrasound Examinations and Transvaginal Ultrasound-guided Follicle Aspirations

Ovarian ultrasound examinations were performed transrectally using a 7.5-MHz linear-array transducer (MyLab™ Alpha on replicate one and MyLab™ Five on replicate two; Esaote Canada, Indianapolis, IN, USA) in all animals at the time of the first FSH treatment, on Day 2.5 (5^{th} FSH treatment), at the time of the last FSH treatment, at the time of pLH treatment and immediately before oocyte collection. Animals in the Long treatment were also examined on Day 5.5 (11^{th} FSH treatment). Cine-loops from 7 to 30 seconds were recorded for each ovary and follicles were measured and counted. For examinations at the time of wave emergence, all follicles ≥ 1mm were counted for each ovary while for ultrasound recording after gonadotropin treatments, follicles ≥ 3mm were counted.

All oocyte collections were done in animals in a standing position under caudal epidural anesthesia (2% lidocaine HCl and epinephrine USP; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) by using a custom-designed transducer handle fitted with a needle guide (thin design to fit in calf vagina). The handle was designed to attach around a 5 MHz transvaginal transducer, and the same system was used to collect heifers. A single experienced operator performed all collections and no injuries occurred in any of the calves. Follicular contents were aspirated using an 18 G needle attached to a vacuum pump with a flow-rate set at 20-25 mL/min. Contents were collected in a 50 mL centrifuge tube containing 5 mL of collection medium at 36°C. Collection medium consisted of Dulbecco's phosphate-buffered saline (DPBS 1X containing calcium and magnesium; Gibco, Carlsbad, CA, USA) with ET surfactant (0.3% Pluronate; Bioniche Animal Health, Belleville, ON, Canada) and sodium heparin (2 IU/mL, Heparin Sodium Injection USP; Sandoz Canada Inc.).

5.3.3. Cumulus-Oocyte-Complex (COC) Classification

Fluid containing follicular contents was filtered through an embryo filter (EmCon Filter) immediately after collection and COC were searched under a stereomicroscope (10x). Since COC

were collected 24 hours after pLH treatment and were expected to be matured *in vivo*, classification consisted of visual observation (40 to 80x) of the cumulus cell expansion in four categories: expanded, partially expanded, compact and denuded. Expanded COC were considered when full expansion of cumulus cells was observed, partially expanded when only the outer most layers of cumulus cells were expanded, compact when no cumulus cell expansion was observed and denuded when cumulus cells were absent.

5.3.4. Blood Collection and Hormone Analyses

Blood samples were collected in heparinized tubes (BD Vacutainer®, BD Franklin Lakes, NJ, USA) via jugular venipuncture at the time of the first FSH treatment (36 hours after follicular ablation), at the time of the last FSH treatment, at the time of pLH treatment and immediately before COC collection, as well as 3, 5 and 7 days after COC collection in all animals. Blood samples were centrifuged, and the plasma was stored at -20°C until analysis. Plasma concentrations of FSH and AMH were measured at the time of the first FSH treatment, while plasma LH concentrations were measured at the time of the last FSH treatment, at the time of pLH treatment and immediately before oocyte collection. Plasma progesterone concentrations were measured at the time of pLH treatment, immediately before COC collection and 3, 5 and 7 days after COC collection.

Anti-Müllerian hormone concentrations were analyzed using a commercial ELISA kit (Anti-Müllerian Hormone ELISA Sample Test Kit for Bovine Blood Serum; Mofa®, Verona, WI, USA) according to manufacturer's instructions. Plasma FSH concentrations were measured in duplicates using validated radioimmunoassay (RIA; [164, 165]), the range of the standard curve was 0.1 to 8 ng/mL, and the intraassay coefficients of variation were 3.3 and 3.1% for plasma with concentrations of 0.33 and 1.44 ng/mL, respectively.

Plasma LH concentrations were measured in duplicates using a validated double-antibody RIA [2]. The range of the standard curve was 0.06 to 8 ng/mL, and the intraassay coefficients of variation were 3.2 and 4.1% for plasma with concentrations of 0.66 and 1.29 ng/mL, respectively, in Replicate 1 and 3.7 and 4.3% for plasma with concentrations of 0.69 and 1.36 ng/mL, respectively, in Replicate 2.

Plasma progesterone concentrations were measured using a commercial radioimmunoassay kit (ImmunoChem™ Progesterone ^{125} RIA kit, MP Biomedicals, Costa Mesa, CA, USA). The range

of the standard curve was 0.15 to 80 ng/mL, and the interassay coefficients of variation were 9.4%, 7.3% and 6.0% for low (0.83 ng/mL), medium (4.30 ng/mL) and high (8.76 ng/mL) reference serum, respectively, in Replicate 1 and 12%, 4.5% and 9.8% for low (0.90 ng/mL), medium (3.86 ng/mL) and high (9.82 ng/mL) reference serum, respectively, for Replicate 2.

5.3.5. Intrafollicular Estradiol and Progesterone Concentrations

At the time of COC collection, the contents of two follicles from one ovary of each animal were aspirated using an empty 6 mL syringe. Follicular fluid was transferred to a 35 mm petri dish, and the COC were searched. Immediately after COC removal, undiluted follicular fluid was centrifuged at 8000 rpm for 8-10 min, and the supernatant was stored at -20°C until hormone analysis. Estradiol concentrations were estimated in duplicates from diluted samples in PBS (1:50, 1:100, 1:200, and 1:500) using the previously described radioimmunoassay procedure [166]. Dilutions were increased until the majority of the samples were in the range of the standard curve. The range of the standard curve was 2 to 1000 pg/mL, and the intraassay coefficients of variation were 16.1 and 2.2% for follicular fluid with concentrations of 20 and 44.7 pg/mL, respectively. Progesterone concentrations were measured in duplicates from undiluted and diluted samples in PBS (1:10) using the same assay as for plasma. The range of the standard curve was 0.15 to 80 ng/mL, and the intraassay coefficients of variation were 3.3, 1.4 and 2.7% for follicular fluid with low (1.1±0.2 ng/mL), medium (4.8±0.7 ng/mL) and high (9.1±0.9 ng/mL) reference levels, respectively. Reported values represent the 1:100, 1:200 and 1:500 corrected for dilutions for estradiol and 1:10 corrected for dilutions for progesterone. When intrafollicular hormone concentrations were above the standard curve at the given dilutions, values were excluded from data analyses.

5.3.6. Statistical Analysis

Pearson's correlation (for numerical values of endpoints; denoted by 'r' in the manuscript) and Spearman's rank-order correlation (animal ranks based on the numerical values of endpoints; denoted by 'r_s') coefficients were used to determine the relationship between follicle counts and plasma concentrations of AMH and FSH at the time of wave emergence, while regression analysis was used to determine the association between these variables. Plasma concentrations of AMH and FSH, the ovarian response (number of follicles and recovered oocytes, recovery rate) and

intrafollicular concentrations of estradiol and progesterone were analyzed for age, treatment effect, and their interactions using repeated measures for comparisons between calves at 4 months and 7 months of age and using General Linear Model (GLM) for comparisons among the three age groups (4-month calves, 7-month calves and pubertal heifers). Plasma concentrations of LH and progesterone were analyzed using repeated measures for age, treatment and day effects. Differences with $P \leq 0.05$ were considered significant, while P values between 0.05 and 0.10 were considered as tendencies. If the main effects or their interactions were significant or had a tendency for a difference, multiple comparisons were performed by Tukey's test. Log transformation was performed when data did not meet the assumptions of equal variances and normality of the residuals, but the mean and the standard error of the mean (Mean±SEM) of raw data are reported and used to illustrate all response variables in figures.

5.4.Results

5.4.1. Number of Follicles and Plasma Concentrations of AMH and FSH at Wave Emergence

The number of follicles ≥ 1 mm and plasma concentrations of AMH and FSH at the time of wave emergence, their correlations, and regression equations are presented in Figures 5.1 and Table 5.1. The AMH levels were higher than the mean + 3x Std. Dev in four calves (Calf # 6, 125 and 145 at 4 months and Calf # 175 at 7 months), and as they were considered outliers and their data were excluded from all data analyses. The number of follicles ≥ 1 mm at the time of wave emergence was almost two times higher at 4 months than at 7 months of age (31.1±4.0 vs. 16.2±1.8; $P<0.001$; a range of 10 to 72 and 6 to 33 follicles, respectively; Fig. 5.1A). The plasma AMH concentrations were also higher at 4 months compared to 7 months of age (606.4±90.5 vs. 279.6±28.3 pg/mL; $P=0.001$; a range of 140.4 to 1296.2 and 101.6 to 604.2 pg/mL, respectively; Fig. 5.1B). Plasma FSH concentrations did not differ between the age groups (0.54±0.05 vs. 0.51±0.03 ng/mL; $P=0.4$; range of 0.23 to 0.96 and 0.27 to 0.84 ng/mL, respectively; Fig. 5.1C).

As samples were collected before the initiation of FSH treatment, there was no difference ($P > 0.6$) between the 4-day and 7-day FSH treatment groups in any of the endpoints confirming that calves were assigned to treatment groups randomly. When data were combined between age groups, plasma AMH and the number of follicles ≥ 1 mm showed a strong relationship ($r=0.827$;

P<0.001; Fig 5.1D), but plasma FSH concentrations were not correlated with either the number of ≥ 1 mm follicles at the time of wave emergence (r=-0.143; P=0.4; Fig. 1E) or the plasma AMH concentrations (r=-0.113; P=0.5; Fig. 5.1F).

When data were analyzed separately for the 4-month and 7-month-old calves, plasma AMH concentration was correlated with the number of follicles ≥ 1 mm at 4 months (r=0.813, P<0.001) and at 7 months of age (r=0.781, P=0.0001). Likewise, the animal ranks between AMH and the number of follicles ≥ 1 mm at 4 months (r_s=0.841; P<0.001) and at 7 months were correlated (r_s=0.658; P=0.003). Plasma FSH concentration was not correlated significantly to either the number of ≥ 1mm follicles or the plasma AMH (Table 5.1). The number of follicles ≥ 1 mm, plasma AMH, and plasma FSH concentrations at 4 months vs. 7 months of age were correlated (Table 5.1).

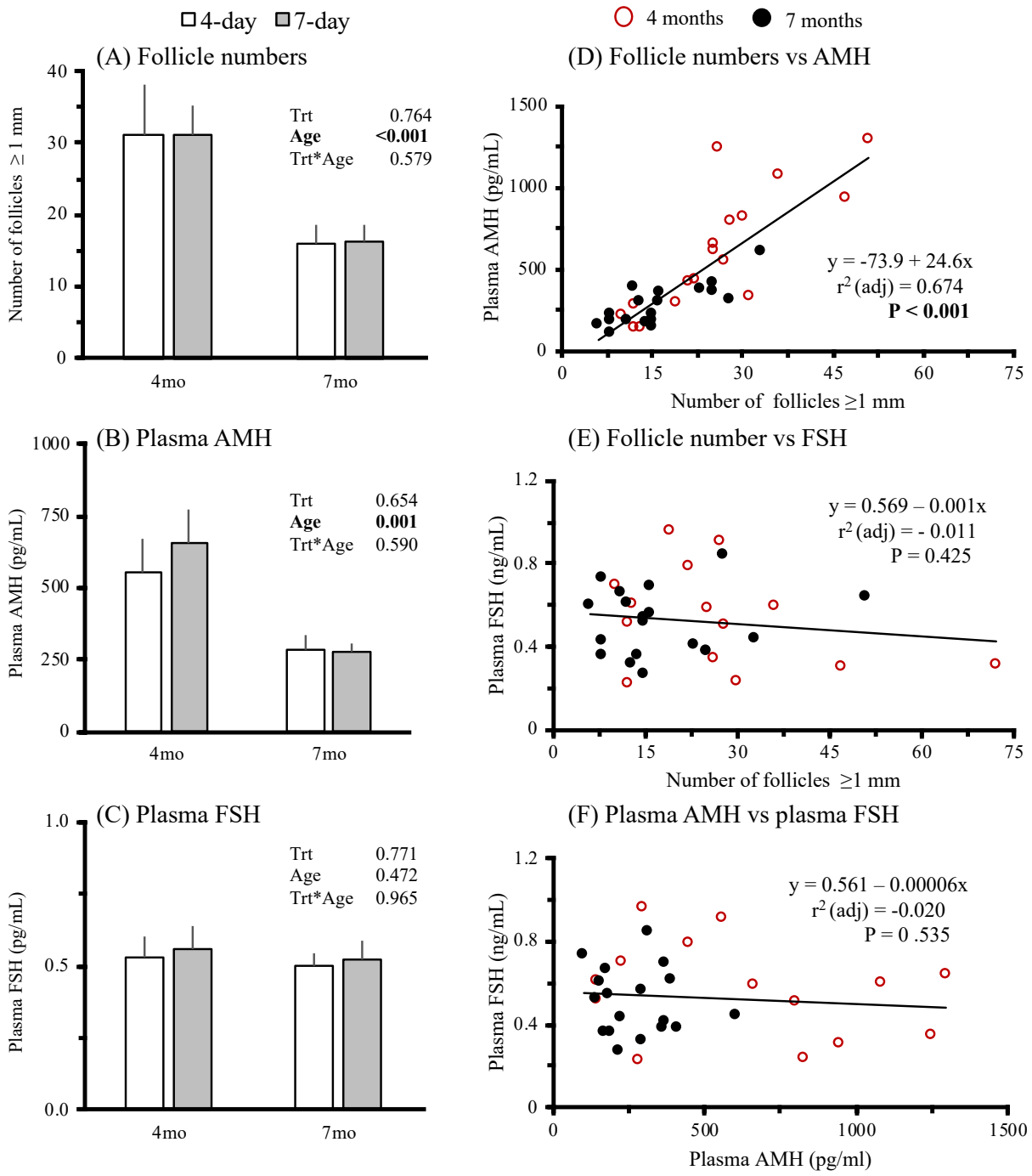


Fig. 5.1. The number of follicles ≥ 1 mm and plasma concentration of AMH and FSH at the time of wave emergence (i.e., 36 hours after follicular ablation); 4-day and 7-day FSH treatments were initiated after these data were recorded. (A) The number of follicles ≥ 1 mm; (B) Plasma AMH concentrations; (C) Plasma FSH concentrations in calves at 4 and 7 months of age in 4-day and 7-

day groups. Linear regression between the (D) Number of follicles ≥ 1 mm and plasma AMH concentrations, (E) Number of follicles ≥ 1 mm and plasma FSH concentrations and (F) Plasma AMH and plasma FSH concentrations. For analyses presented in the right column, the regression equation and adjusted regression coefficient (r^2(adj)) were obtained by combining data from 4 months (red open dots) and 7 months (solid black dots) calves.

Table 5.1. Pearson correlation (between numerical endpoints) and Spearman correlation (between animal ranks) coefficients between endpoints recorded in prepubertal calves at 4 and 7 months of age. Ultrasonographic data and blood samples were obtained at the time of wave emergence (i.e., 36 hours after follicular ablation).

First end point	Second end point	Pearson correlation coefficient (r) (P-value)	Spearman correlation coefficient (r_s) (P-value)
4mo – follicle count*	7mo – follicle count*	0.747 (0.0004)	0.656 (0.003)
4mo – follicle count*	4mo – plasma AMH	0.813 (<0.001)	0.841 (<0.001)
4mo – follicle count*	4mo – plasma FSH	-0.301 (0.250)	-0.311 (0.241)
4mo - plasma AMH	7mo – plasma AMH	0.535 (0.033)	0.485 (0.057)
4mo – plasma AMH	4mo – plasma FSH	-0.297 (0.302)	-0.244 (0.401)
4mo – plasma FSH	7mo – plasma FSH	0.492 (0.053)	0.564 (0.023)
7mo– plasma AMH	7mo – plasma FSH	-0.146 (0.550)	-0.056 (0.759)
7mo – follicle count*	7mo - plasma AMH	0.781 (0.0001)	0.658 (0.003)
7mo – follicle count*	7mo – plasma FSH	-0.056 (0.825)	-0.011 (0.965)

5.4.2. Number and Size of Follicles at the End of Treatment

The numbers of follicles ≥ 3, ≥ 6 and ≥ 9 mm (combined counts of left and right ovaries) for calves at 4 and 7 months of age and heifers are presented in Figure 5.2. Follicles were counted and measured using the ultrasound cine-loops recordings of each ovary immediately before COC collection (i.e., 24 hours after pLH treatment). The numbers of follicles ≥ 3 and ≥ 6 mm were two times greater ($P<0.003$) at 4 than at 7 months of age (36.4±5.7 vs. 16.2±2.1 and 32.4±5.4 vs. 14.9±2.0, respectively). The numbers of follicles ≥ 3 and ≥ 6 mm were also higher in heifers (24.7±2.7 and 22.0±2.3, respectively) than in calves at 7 months of age, but not different (albeit numerically lower) than the calves at 4 months of age. The number of follicles ≥ 6 mm (data combined among three age groups) was higher ($P=0.041$) in the 7-day than in the 4-day FSH treatment groups (27.3±3.8 vs. 18.8±2.4).

There was a tendency for a treatment by age interaction for the number of follicles ≥ 9 mm ($P=0.07$) resulting from a higher number of follicles in the 7-day FSH group at 4 months of age (36.2±6.9) than the 4-day FSH group at 4 months of age (10.4±2.9), both groups at 7 months of age (4-day = 6.9±1.9 and 7-day = 10.5±2.4) and the 4-day FSH treatment group in heifers (13.1±1.8), but not from the 7-day FSH group in heifers.

The number of follicles ≥ 1 mm at the time of wave emergence was correlated with the number of ≥ 6 mm follicles at the time of oocyte collection at 4 months ($r=0.697$; $P=0.0009$) and at 7 months ($r=0.727$; $P=0.006$) of age. Plasma AMH concentrations at the time of wave emergence were correlated with the number of follicles ≥ 6 mm at the time of oocyte collection at 4 months ($r=0.634$; $P=0.008$) and at 7 months ($r=0.473$; $P=0.04$) of age.

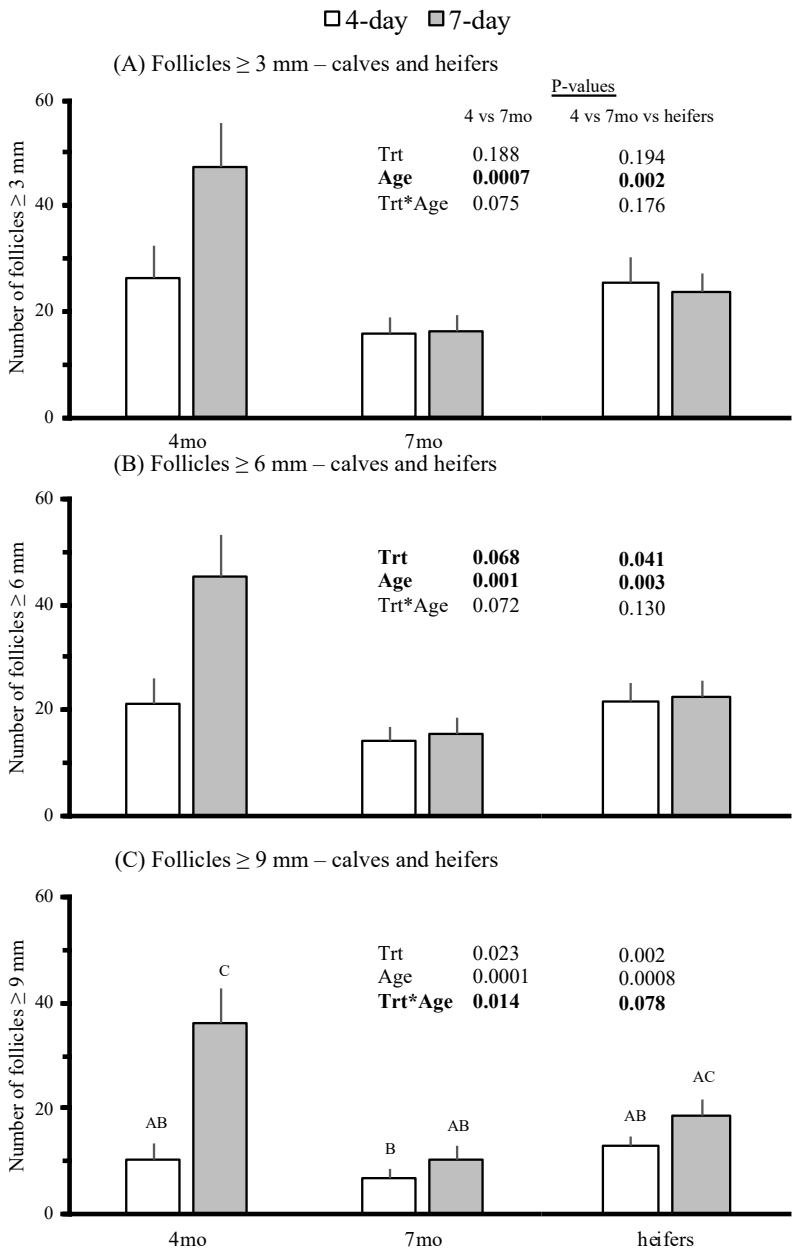


Fig. 5.2. Number and size of follicles at the time of oocyte collection (i.e., 24 hours after pLH treatment) in calves at 4 and at 7 months of age and in heifers that were given the 4-day (white bars) or the 7-day (grey bars) FSH treatment. The number of (A) follicles ≥ 3 mm, (B) follicles ≥ 6 mm and (C) follicles ≥ 9 mm. Repeated measures mixed model factorial analysis was used for comparisons between calves at 4 and at 7 months of age (P-values in the left column) and general linear model factorial analysis to compare calves and heifers (P-values in the right column). Bars with uncommon letters in Fig. C indicate statistical difference ($P<0.05$).

5.4.3. Intrafollicular Concentrations of Estradiol and Progesterone

The intrafollicular concentrations of estradiol and progesterone and E2:P4 ratios are presented in Figure 5.3. Overall, the intrafollicular concentrations of estradiol were higher in the 4-day than in the 7-day FSH groups (144.5±29.5 vs. 23.7±4.5 ng/mL; P<0.0001), and were higher in heifers than in calves at 4 and at 7 months (132.6±41.1 vs. 63.8±27.5 vs. 67.2±22.4 ng/mL; P=0.01), while progesterone concentrations tended to be higher in the 7-day than 4-day FSH groups (217.5±29.3 vs. 157.0±33.9 ng/mL; P=0.07). The estradiol to progesterone ratio was higher in the 4-day than 7-day FSH groups (1.04±0.3 vs. 0.3±0.1; P=0.005).

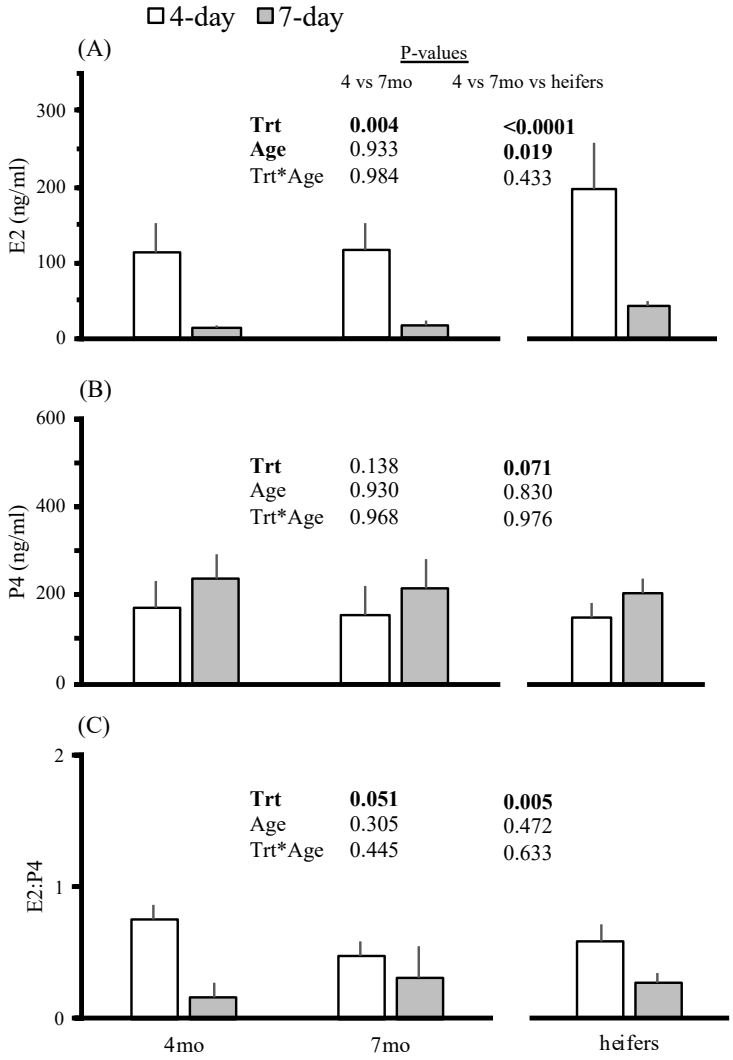


Fig. 5.3. Intrafollicular concentrations of (A) Estradiol (E2) and (B) Progesterone (P4) and (C) E2:P4 ratio in calves at 4 months and at 7 months of age and in heifers that were given the 4-day (white bars) or 7-day (grey bars) FSH treatment. Follicular fluid was aspirated from two follicles in each animal immediately before oocyte collection. Repeated measures mixed model factorial analysis was used for comparisons between calves at 4 and at 7 months of age (P-values in the left column) and general linear model factorial analysis to compare calves and heifers (P-values in the right column).

5.4.4. Number of Recovered Oocytes and Morphological Evaluation of COC

The number of recovered COC (Fig. 5.4) was higher in calves at 4 months (P=0.008) than at 7 months of age and in heifers (13.4±2.6 vs. 5.8±1.1 vs. 6.0±1.0), while recovery efficiency (i.e., number of recovered oocytes/number of follicles ≥ 6 mm) was not affected by treatment or animal age (overall 37.2±2.9%). The proportion of expanded COC recovered (Fig. 5.5) tended to be higher (P=0.07) in the 7-day than in the 4-day FSH groups (50.1±7.7% vs. 31.9±6.8%) and was higher (P=0.058) in calves at 7 months of age (57.7±9.4%) than in heifers (30.8±8.4%), but did not differ from calves at 4 months of age (33.2±8.2%). The proportion of compact COC tended to be higher (P=0.08) in the 4-day than in the 7-day FSH groups (18.2±4.2 vs. 8.9±2.9).

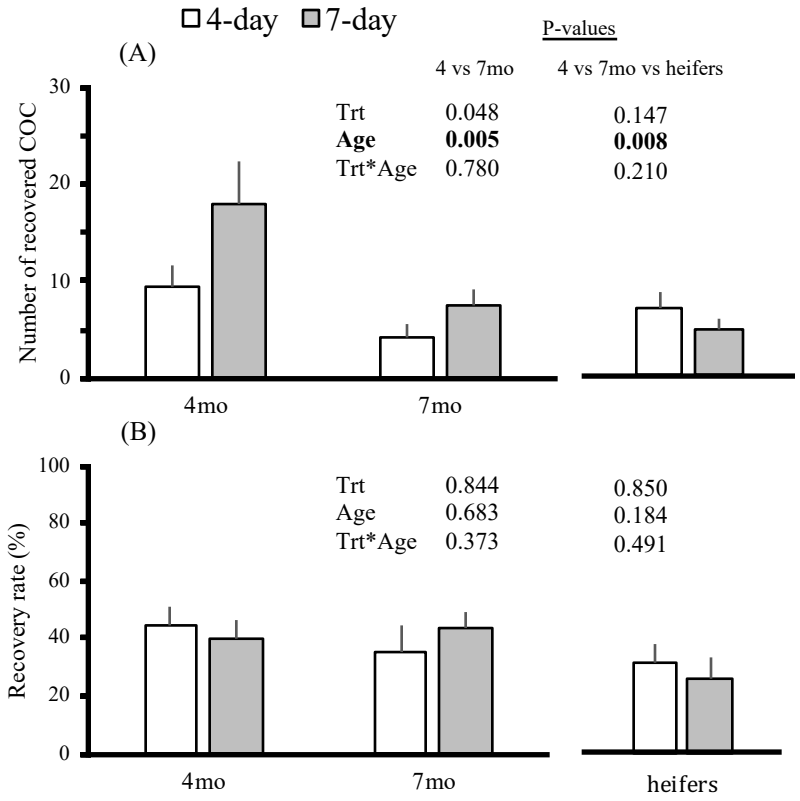


Fig. 5.4. (A) The number of cumulus-oocyte complexes (COC) recovered after transvaginal follicular aspiration and the (B) COC recovery efficiency (%), based on the number of follicles ≥ 6 mm in calves at 4 months and at 7 months of age and in heifers that were given the 4-day (white bars) or 7-days (grey bars) FSH treatment. Repeated measures mixed model factorial analysis was used for comparisons between calves at 4 and at 7 months of age (P-values in the left column) and general linear model factorial analysis to compare calves and heifers (P-values in the right column).

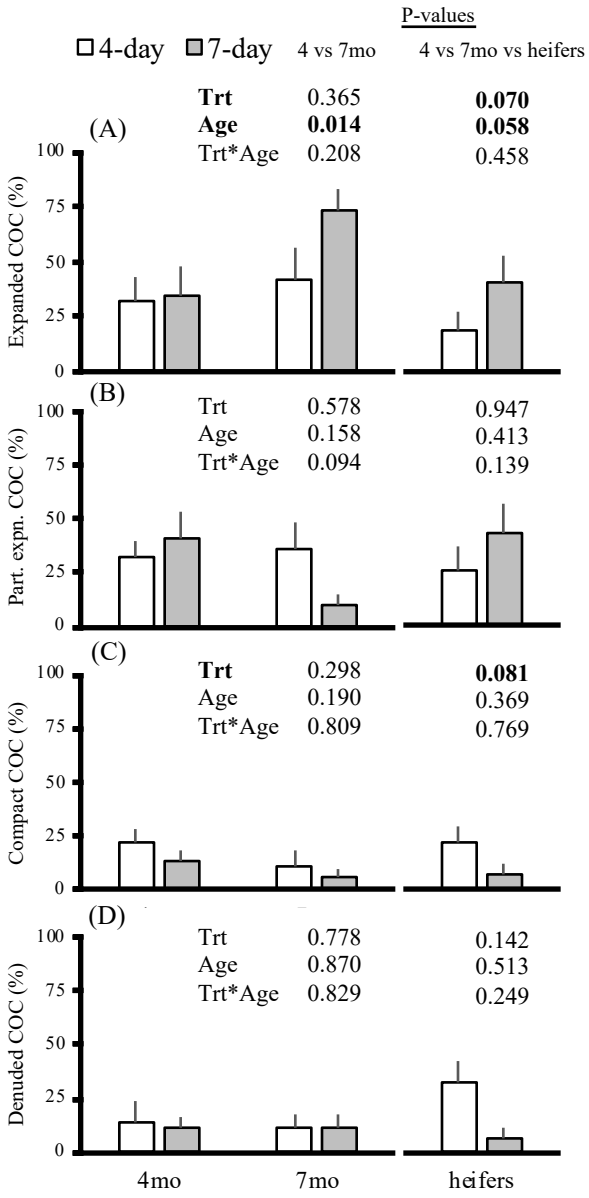


Fig. 5.5. Morphological evaluation of recovered COC in calves at 4 months and at 7 months of age and in heifers that were given the 4-day (white bars) or 7-day (grey bars) FSH treatment. COC were collected at 24 hours after pLH treatment and classified based on the characteristics of the cumulus cells in (A) Expanded; (B) Partially expanded; (C) Compact or (D) Denuded categories. Repeated measures mixed model factorial analysis was used for comparisons between calves at 4 and at 7 months of age (P-values in the left column) and general linear model factorial analysis to compare calves and heifers (P-values in the right column).

5.4.5. Plasma Concentrations of LH and Progesterone

Plasma LH concentrations were measured at the time of the last FSH treatment, immediately before pLH treatment and COC collection (Fig. 5.6A). The levels of plasma LH increased over time, from the time of pLH treatment to COC collection and were higher in heifers than in calves (0.30±0.07 vs. 0.22±0.04 ng/mL; Age*Day interaction P=0.05).

Plasma progesterone concentrations were measured on the day of COC collection, and 3 and 7 days after oocyte collection to investigate possible progesterone production for luteal structures formed after follicular aspiration. No corpora lutea were observed in ultrasonographic examinations at the time of COC collection and the plasma progesterone concentrations were low at that time (Fig. 5.6B), but plasma progesterone levels increased over time and were higher in the animals that were given the 7-day than the 4-day FSH treatment (9.5±1.8 vs. 4.0±0.9 ng/mL).

In calves at 4 months of age, plasma progesterone concentrations remained below 2 ng/mL from the time of pLH treatment to COC collection. Three calves in the Long FSH treatment group had progesterone concentrations between 1 and 2 ng/mL by the time of COC collection, one of which lost her progestin device during gonadotropin treatment and possibly ovulation was triggered, although luteal structures were not identified in ultrasonographic images at that time. When data were analyzed for calves alone, calves in the 7-day FSH group had higher progesterone levels on Day 7 after COC collection than the calves in 4-day FSH group (data not shown).

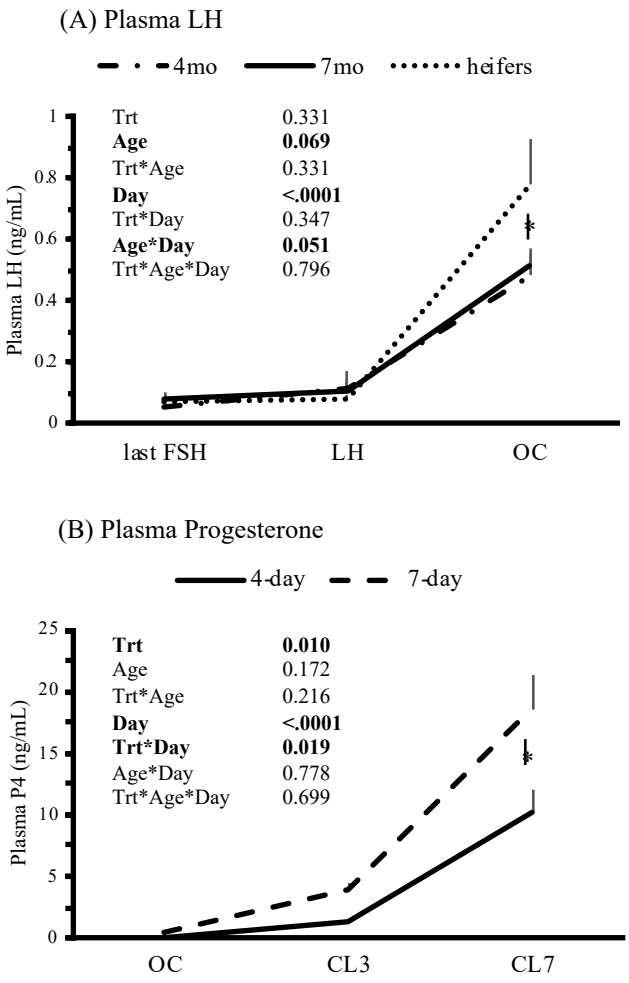


Fig. 5.6. Plasma concentrations of LH and progesterone (P4). Plasma concentrations of (A) LH at the time of the last FSH treatment (last FSH), immediately before pLH treatment (LH) and immediately before oocyte collection (OC). Repeated measures mixed model factorial analysis was used to evaluate the effect of treatment, age, and day on the plasma concentrations of LH. Graph illustrates the main significant effects (animals of the 4-day and the 7-day treatment were combined in each age group); and (B) Plasma progesterone concentrations (P4) immediately before COC collection (OC), and three days (CL3) and 7 days (CL7) after COC collection in calves at 4 months and at 7 months of age and in heifers that were given the 4-day and the 7-day exogenous FSH treatment. Repeated measures mixed model factorial analysis was used to evaluate the effect of treatment, age, and day on the plasma concentrations of LH. The graph illustrates the main significant effects (all animals that were given the 4-day or 7-day treatment combined).

5.5.Discussion

Investigating the relationship between AFC and plasma concentrations of AMH and FSH at the time of wave emergence in calves at 4 months and 7 months of age, we found a significant positive correlation between the number of follicles ≥ 1 mm and the plasma concentrations of AMH in calves at both ages. The number of ≥ 1 mm follicles and the plasma concentrations of AMH were greater in calves at 4 months than at 7 months of age, while plasma FSH concentrations did not differ between ages. We also found that the number of ≥ 1 mm follicles and the plasma concentrations of AMH at the time of wave emergence were both correlated with the number of follicles ≥ 6 mm at the time of COC collection, suggesting that plasma AMH concentrations can be used as a marker of the AFC and potential ovarian response to FSH treatments in prepubertal calves, as has been shown in adult cattle [161]. At the time of oocyte collection, the number of ≥ 6 mm follicles observed in calves at 4 months of age was more than double of the number of follicles observed in calves at 7 months of age, reflecting the high AFC that was observed at wave emergence and the major phase of antral follicular development that is observed in prepubertal cattle from birth to 4 months of age [2, 126]. Prolongation of the follicular growth under exogenous FSH support by three days resulted in a greater number of large-sized follicles available for aspiration and recovered COC than the conventional 4-day treatment. A higher level of follicular maturity, characterized by lower E2:P4 ratio and a higher proportion of fully expanded COC recovered, was also observed in the 7-day than in the 4-day treatment and in agreement with previous results presented for sexually mature animals only [13, 14]. Luteal structures capable of progesterone synthesis were formed in all animals after follicular aspiration and plasma progesterone concentrations at 7 days after oocyte collection was higher in the animals that were given the 7-day than the 4-day FSH treatment.

The number of follicles growing in each follicular wave is highly repeatable among adult cattle [128, 129] and similar findings were observed in the prepubertal calves in this study. Despite a decline in the number of follicles ≥ 1 mm from 4 to 7 months of age, a positive correlation was observed between the number of follicles ≥ 1 mm at the time of wave emergence and animal ranks based on AFC at 4 months and at 7 months of age. Different physiological states may result in differences in the number of recruited follicles in sexually mature cattle [167-169], but AFC in calves may also be influenced by a developing hypothalamic-pituitary-gonadal axis. Therefore,

variation in hormone levels and the number of ovarian follicles must be interpreted accordingly. A major phase of postnatal follicular development is observed during the first 4 months of age, this is followed by a decrease and subsequent stabilization in the number of follicles per wave after that [2, 126]. Accordingly, a higher number of follicles at the beginning of the wave was observed in calves of 4 months than at 7 months of age, while no differences in plasma concentrations of FSH were recorded. It is noteworthy that each wave of follicular growth is preceded by a surge in FSH levels in adult and prepubertal cattle [2, 44, 128], and that follicular products, e.g., estradiol and inhibin, subsequently suppress FSH release. Our hypothesis that FSH levels would be negatively associated with the number of recruited follicles was not supported and could be explained by the random selection of calves to the study, regarding the antral follicle population. Previous observations showing this negative correlation were demonstrated in animals that represented the AFC extremes of the herd [128, 129, 145]. However, AFC was highly correlated with plasma concentrations of AMH which are in agreement with the literature for adult cattle [151]. In this regard, plasma AMH concentrations at the time of wave emergence were also correlated with the number of follicles ≥ 6 mm observed at the time of oocyte collection. The higher AFC may explain the higher levels of AMH in calves at 4 months than at 7 months of age; plasma AMH levels were positively correlated at 4 and 7 months of age, and according to a recent study [170], plasma AMH levels in prepubertal calves is positively correlated with plasma AMH levels post-puberty. Therefore, AMH levels in 4-month old calves may be a reliable marker of ovarian reserve for the entire reproductive life of the animal.

While gonadotropin treatment has been reported to increase the numbers and diameters of follicles available for oocyte recovery at the end of treatment in calves [17, 89, 93], others have reported only an increase in follicle sizes [87, 94]. We have shown previously that both dose and length of gonadotropin treatment in prepubertal calves can influence the number and the size of follicles growing during the treatment (*Unpublished, Chapter 3*), perhaps due to direct effects on follicle growth rate as has been reported in adult cattle [132]. It is interesting to note that the 7-day FSH treatment resulted in a higher number of large follicles at the end of treatment than the traditional 4-day regimen when the cumulative dose per animal was higher, but the FSH dose per treatment was the same [13, 133]. In contrast, when total FSH dose was the same, follicular growth rate was slower in the 7-day treatment group as compared to the 4-day treatment, possibly due to a lower FSH dose per treatment [132]. Based on these studies, the 7-day FSH treatment was

expected to result in a higher number of large follicles, regardless of age, since the same dose per injection was given. However, this effect was observed only in calves at 4 months of age, and thus this hypothesis was not fully supported. The number of large-sized follicles (≥ 9 mm) was more than three times higher after the 7-day than the 4-day FSH treatment in 4-month-old calves, but a similar result was not observed in 7-month-old calves or pubertal heifers, which showed only slight numerical differences in follicle numbers between 4-day and 7-day FSH treatments.

It appears that oocytes from follicles > 6 mm in diameter have greater developmental capacity than oocytes from smaller follicles [15], thereby, a higher number of large follicles is the desired outcome of gonadotropin treatment. In this regard, we observed that prolonged follicular growth under exogenous FSH support resulted in overall higher degree of follicular maturation in our study, characterized by lower intrafollicular E2:P4 ratio and higher proportion of fully expanded cumulus-oocyte complexes recovered and similar findings were also observed in sexually mature cattle that were given the same duration and dose per injection of FSH treatment [13, 14]. It was shown that the 7-day treatment activated molecular mechanisms that are associated with LH responsiveness and increased the expression of markers related to oocyte competence in granulosa cells of cows when compared to the 4-day treatment [14]. Indeed, in heifers, the 7-day treatment resulted in 2.5 more transferable embryos per animal than the 4-day treatment [13]. On the other hand, a negative impact of artificially extending follicular growth has been shown through FSH coasting, in which FSH support is discontinued to induce follicular and oocyte maturation [86]. Oocyte collection performed between 44 and 68 hours of coasting produced the oocytes with higher developmental competence, while prolonged interval of coasting was associated with a reduced developmental capacity [97]. A progressive and gradual hypoxia associated with an increase in apoptosis and inflammation were observed in the transcriptome of granulosa cells from follicles of the optimal oocyte competence window [171] and were shown to be compromised in granulosa cells of 8-month-old prepubertal calves that were given the same treatment, suggesting that ovulation signaling may be weaker at this age [172].

The intrafollicular E2:P4 ratio can be used to distinguish growing from atretic and pre-ovulatory follicles [54, 55, 173]. Atretic follicles have a reduced capacity to produce estradiol, due to a decrease in the number of granulosa cells and gonadotropin receptors [138, 173], while preovulatory follicles are marked by high steroid concentrations in the follicular fluid (both E2 and P4), despite reduction in the expression of key enzymes of androgen and estrogen production in

theca (P450C17) and granulosa (P450 aromatase) cells [174]. Spontaneous or induced preovulatory gonadotropin surge is the endocrine signal for terminal differentiation of granulosa into luteal cells. The LH surge leads to a reduction in the capacity of follicular cells to produce estradiol, without a reduction in the capacity to produce progesterone. In the interval from the LH surge to ovulation, progesterone becomes the dominant steroid in follicular fluid [175], and follicles are categorized as estrogen inactive when estradiol:progesterone is below 1. Ovarian function is affected by FSH treatment through an increase in the number of estradiol-active follicles as compared to non-treated animals since single follicle dominance is prevented by exogenous FSH treatment [176]. We expected that both of our treatments would result in overall E2:P4 ratio below 1 after the LH treatment, but it was observed only in the 7-day FSH treatment. Although there is evidence that a rise in intrafollicular concentrations of progesterone is not necessary for ovulation in cattle [177], by 25 hours after a GnRH-induced LH surge, estradiol concentrations dropped markedly while progesterone concentrations increased in FSH treated and non-treated cows [175].

Spontaneous or induced LH surge that precedes ovulation causes irreversible changes in the follicle since the LH surge is responsible for hormonal changes in the intrafollicular environment and chromosomal configuration and morphological changes in the oocyte [178]. Cumulus cells are responsible for the exchange of metabolites, nutrients, and signaling molecules during growing and maturation of the oocyte [156]. Expansion of the cumulus cells surrounding the oocyte is a morphological indicator of oocyte maturation and *in vivo* maturation is associated with higher rates of development to the blastocyst stage in adult animals [179, 180]. In the current study, calves at 7 months of age produced a higher proportion of fully expanded oocytes despite no difference in recovery rates. Since all the animals were given the same amount of pLH (12.5 mg), individual differences in follicle number and size or the inherent differences due to calf age may be attributed to this result. Longer FSH treatment resulted in an overall higher proportion of fully expanded COC and lower proportion of compact COC than the 4-day treatment, results that are in agreement with previous findings in adult beef cattle [13, 133].

In conclusion, AFC and plasma concentrations of AMH at the time of wave emergence were correlated in prepubertal calves. The AFC and the plasma concentrations of AMH were also correlated with the number of follicles ≥ 6 mm at the time of oocyte collection, suggesting that AMH can be used as a marker of the AFC and the potential ovarian response to FSH treatment in prepubertal calves. Ovarian response was greater in calves at 4 months of age than at 7 months and

was characterized by a higher number of medium to large (i.e., ≥ 6 mm) sized follicles and a higher number of recovered oocytes following ultrasound-guided aspiration. Prolonged follicular growth resulted in a higher number of large follicles at the end of the treatment and a greater degree of follicular maturation, characterized by lower intrafollicular E2:P4 ratio and a higher proportion of fully expanded COC after LH treatment.

5.6.Supplementary Information

Table 5.2. Embryo development of *in vivo*-matured oocytes from 4-month-old calves and sexually mature heifers given 4 or 7 days of exogenous FSH treatment. Cleavage rate[a] 48 hours after in vitro fertilization (IVF), and blastocyst rate[b] at days 7 and 9 of culture. Oocytes were induced to mature *in vivo* by LH treatment and after collection given 6 hours of *in vitro* maturation before IVF.

	Cleavage (%)	Blast Day 7 (%)	Blast Day 9 (%)
4-day/calves	43 (40/93)	2.2^{B} (2/93)	4.3^{B} (4/93)
7-day/calves	56.5 (91/161)	5.6^{B} (9/161)	6.2^{B} (10/161)
4-day/heifers	53.7 (22/41)	7.3^{AB} (3/41)	7.3^{AB} (3/41)
7-day/heifers	44.4 (8/18)	33.3^{A} (6/18)	33.3^{A} (6/18)
Txt	0.784	**0.018**	**0.034**
Age	0.928	**0.006**	**0.016**
Txt*Age	0.159	0.451	0.154

[a]Total number of cleaved embryos/total number of recovered oocytes

[b]Total number of blastocysts/total number of recovered oocytes

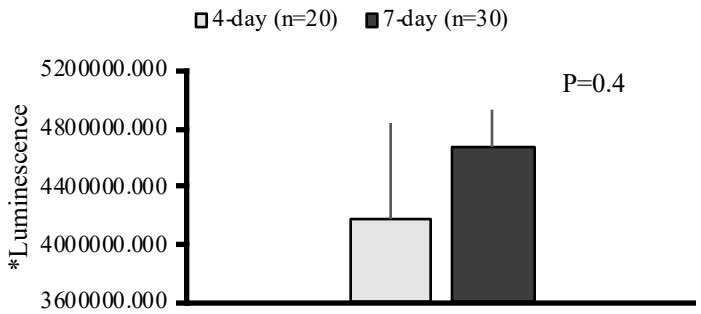
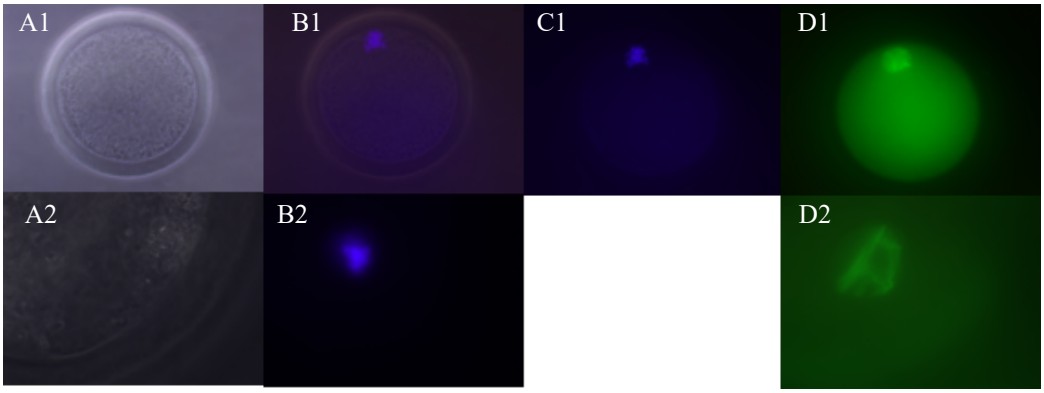


Fig. 5.7. ATP content in *in vivo* matured oocytes collected from 7-month-old calves given 4 or 7 days of exogenous FSH treatment, expressed by values of luminescence.

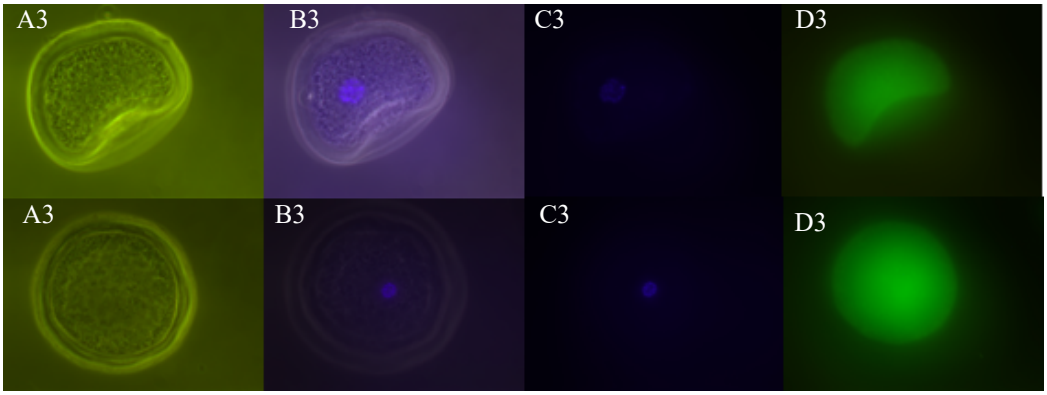
Table 5.3. Number of stained *in vivo* matured oocytes from 7-month-old calves and sexually mature heifers given 4 or 7 days of exogenous FSH treatment for lamin/DAPI (nuclear staining).

Txt/Cat	Calves	Heifers
4-day	10 oocytes/5 calves	5 oocytes/2 heifers
7-day	30 oocytes/9 calves	2 oocytes/2heifers

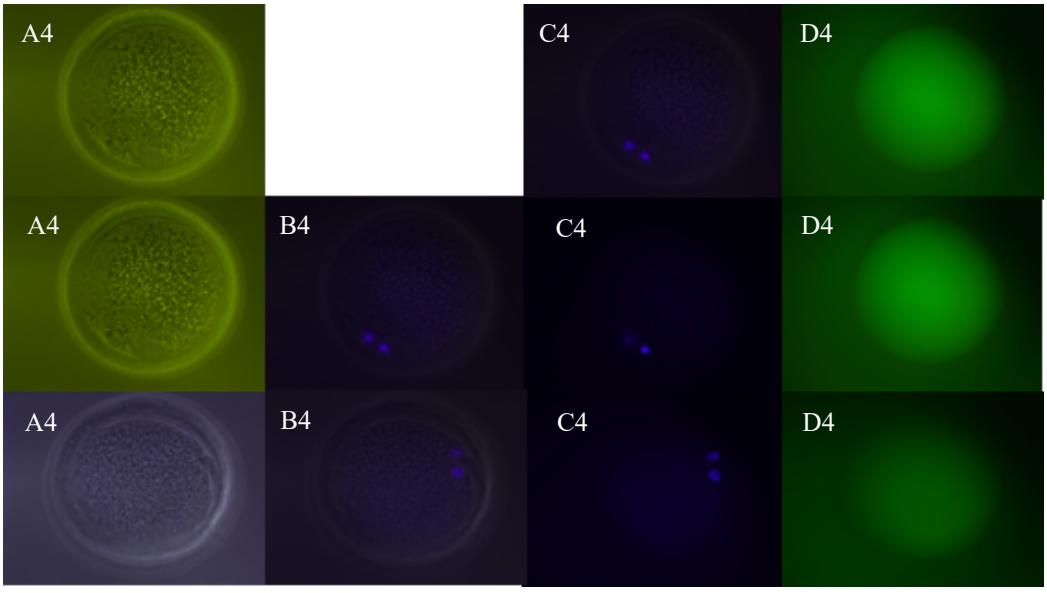
GV (1) and GVBD (2) oocytes



MI oocytes



MII oocytes



(A) phase contrast 40x; (B) phase contrast/DAPI 40x; (C) DAPI 40x; (D) A/C lamin 40x.

CHAPTER 6

6. THE STEROIDOGENIC AND OVULATORY CAPACITY OF OVARIAN FOLLICLES DURING AND AFTER EXOGENOUS FSH TREATMENT IN PREPUBERTAL HEIFER CALVES

Krause ART, Dias FCF, Adams GP, Mapletoft RJ, Singh J

Relationship of this study to the dissertation

The prepubertal period was shown to be associated with little or no ovarian stimulation by the pituitary gland, rather than an inability of the ovary to respond to an exogenous stimulus. Indeed, spontaneous ovulations occurred during gonadotropin treatment in earlier studies. In this study three methods of controlling endogenous LH release were evaluated to prevent ovulations during 7 days of exogenous FSH treatment and to compare the steroidogenic and ovulatory capacity of ovarian follicles during and after treatment.

Authors' contribution: ARTK, FCFD and JS conceived and designed the experiment. ARTK conducted and coordinated the experiment, collected and analyzed the data. ARTK wrote the draft of the manuscript. FCFD, GPA, RJM and JS reviewed the manuscript.

6.1. Abstract

The objective was to evaluate three methods of controlling endogenous LH release to prevent ovulation during 7 days of exogenous FSH treatment in prepubertal heifer calves. Calves (n=20, 137.5±0.9 days of age; 202.6±2.8 Kg) underwent transvaginal follicular ablation (Day -1) and FSH treatments (14 im injections of 25 mg of pFSH at 12 hours intervals) started 36 hours later. Calves were randomly assigned to one of four treatment groups (n=5/group): Control – no treatment to prevent endogenous LH release; Sheep-CIDR – CIDR device containing 0.3 grams of progesterone was placed in vagina immediately after follicular ablation; Long-acting progesterone – an administration of 600 mg i.m. of progesterone in 2 mL of a sustained-release formulation was given immediately after follicular ablation; and Cetrorelix group – calves were given administrations of 20μg/Kg i.m. of Cetrorelix Acetate at Days 2, 4 and 6 of FSH treatment. Daily blood samples were collected, and ovarian ultrasound examinations were performed from the time of the first FSH injection (Day 0.5) until Day 13. At the time of follicular ablation, plasma concentrations of LH (0.09±0.02 ng/mL; P=0.9) and progesterone (0.4±0.1 ng/mL; P=0.9) did not differ among groups. At the time of the first FSH treatment, plasma concentrations of progesterone were higher in the Long-acting P4 group (4.7±0.7 ng/mL; P<.0001) than in the other groups (0.03±0.03, 0.64±0.1 and 0.65±0.6 for Control, Sheep-cidr and Cetrorelix groups, respectively). From the time of follicular ablation until Day 8 (i.e., 24 hours after last FSH injection), the area under the curve of LH tended (P=0.08) to be higher in the Control (83.7±26.8) and Sheep-cidr (75.2±21.0) groups than in the Long-acting P4 (32.2±24.8) and Cetrorelix (11.8±3.0) groups. Plasma concentrations of estradiol at Day 2 tended to be lower (Treatment*Day, P=0.06) in the Long-acting P4 group than in the Control group (2.5±0.2 vs. 5.7±1.4 pg/mL). On Day 8, luteal structures were detected by ultrasonography in 7 calves (7/20), confirming that spontaneous ovulations occurred during FSH treatment in calves in the Control (4/5) and Sheep-cidr (3/5) groups and plasma concentrations of progesterone were higher (P=0.008) in the Control (2.0±0.8 ng/mL) and Sheep-cidr (2.3±1.2) groups than in the Cetrorelix group (0.02±0.01), while the Long-acting P4 group (0.6±0.1) was intermediate. On Day 13, luteal structures were observed in calves (10/20), of the Control (4/5), Sheep-cidr (4/5) and Long-acting P4 (2/5) groups. Among calves that ovulated, the number of luteal structures was greater (P=0.01) in the Long-acting P4 group

(19.5±4.5) than in the Control group (5.8±2.0), while the Sheep-cidr group was intermediate (11.5±1.4). Plasma progesterone concentrations on Day 13 tended to be higher in the Sheep-cidr group (22.9±7.0 ng/mL) than in the Cetrorelix group (0.01±0.01), while the Control (8.1±3.5) and Long-acting P4 (17.0±10.4) groups were intermediate. A significant positive correlation was observed between the number of luteal structures and plasma progesterone concentrations (r=0.8; P=0.0006). In summary, the Long-acting P4 and the Cetrorelix treatments were effective in preventing endogenous LH release and spontaneous ovulations during 7 days of exogenous FSH treatment in prepubertal calves.

Keywords: progesterone, estradiol, GnRH antagonist, LH, FSH, ovulation.

6.2. Introduction

Follicular development in prepubertal calves is characterized by a wave pattern of follicular growth, similar to that observed in sexually mature animals and this wave pattern has been observed in calves as young as two weeks of age. An FSH surge precedes each wave of follicle development in prepubertal and pubertal cattle and dominant and subordinate follicular growth comprise distinct growing, static, and regression phases [1, 2, 16]. Prepubertal calves have a low pulse frequency of LH during these phases of follicle development, resulting in successive anovulatory waves of follicular growth and the absence of corpora lutea, characterizing their sexual immaturity. An increase in the mean concentrations and pulse frequency of LH and a decrease in the concentration of estradiol receptors in the hypothalamus and pituitary has been observed in heifers as puberty approached [68, 181].

It has been shown that surges in LH, similar to a preovulatory LH surge, can be induced in prepubertal calves by GnRH [61, 62], estradiol [182], LH [60] or progesterone [183] treatment, supporting the hypothesis that the prepubertal period is characterized by lack of pituitary stimulation, rather than responsiveness. In our previous studies, spontaneous ovulations were observed in prepubertal calves treated with exogenous FSH under uncontrolled endogenous LH release conditions (*Unpublished, Chapter 3 and 4*). The insertion of a progestin ear implant containing 0.3 mg of Norgestomet prevented spontaneous and induced (pLH treatment) ovulations

after 4 or 7 days of gonadotropin treatment (*Unpublished, Chapter 5*), but a transvaginal device containing 0.3 grams of progesterone did not (*Unpublished, Chapter 6 and 7*).

In the later stages of the follicular phase, estradiol production by the dominant follicle increases and a rise in circulating estradiol concentrations cause a marked suppression in GnRH and LH pulse frequency, that is followed by a high amplitude surge of these hormones to cause ovulation [57, 184]. During the luteal phase, the corpus luteum maintains elevated circulating concentrations of progesterone, suppressing GnRH and LH pulse frequency [48, 56, 65]. Exogenous FSH treatment is characterized by the simultaneous growth of multiple estrogen-active follicles and intrafollicular concentrations of estradiol at the end of FSH treatment in adult cattle are similar to those in a single dominant follicle [185], resulting in higher than physiological plasma levels of estradiol. The increase in estradiol production by the dominant follicle has been shown to precede the increase in the number of gonadotropin receptors [138] and the physiological increase in circulating concentrations of estradiol stimulates GnRH secretion which induces the preovulatory LH surge [65]. Therefore, it is plausible that spontaneous ovulations observed during the course of FSH treatment in 6 and 7 months old calves (*Unpublished, Chapter 3, 4 and 7*) occured due to release of endogenous LH resulting from no- or low-levels of circulating progesterone (lack of negative feedback) and pharmacological levels of plasma estradiol (positive feedback).

The endogenous release of LH during exogenous FSH treatment has interfered with hypothesis testing in previous studies in prepubertal calves; it was possible to assess ovarian response, but the collection of oocytes for *in vitro* embryo production was compromised (*Unpublished, Chapter 3 and 7*). Therefore, the overall aim of this study was to evaluate three methods of controlling endogenous LH release and prevent ovulations during 7 days of exogenous FSH treatment in prepubertal calves. The specific objective was to determine the effectiveness of a long-acting progesterone injection, an intravaginal progesterone releasing device and a GnRH antagonist in controlling endogenous LH release. By design, we also evaluated the endocrine profile during the treatment period to gain an understanding of the underlying mechanisms. We hypothesized that controlling plasma concentrations of LH during FSH treatment by maintaining higher circulating levels of progesterone or by GnRH antagonist treatment would prevent ovulations in prepubertal calves.

6.3.Material and Methods

6.3.1. Animals and Experimental Design

The experiment was conducted during August at the Goodale Research Farm (52° North and 106° West), University of Saskatchewan, Saskatoon, SK, Canada. All animal procedures were approved by the University of Saskatchewan's Protocol Review Committee and in accordance with the Canadian Council on Animal Care (CCAC). A group of heifer calves (n=20) was selected (137.5±0.9 days of age; 202.6±2.8 Kg) from a larger group of spring-born Hereford crossbred calves. Selection of the calves was based on the date of birth, dam breed (Hereford cow) and sire breed (Simmental bull). The emergence of a new follicular wave (defined as Day 0) was induced by transvaginal ultrasound-guided follicular ablation of all follicles ≥ 5 mm in diameter under caudal epidural anesthesia (Lurocaine – 20 mg lidocaine as hydrochloride; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada) on Day -1. Wave emergence was expected to occur within 24 to 36 hours after follicle ablation [91]. Calves were randomly assigned to one of four treatment groups (n=5/group): Control – no prevention of endogenous LH release; Sheep-cidr group – an intravaginal progesterone device containing 0.3 gram of progesterone (Eazi-Breed™ CIDR® Sheep) was introduced immediately after follicular ablation and removed on Day 13; Long-acting progesterone group – an i.m. administration of 600 mg of progesterone in 2 mL of a sustained-release formulation (BioRelease Technologies, Lexington, KY, USA) was given immediately after follicular ablation; and Cetrorelix group – calves were given 3 i.m. administrations of 20μg/Kg body weight of Cetrorelix Acetate (C5249 – Sigma-Aldrich, Co., St. Louis, MO, USA) on Days 2, 4, and 6 of gonadotropin treatment. Exogenous FSH treatment started 36 hours after follicular ablation (Day 0.5) and consisted of 14 i.m. treatments of 25 mg of NIH-FSH-P1 (Folltropin®-V; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada) at 12-hour intervals (6 am and 6 pm). Calves were handled daily for FSH treatments, blood collection, and ovarian ultrasound examinations during the seven days of exogenous FSH treatments and six days after the last FSH treatment.

6.3.2. Blood samples

Blood samples were collected by jugular venipuncture using a 21G x 1" needle in a 10 mL heparinized tube (Becton Dickison Vacutainer® Systems, Franklyn Lakes, NJ, USA) on the day of the follicular ablation (Day -1), at the time of the first FSH injection (i.e., Day 0.5, 36 hours after

follicular ablation) and daily thereafter for 13 days. Blood samples were also collected 12 hours after Cetrorelix treatments in all calves (Days 2.5, 4.5, and 6.5). Samples were immediately centrifuged at 3000 x g for 15 minutes, and the plasma was stored at -20°C until analyses.

6.3.3. Ovarian ultrasound examinations

Cine-loops of ovarian ultrasound examinations of each ovary were recorded at the time of the first FSH injection (i.e., at the time of wave emergence – Day 0.5) and daily starting on Day 2 of FSH treatment until Day 13. For each examination day, ovarian structures (follicles and luteal structures) in each ovary were measured and counted.

6.3.4. Hormone Analyses

Plasma LH concentrations were measured daily from the day of the follicular ablation until Day 10, including the 12-hour intervals after Cetrorelix treatments, in all animals (Days -1, 0.5, 1, 2, 2.5, 3, 4, 4.5, 5, 6, 6.5, 7, 8, 9, and 10) in duplicates using a validated double-antibody radioimmunoassay [1]. The range of the standard curve was 0.06 to 8 ng/mL, and the intraassay coefficients of variation were 2.7 and 2.3% for plasma with concentrations of 0.80 and 1.59 ng/mL, respectively.

Plasma estradiol concentrations were measured every other day starting on Day 2 until Day 12 (Days 2, 4, 6, 8, 10, and 12) in duplicates using previously described radioimmunoassay [186]. The range of the standard curve was 0.5 to 20 pg/mL, and the intraassay coefficient of variation of all samples was below 15%.

Plasma progesterone concentrations were measured daily from the day of follicular ablation until Day 13, using a commercial radioimmunoassay kit (ImmunoChen ™ Progesterone^{125} kit, MP Biomedicals, Costa Mesa, CA, USA). The range of the standard curve was 0.15 to 80 ng/mL, and the intraassay coefficient of variation was 12.1, 10.4 and 8.7% for low (0.9 ng/mL), medium (3.5 ng/mL) and high (7.7 ng/mL) reference plasma, respectively.

6.3.5. Statistical Analysis

All analyses were performed in SAS® Enterprise Guide 6.1 (SAS® 9.4; SAS Institute Inc., Cary, NY, USA). Plasma hormone concentrations on specific days, the area under the curve of LH and the number of visible luteal structures were compared among groups by one-way analysis of

variance (ANOVA). Estradiol and progesterone concentrations over time were compared using repeated measures Proc Mixed. Log transformation was used to analyze data that did not have equal variances and normality of the residuals. Multiple comparisons were made using Tukey's post-hoc test, and mean ± standard error of the mean (SEM) of raw data are reported to describe all response variables. Pearson correlation was used to evaluate the association between plasma progesterone concentrations and the number of luteal structures. Statistical significance was assumed when P values were < 0.05, while P values between 0.05 and 0.1 were considered as tendencies.

6.4.Results

Four calves out of 20 (1/5 from Sheep-cidr, 2/5 from Long-acting P4 and 1/5 from Cetrorelix group) showed clinical signs of foot rot during the experiment and were examined and treated by a veterinarian. Animal handling was decreased for these calves, but their data collection was maintained, and their data were included in all statistical analyses.

6.4.1. Plasma LH Concentrations

At the time of follicular ablation, plasma concentrations of LH were not different among treatment groups (0.09±0.02 ng/mL; P=0.9). Figure 6.1A shows the plasma LH concentration profile for each treatment group from the day of follicular ablation (Day -1) until Day 8 (i.e., 24 hours after the last FSH treatment) wherein it is possible to observe rises in LH concentrations. Figure 6.2 (left panel) shows the LH profile of individual calves grouped by treatment (Fig. 6A, C, E, and F). Because frequent sampling was not undertaken, an LH rise was assumed to have occurred when the levels of LH were 20 times higher (i.e., 1.8 ng/mL) than the mean value of LH for all animals at the time of follicular ablation. Based on this criterion, an LH rise was detected during the FSH treatment (from Day 0.5 until Day 7) in 8 calves (8/20; Control = 3, Sheep-cidr = 4, Long-acting P4 = 1). The area under the curve of LH from the day of follicular ablation until Day 8 tended (P=0.08) to be greater in the Control (83.7±26.8 arbitrary units) and Sheep-cidr (75.2±21.0) treatment groups than in the Long-acting P4 (32.2±24.8) and Cetrorelix (11.8±3.0) groups.

6.4.2. Plasma Estradiol Concentrations

Estradiol concentrations from Day 2 of FSH treatment until Day 8 (i.e., 24 hours after the last FSH treatment) are shown in Figure 6.1B. A tendency for a Treatment*Day interaction was observed (P=0.06), in which the estradiol concentrations in the Sheep-cidr group were lower on Day 2 than on Day 6 (2.8±0.2 vs. 7.3±1.5 pg/mL). The concentrations of estradiol were lower in the Long-acting P4 group (2.5±0.3) than in the Control group (5.7±1.4) on Day 2, and the Sheep-cidr group on Days 4 (7.8±2.3) and 6 (7.3±1.5).

6.4.3. Plasma Progesterone Concentrations

Concentrations of progesterone at the time of follicular ablation (i.e., immediately before exogenous progesterone treatment in the Sheep-cidr and Long-acting P4 groups) were not different among treatment groups (0.4±0.1 ng/mL; P=0.9). At the time of first FSH treatment (Day 0.5; 36 hours after ablation), plasma concentrations of progesterone were higher (P<.0001) in the Long-acting P4 group (4.7±0.7ng/mL) than in the other groups (0.03±0.03, 0.6±0.08 and 0.6±0.6 for Control, Sheep-cidr and Cetrorelix groups, respectively). Figure 6.1C shows plasma progesterone concentrations from the day of follicular ablation (D-1) until Day 8 (i.e., 24 hours after the last FSH treatment).

We sought to distinguish between endogenous plasma progesterone rise (coming from possible luteal structures formed due to spontaneous ovulation after Day 2) from the exogenous progesterone level (resulting from Sheep-cidr and Long-acting P4 given at the beginning of the experiment; declining over time). For this purpose, the endogenous progesterone increase was defined as an increase above 1 ng/mL, where the previous two samples (24 and 48 hours before) were below 1 ng/mL. Two calves (Control = 1/5 and Sheep-cidr =1/5) showed endogenous production of progesterone during FSH treatment. On Day 8 (24 hours after the last FSH injection), plasma concentrations of progesterone were higher (P=0.008) in the Control (2.0±0.8 ng/mL) and Sheep-cidr (2.3±1.2) treatment groups than in the Cetrorelix group (0.02±0.01), while the Long-acting P4 group (0.6±0.1) was intermediate (i.e., not different from any group). On Day 13 (i.e., six days after the last FSH injection), progesterone concentrations were higher (P=0.06) in the Sheep-cidr group (22.9±7.0 ng/mL) than in the Cetrorelix group (0.01±0.01), while the Control (8.1±3.5) and the Long-acting P4 (17.0±10.4) groups were intermediate. Figure 2 (right panel) shows plasma progesterone concentrations of individual calves from Day 7 (i.e., last FSH

treatment) until Day 13, in which a marked increase in plasma progesterone concentrations can be observed in calves that ovulated.

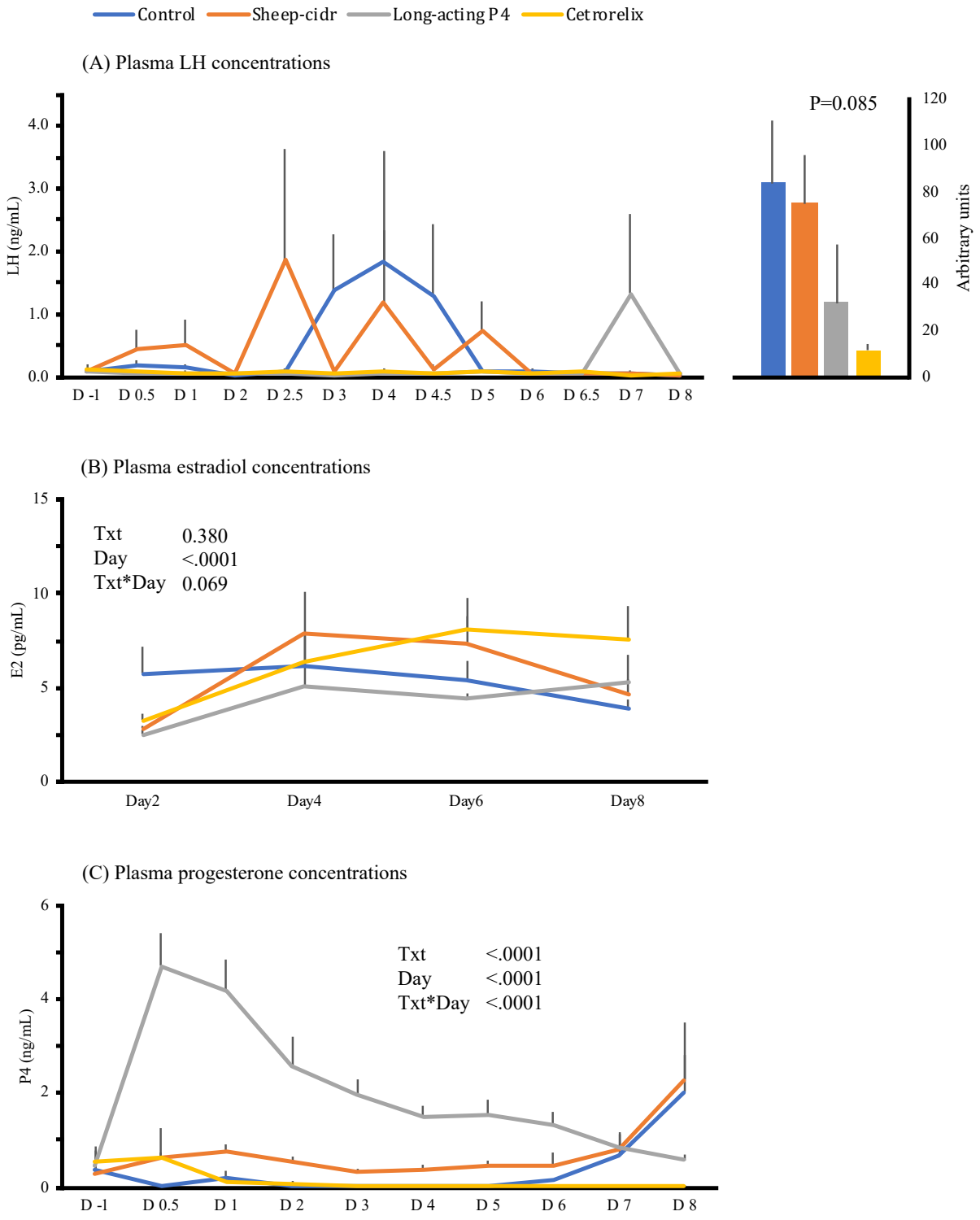


Fig. 6.1. Mean plasma concentrations of (A) LH from the day of follicular ablation (D-1) until D8 (i.e., 24 hours after the last FSH injection; left panel) and the area under the curve of LH during those days (right panel); (B) Estradiol from D2 until D8; and (C) Progesterone from the day of follicular ablation (D-1) until D8 in prepubertal calves given 7 days of exogenous FSH treatment and the following treatments to control endogenous LH release: Control; no treatment (blue), Sheep-cidr (orange), Long-acting progesterone (grey) and Cetrorelix (yellow).

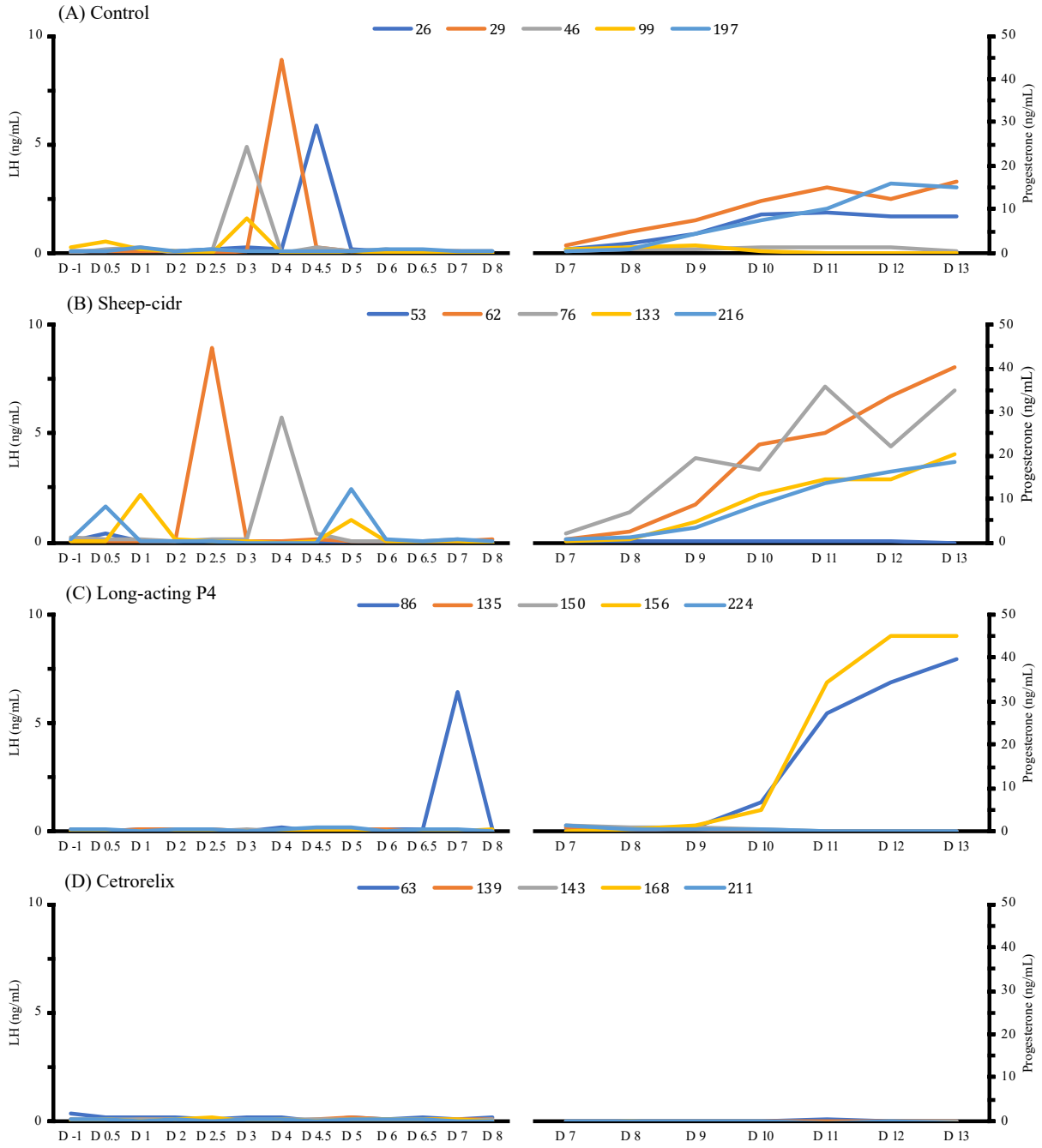


Fig. 6.2. Individual concentrations of plasma LH and progesterone in calves of the (A) Control; (B) Sheep-cidr; (C) Long–acting P4, and (D) Cetrorelix groups. Plasma LH concentrations are shown from the day of follicular ablation (D-1) until D8 (i.e., 24 hours after the last FSH injection) and plasma progesterone concentrations from D8 until Day 13 are shown.

6.4.4. Visible Luteal Structures

At Day 8 (i.e., 24 hours after last FSH treatment), luteal structures were detected by ultrasonography in 7 calves, confirming that spontaneous ovulations had occurred during the FSH treatment in Control (4/5) and Sheep-cidr (3/5) groups, while no luteal structures were observed in calves in the Long-acting P4 (0/5) and Cetrorelix (0/5) groups. On Day 13 (i.e., six days after the last FSH treatment) luteal structures were observed in 10 out of 20 calves, confirming that spontaneous ovulations occurred in calves of the Control (4/5), Sheep-cidr (4/5) and Long-acting P4 (2/5) groups. No luteal structure was observed in calves in the Cetrorelix group (0/5). A greater proportion of calves ovulated spontaneously (Fisher's Exact test p=0.047) in the Control and Sheep-cidr group than in the Cetrorelix group, while Long-acting P4 group did not differ from any group. By analyzing data from the subset of calves that had luteal structures on Day 13 (n=10), on average the number of luteal structures was higher (P=0.01) in the Long-acting P4 (19.5±4.5, n=2 calves) than in the Control (5.8±2.0; n=4 calves) group, while the Sheep-cidr (group 11.5±1.4; n=4 calves) was intermediate. Among calves that ovulated, there was a positive correlation between the number of luteal structures formed and the plasma concentrations of progesterone (r=0.8; P<0.001).

To evaluate differences in ovulation time among treatment groups during and after FSH treatment, the time (in hours) from the first FSH injection until an endogenous increase in plasma progesterone concentrations was detected for individual calves was compared. Calves in the Control group had endogenous progesterone concentrations above 1 ng/mL at 184±12 (n=4/5) hours compared to Sheep-cidr group at 174±15 hours (n=4/5) and the Long-acting P4 group at 216±12 hours (n=2/5); no difference (P=0.2) in the timing of the rise in endogenous progesterone was recorded.

6.5.Discussion

In this study, different methods of preventing endogenous LH release and ovulations during the seven days of gonadotropin treatment were compared in prepubertal heifer calves. However, plasma concentrations of progesterone resulting from a sheep-cidr did not reach values above 1 ng/mL and therefore, did not prevent spontaneous ovulations during the FSH treatment. The Long-acting P4 treatment resulted in an average mean progesterone concentration of 4.7±0.7 ng/mL at 36 hours after injection, an increase of almost ten times when compared to basal levels before

treatment. Plasma progesterone concentrations in the Long-acting P4 group were maintained above 1 ng/mL until Day 6 of FSH treatment. Calves in the Sheep-cidr and Control groups had increases in LH concentrations during the FSH treatment. Cumulative estradiol production by the growing follicles (based on plasma concentrations of estradiol) was similar among groups beginning four days after initiation of exogenous FSH treatment. Spontaneous ovulations occurred during FSH treatment in 7 of 10 calves of Control and Sheep-cidr groups. Ovulations occurred in 2 of 5 calves after the end of gonadotropin treatment in the Long-acting P4 treatment group, while no ovulations were recorded after Cetrorelix treatment. Based on these results, both the Long-acting progesterone and the Cetrorelix treatments can be used to prevent spontaneous ovulations for the purpose of oocyte retrieval; however, oocyte competence after these treatment remains to be evaluated.

Cattle are a spontaneous ovulating species, in which the preovulatory LH surge and ovulation of the dominant follicle is triggered by increasing circulating concentrations of estradiol under low plasma progesterone levels after luteolysis [187]. Estradiol acts at the hypothalamus to stimulate GnRH release and at the pituitary to increase its responsiveness to GnRH [188]. In earlier studies (*Unpublished, Chapter 3 and 4*), calves treated with exogenous FSH without any concurrent progesterone treatment (i.e., under uncontrolled LH release) spontaneously ovulated during treatment and in another study (*Unpublished, Chapter 7*), when an intravaginal device containing 0.3 grams of progesterone was used along with the FSH treatment, calves also ovulated spontaneously during the 7-day treatment period, but because daily blood sampling was not done, it was not possible to determine circulating progesterone concentrations resulting from the device. In sexually mature animals, LH surge and consequently ovulations are suppressed by luteal phase progesterone concentrations (> 2 ng/mL) [189, 190], but it was shown that even sub-luteal concentrations of progesterone (1-2 ng/mL) were effective in suppressing the occurrence of a spontaneous preovulatory LH surge [191]; although not investigated in this study, we speculated that in prepubertal calves, the requirements are likely the same. In this study, progesterone concentrations after 36 hours of Sheep-cidr (containing 0.3 g progesterone) insertion were below 1 ng/mL in all animals (mean of 0.64±0.1), and calves had risen in plasma LH concentrations during the FSH treatment that were twenty times higher than the basal LH concentrations. Frequent bleeding for an extended period of time to determine the exact magnitude of LH surge [181] was beyond the scope of this study, therefore the reported rises in LH secretion in this study cannot be

considered LH surge levels, but data are useful to demonstrate that rises in plasma LH concentrations indeed occurred during those time periods.

Exogenous progesterone administered during proestrus, i.e., after luteolysis has occurred, can prevent the estradiol-induced LH surge, but not after the onset of the surge [192] and both progesterone concentrations and duration of the exposure to progesterone can delay the estradiol-induced LH surge [193]. Moreover, progesterone exposure is essential for the full expression of the positive feedback effect of estradiol in inducing the preovulatory GnRH surge [194] and in agreement, a greater number of ovulations and progesterone production was observed in the calves in the Sheep-cidr and Long-acting P4 groups than in calves of the Control group. In prepubertal calves implanted with one or three progestin devices containing 6 mg of Norgestomet for ten days, ovulations also occurred, calves exposed to one device ovulated during the exposure period (6/16) and after device withdrawal (4/10), while calves exposed to three devices ovulated after device withdrawal (13/16) [183]. LH pulse frequency was reduced during device exposure but increased after device removal in calves receiving three devices in comparison to non-treated calves, while LH pulse amplitude was reduced in calves during and after device exposure. In calves, LH secretion has been shown to be more sensitive to estradiol feedback than in mature animals [67]. The current data suggest that the circulating concentrations of estradiol resulting from the growth of multiple follicles were sufficient in the Control, Sheep-cidr and Long-acting P4 groups to elicit LH responses when plasma progesterone levels were below 1 ng/mL. Interestingly, circulating concentrations of estradiol were not different among treatment groups during the treatment period, and the Long-acting P4 treatment resulted in levels of progesterone during the 7-day FSH treatment that were sufficient to prevent ovulations, even under the rising levels of estradiol. The slow decline in progesterone concentrations in this group resulted in estradiol positive feedback and LH rise (detected in one calf) and ovulations (detected in 2 animals) only after the end of the FSH treatment. Six days after the last FSH treatment, the number of visible luteal structures in calves of the Long-acting P4 group (2/5) was higher than in calves of the Control group (4/5).

Treatment with the competitive GnRH receptor antagonist Cetrorelix prevented ovulations during FSH treatment and ovulations after the treatment. In sheep, use of a GnRH receptor antagonist before or at any time during the LH surge resulted in no further LH release and prevented the generation of an LH surge of normal duration and amplitude [64]. That is, GnRH receptor antagonists act by blocking LH release from gonadotrophs in adenohypophysis even in the

presence of GnRH pulses, explaining why plasma levels of LH did not change during the superstimulatory window after Cetrorelix treatment. It has been shown that removal of LH support to FSH treated follicles by a GnRH antagonist treatment stops follicular growth and upregulate genes associated with atresia, resembling a gene expression pattern similar to early atretic follicles [195]. When follicles enter atresia, there is a window in which both live and dead cells are present, and steroid production continues in the living cells changing intrafollicular and consequently plasma steroid hormone concentrations later than the onset of atresia [196, 197]. An additional observation in the current study was a decrease in follicular size after FSH treatment in the Cetrorelix-treated calves that resembled regressing follicles. Folltropin-V preparation contains some residual LH that may have provided sufficient support to the follicles in the Cetrorelix treated animals. Although plasma levels of estradiol were high until the end of exogenous FSH treatment (i.e., follicles were estradiol-active), no LH rises, or ovulations were recorded in the Cetrorelix treated calves. GnRH antagonist treatment blocks the pulsatile secretion of LH and dramatically reduces basal concentrations of LH, almost eliminating circulating concentrations of LH [198, 199], as was observed in this study. Therefore, we hypothesized that follicles were in an atretic phase and lost their ability to ovulate in this group by the end of gonadotropin treatment or immediately thereafter. The exact timing of the loss of ovulatory capacity can only be confirmed by giving exogenous stimulus to induce ovulation at the end of treatment.

As with our previous studies with calves (*Unpublished, Chapter 4, 5 and 7*), spontaneous ovulations resulted in the formation of luteal structures capable of progesterone synthesis. Therefore, the number of ovulations, based on the number of visible luteal structures formed and endogenous progesterone concentrations were used to determine the biological effects of the rises in LH secretion. Luteal structures were visible at 24 hours after the last FSH treatment in all calves in the Control and Sheep-cidr treatment groups that had risen in LH concentrations during the treatment, but a higher number of ovulations that were associated with higher progesterone production were observed in calves in the Long-acting P4 group on Day 13. Based on at least two of the endpoints of LH rise, endogenous progesterone concentrations above 1 ng/mL during FSH treatment and visible luteal structures on Day 8 combined, we concluded that ovulation during FSH treatment occurred in 80% (4/5) of calves in the Control group, 60% (3/5) in the Sheep-cidr group and 0% (0/5) in the Long-acting P4 and Cetrorelix groups.

We have demonstrated that 7 days of exogenous FSH treatment results in a higher number of large-sized follicles at the end of the treatment than 4 or 6 days (*Unpublished, Chapter 7*). The result of this study supports the hypothesis that at the end of the treatment and under controlled endogenous LH release, prolonged follicular growth was not detrimental for follicle health since the ovulatory capacity was retained as compared to those in the Cetrorelix group where no ovulations were recorded even after the end of treatment.

In conclusion, endogenous LH release and spontaneous ovulations during seven days of exogenous FSH treatment were prevented by a Long-acting P4 treatment which resulted in plasma concentrations of progesterone above 1 ng/mL or by treatment with the GnRH antagonist Cetrorelix in prepubertal calves. Calves in the Long-acting P4 group ovulated after FSH treatment, demonstrating that follicles were still responsive to LH by the end of the treatment, while no ovulations were observed in calves of the Cetrorelix group, suggesting either that follicles had lost their capacity to respond to endogenous LH release, or that endogenous LH release was completely suppressed in calves of this group. Ovulations during the seven days of FSH treatment were not prevented by plasma progesterone concentrations arising from the use of a sheep-cidr. Results suggest that treatment with a Long-acting P4 formulation or with the GnRH antagonist Cetrorelix can be used effectively to prevent spontaneous ovulations during seven days of exogenous FSH treatment in prepubertal calves.

CHAPTER 7

7. DURATION OF EXOGENOUS FSH TREATMENT INFLUENCES FOLLICULAR GROWTH AND OOCYTE DEVELOPMENTAL COMPETENCE IN PREPUBERTAL CALVES

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Relationship of this study to the dissertation

The competence of bovine oocytes to developed to the blastocyst stage *in vitro* was shown to be greater when oocytes were collected from follicles larger than 6 mm in diameter than from smaller follicles and a linear relationship between follicle size and oocyte competence appeared to exist until the follicle reaches 10 mm. Prepubertal calf oocytes have been shown to have poor developmental capacity when compared to oocytes from sexually mature animals and there are reports that oocytes from prepubertal calves have perturbed or impaired cytoplasmic maturation when compared to adult oocytes. Maturation of bovine oocytes *in vivo* have been associated with higher rates of embryo development in oocytes from sexually mature animals, but not in prepubertal calves. Prolonged follicular growth following FSH treatment for 7 days has resulted in a greater number of follicles ≥ 9 mm, a greater proportion of fully expanded COC collected following a LH stimulus and 2.5 times more transferable embryos produced than 4 days of treatment. The aim of this study was to compare oocyte developmental competence following 4 vs. 7 days of exogenous FSH treatment and *in vitro* vs. *in vivo* oocyte maturation. Based on the results of this and previous studies combined, a second study aiming to compare oocyte developmental competence following 4, 6 or 7 days of exogenous FSH treatment under controlled endogenous LH release and *in vitro* oocyte maturation in prepubertal calves was conducted.

Authors' contribution: ARTK, FCFD and JS conceived and designed the experiment. ARTK conducted and coordinated the experiment, collected and analyzed the data. ARTK wrote the draft of the manuscript. FCFD, GPA, RJM and JS reviewed the manuscript.

7.1. Abstract

Two studies were conducted to compare ovarian response and oocyte developmental competence following different durations of exogenous FSH treatments in prepubertal calves. *In vivo* vs. *in vitro* oocyte maturation was also evaluated among calves in Study I. Calves (n=26; 161.7±0.8 days of age; 217.4±4.3 Kg) were randomly assigned to 4 (n=13) or 7 (n=13) days of exogenous FSH treatment and to *in vivo* (n=16) or *in vitro* (n=10) oocyte maturation groups, along with sexually mature heifers (n=10; 17.3±0.1 months, 493.3±11.9 Kg), that were given 4 (n=5) or 7 (n=5) days of FSH treatment prior to *in vivo* oocyte maturation. Follicular ablation to synchronize wave emergence was followed by the insertion of a progestogen device in all animals and FSH treatments were initiated 36 hours later (25 mg pFSH 12-hour intervals). Heifers received two doses of prostaglandin concomitant with the last two FSH treatments, and progestogen devices were removed at the time of the last FSH treatment in all animals. pLH was administered 12 hours after the last FSH treatment in the *in vivo* maturation groups. Transvaginal ultrasound guided COC collection was performed 8 hours after the last FSH injection in the *in vitro* groups and 20 hours after LH treatment in the *in vivo* groups. Spontaneous and induced ovulations were observed in calves (9/26) at the time of COC collection and were added to follicle numbers for treatment comparisons. The number of follicles ≥ 6 mm was not different between the 4- and 7-day treatment groups (23.6±3.4 vs. 26.0±3.3; P=0.5), but was higher (P=0.01) in the *in vivo* calf (27.4±3.7) and heifer groups (29.1±3.4) than in the *in vitro* calf group (16.3±4.1), while the number of COC collected was higher in the *in vivo* than in the *in vitro* calf groups (12.1±1.7 vs. 6.8±1.0; P=0.03). Cleavage and blastocyst rates were not different between the 4-day *in vitro* calf and 7-day *in vivo* heifer group (71.8 vs. 69.2% and 33.3 vs. 48.1%, respectively), but were higher than the 7-day *in vivo* calf group (24.5 and 1%, respectively; P=0.0009). In Study II, calves (n=21; 144.09±0.7 days of age; 204.9±4.6 Kg) were randomly assigned to 4 (4-day), 6 (6-day) or 7 (7-day) days of exogenous FSH treatment (25 mg pFSH 12-hour intervals). Follicular ablation to synchronize wave emergence was performed in all calves, followed by 600 mg of progesterone in 2 mL of a sustained release formulation given intramuscularly. Exogenous FSH treatments were initiated 36 hours later, and transvaginal oocyte collection was conducted 8 hours after the last FSH injection. No ovulations were observed during FSH treatments and all recovered COC were submitted to *in vitro* maturation. At the time of COC collection, the number of follicles ≥ 6 mm was greater (P=0.03) in

the 7-day (37.3±5.5) than in the 4-day (14.7±2.5) treatment groups, but the number of COC collected did not differ among groups (0.1). Cleavage rate was higher (P=0.01) in the 6-day (73.2%) than in the 4-day (51.3) or 7-day (47.2) treatment groups, while the proportion of oocytes that reached the morula and blastocyst stage after 7 days of culture did not differ (P=0.5). At Day 9 of culture, 6 days of FSH treatment group resulted in a higher (P=0.02) blastocyst rate (40.9%) than the 4-day (20.5) or 7-day (20.2) groups. In summary, similar rates of embryo development were observed among *in vitro* matured calf oocytes and *in vivo* matured heifer oocytes following 4 or 7 days of exogenous FSH treatment, respectively in Study I. In Study II, 6 days of exogenous FSH treatment under controlled endogenous LH release when compared to 4 or 7 days was associated with the greatest developmental competence of oocytes collected from 5 months old calves.

Keywords: ovarian response, embryo development, FSH, LH, progesterone, estradiol.

7.2. Introduction

In vitro embryo production from calf oocytes and transfer to adult recipients can reduce the generation interval and accelerate the rate of genetic gain. Embryos have been produced successfully after superovulation of calves as young as one month of age [77, 82], while *in vitro* embryo production from calves under six months of age have been reported from several other studies [6, 11, 85, 87, 88, 92, 94, 154, 200, 201]. Gonadotropin treatment of prepubertal calves results in an increase in the number and diameter of follicles available for aspiration and an improved developmental competence of recovered oocytes [11, 17, 87, 88, 91-94, 154]. Although full-term pregnancies have been reported after the transfer of *in vitro* produced embryos [6, 87, 93, 154, 200], prepubertal calf oocytes do not develop into blastocysts at rates similar to adult cow oocytes. The low developmental competence of prepubertal calf oocytes has been attributed to incomplete or perturbed cytoplasmic maturation [104-106] and the age of the calf at the time of the treatment [11, 94, 201]. A study that examined the relationship between donor age and oocyte developmental competence found that although a higher number of oocytes were recovered after FSH stimulation of calves between 5 and 8 months of age than from sexually mature animals, this

advantage was not reflected in embryo production because of the lower rate of embryonic development in prepubertal calf oocytes [11].

Embryo development to the blastocyst stage and rates of survival after vitrification were higher in *in vivo* than in *in vitro* matured oocytes obtained from sexually mature cattle [180]. A higher level of ultrastructural homogeneity was observed in *in vivo* matured oocytes than in *in vitro* matured oocytes; the latter showed asynchrony between nuclear and cytoplasmic maturation and incomplete cumulus cell expansion [202]. A varying degree of maturation was observed in oocytes from calves that were given GnRH to induce oocyte maturation after FSH treatment; only 40% of the recovered oocytes exhibited expansion of the cumulus cells. Although direct comparisons were not made, cleavage (24 vs. 21%) and blastocyst (8 vs. 9%) rates did not appear to differ between the GnRH-treated and Control groups [90].

The developmental competence of oocytes has been associated with follicle size in sexually mature cattle [15, 84, 153, 180, 203] and prepubertal calves [87, 96]. Oocytes originating from follicles > 6 mm in diameter had greater developmental potential than oocytes from smaller follicles [15]. Duration of gonadotropin treatment and interval from FSH treatment to oocyte collection (known as 'coasting') also influenced the capacity of oocytes to develop *in vitro*. In this regard, 48 to 54 hours of coasting resulted in embryo production rates as high as 70 to 80% in adult cattle [86, 97]. Although different periods of FSH coasting showed no positive effects during *in vitro* embryo production of prepubertal calf oocytes [11], a 3-day vs. 1.5-day FSH treatment resulted in higher rates of usable oocytes and embryo production in 2 to 6 months old calves [87]. In sexually mature cattle, prolongation of follicular growth (4-day vs. 7-day FSH treatment) resulted in a higher number of large follicles and a greater proportion of cleaved oocytes reaching the morula and blastocyst stages in culture [13]. Therefore, prolongation of follicular development with exogenous FSH to allow the growth and differentiation of follicles for 4 to 7 days along with *in vivo* oocyte maturation may be a key strategy to improve oocyte developmental competence in calves. The additional days of FSH treatment may enable oocytes to complete the synthesis of molecular products required for further development. In the current study, developmental competence of oocytes obtained from prepubertal calves after varying lengths of FSH treatment and after *in vivo* versus *in vitro* oocyte maturation was investigated.

Two studies were performed to address objectives and hypotheses. The objectives of Study I were to compare the developmental competence of *in vitro* vs. *in vivo* matured oocytes collected

after 4 vs. 7 days of FSH treatment in 5- to 6-month-old calves. It was hypothesized that ovarian response and oocyte developmental competence in prepubertal calves will be increased by 7 days of follicular growth under the influence of exogenous FSH as compared to 4 days and that oocyte developmental competence will be increased by *in vivo* vs. *in vitro* oocyte maturation. The experimental design also allowed a comparison of oocyte maturation between prepubertal and sexually mature animals. Based on the results of Study I, the Study II was conducted with the objective of comparing ovarian response and oocyte developmental competence of prepubertal calves that were given 4, 6 or 7-days of exogenous FSH treatment followed by *in vitro* maturation of oocytes.

7.3.Material and Methods

Study I was performed during September and October and Study II during August and September in the following year. Both studies were conducted at the Goodale Research Farm (52° North and 106° West), University of Saskatchewan, SK, Canada. All animal procedures were approved by the University of Saskatchewan's Protocol Review Committee and in accordance with the Canadian Council on Animal Care.

7.3.1. Animals and Experimental Design

In both studies, calves were selected for gonadotropin treatment from a group of spring-born calves based on the date of birth, dam breed (multiparous Hereford cow) and sire breed (Simmental bulls). For Study I, 26 crossbreed Hereford calves (161.7±0.8 days of age; 217.4±4.3 Kg) were selected from a larger group of spring-born calves and sexually mature heifers of similar breeding (n=10; 17.3±0.1 months, 493.3±11.9 Kg) were included for gonadotropin treatments. For Study II, 21 crossbreed Hereford calves (144.09±0.7 days of age; 204.9±4.6 Kg) were selected for gonadotropin treatment. During the experimental periods, calves were housed with their mothers in outdoor corrals, fed silage in the morning, and had free access to hay, water, and mineral block. Calves were separated from mothers in the morning and evening for brief periods for treatment purposes.

7.3.1.1. Study I

Transvaginal ultrasound-guided follicular ablation of all follicles ≥ 5 mm was performed in all animals (Day -1) under caudal epidural anesthesia (2% lidocaine HCl and epinephrine USP) followed immediately by insertion of a progestogen device. Calves received an intra vaginal device containing 0.3g of progesterone (Eazi-Breed™ CIDR® Sheep), while heifers received an intra vaginal device containing 1.38 g of progesterone (Eazi-Breed™ CIDR® Cattle). Calves were randomly assigned to the gonadotropin and oocyte maturation groups: 4-day/*in vivo* (n=8) vs. 4-day/*in vitro* (n=5) vs. 7-day/*in vivo* (n=8) vs. 7-day/*in vitro* (n=5), while heifers were randomly assigned to 4 days (n=5) or 7 days (n=5) of exogenous FSH treatment and *in vivo* oocyte maturation. Gonadotropin treatment were initiated 36 hours after follicular ablation (i.e., on the day of follicle wave emergence) and consisted of 8 (4-day groups) or 14 (7-day groups) treatments of 25 mg i.m. of pFSH (NIH-FSH-P1; Folltropin-V®; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada) at 12-hour intervals. Heifers were given two luteolytic doses of prostaglandin analogue (500 mcg cloprostenol sodium; Estrumate™, Merck Animal Health, Madison, NJ, USA) simultaneous with the last two FSH injections and progestogen devices were removed at the time of the last FSH injection in all animals. Calves and heifers in the *in vivo* group were given pLH treatment (12.5 mg i.m., Lutropin-V®; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada)12 hours after the last FSH injection (Fig. 7.1).

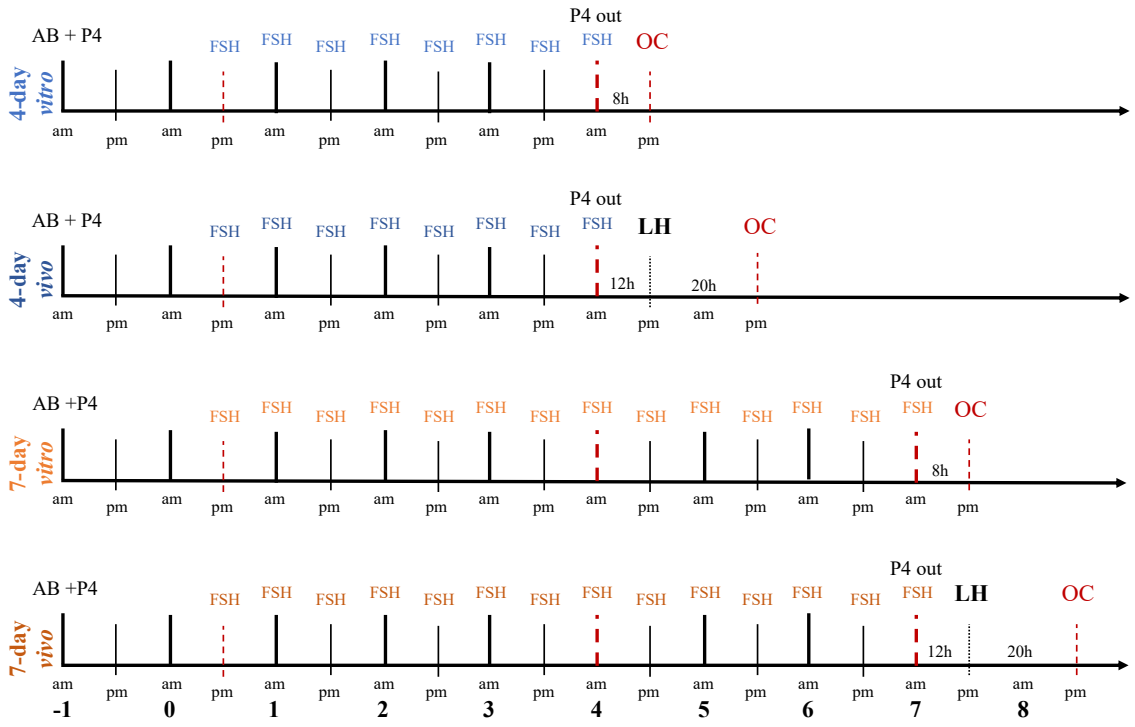

Fig. 7.1. Experimental design Study I. Calves (n=26) were randomly assigned to 4 (4-day; n=13) or 7 (7-day; n=13) days of FSH treatment and *in vivo* (n=16) or *in vitro* (n=10) oocyte maturation groups, along with sexually mature heifers (n=10) that were randomly assigned to 4 (4-day) or 7 (7-day) days of FSH treatment and *in vivo* maturation in a 2x3 factorial design. Transvaginal ultrasound-guided follicular ablation (AB) of all follicles ≥ 5 mm was performed in all animals followed immediately by intravaginal progestogen (P4) device insertion (Eazi Breed™ CIDR® Sheep and Cattle for calves and heifers, respectively). Exogenous FSH treatments were initiated 36 hours later and consisted of 8 (4-day) or 14 (7-day) treatments of 25 mg i.m. of pFSH (FSH) given at 12-hour interval. Heifers received 2 doses of prostaglandin f2alpha analog concomitant with the last two FSH injections, progestogen devices were removed at the time of the last FSH injection in all animals and animals in the *in vivo* groups received a pLH treatment (LH) 12 hours after the last FSH injection. Transvaginal ultrasound-guided oocyte collection (OC) was performed 8 hours after the last FSH injection in the *in vitro* group calves and 20 hours after LH treatment in the *in vivo* group animals.

7.3.1.2. Study II

As spontaneous ovulations were recorded during the gonadotropin treatment period in Study I. Study II was designed using a method of controlling endogenous LH release that was evaluated in calves given the 7-day treatment in another experiment (*Unpublished, Chapter 6*). An intermediate duration of FSH treatment (6 days) was also included.

Transvaginal ultrasound-guided follicular ablation of follicles ≥ 5 mm was performed under caudal epidural anesthesia to synchronize the emergence of a new follicular wave. All calves were given 600 mg i.m. of progesterone in 2 mL of a sustained-release formulation (BioRelease Technologies, Lexington, KY, USA) at the time of follicle ablation to prevent endogenous release and spontaneous ovulations during the FSH treatment. Calves were randomly assigned to one of the three treatment groups, consisting of 8 (4-day), 12 (6-day) or 14 (7-day) days of i.m. treatments of 25 mg of pFSH at 12-hour intervals starting 36 hours after follicle ablation (Fig. 7.2).

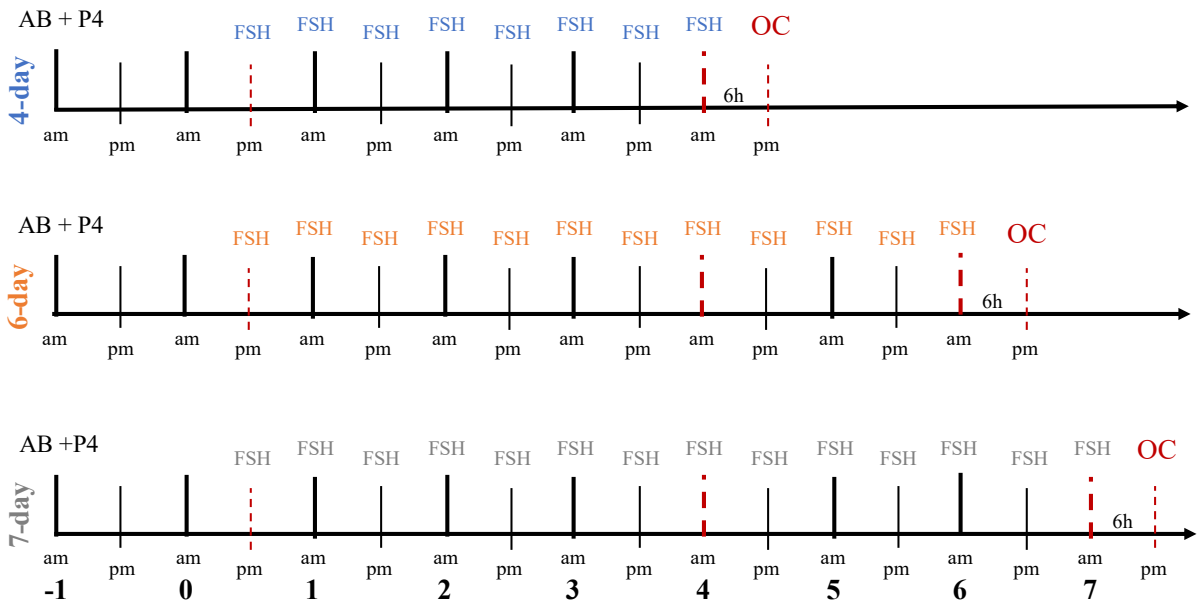

Fig. 7.2. Experimental Design Study II. Calves (n=21) were randomly assigned to 4 (4-day; n=7), 6 (6-day; n=7) or 7 (7-day; n=7) days of FSH treatment. Transvaginal ultrasound guided follicular ablation (AB) of all follicles ≥ 5 mm was performed in all animals and was followed immediately by a treatment of 600 mg i.m. of progesterone in 2 mL of a sustained release formulation (BioRelease Technologies). Exogenous FSH treatments were initiated 36 hours later and consisted

of 8 (4-day), 12 (6-day) or 14 (7-day) treatments of 25 mg i.m. of pFSH (FSH) given 12-hour intervals. Transvaginal ultrasound guided oocyte collection (OC) was performed 6 hours after the last FSH injection.

7.3.2. Collection of cumulus-oocyte-complexes (COC)

Transvaginal ultrasound-guided follicular aspirations to retrieve COC were performed under caudal epidural anesthesia in both studies. In Study I, COC were collected approximately 8 hours after the last FSH injection in the *in vitro* group calves and 20 hours after the pLH treatment in the *in vivo* group calves and *in vivo* group heifers. In Study II, COC were collected approximately 6 to 7 hours after the last FSH injection. Follicular contents were collected in a 50 mL conical tube containing 5 mL of BO OPU (IVF Limited T/A, IVF Biosciences; Falmouth, UK) at 36°C, using a vacuum pump (BV-003D, WTA, Cravinhos, SP, Brazil) set at 18 to 20 mL/min flow rate. The aspirated fluid was filtered immediately through an EmCon embryo filter using the BO OPU medium, and the COC were searched immediately using Nikon SMZ800 stereomicroscope fitted with a heating plate. In Study I, COC were classified according to the cumulus cells characteristics as expanded (fully expanded cumulus cells), partially expanded (outmost layers of cumulus cells expanded, and innermost layers compact), compact (compact cumulus cells) and denuded (absence of cumulus cells). In Study II COC were classified according to the characteristics of the cumulus cells and the cytoplasm as Grade 1 (≥ 3 layers of compact cumulus cells and homogeneous cytoplasm), Grade 2 (1 to 3 layers of compact cumulus cells and homogeneous cytoplasm), Grade 3 (absence of cumulus cells – denuded oocytes) and Grade 4 (expanded and/or partially expanded cumulus cells and/or heterogeneous cytoplasm).

7.3.3. In vitro maturation, fertilization and culture

In vitro maturation (IVM), fertilization (IVF) and culture (IVC) were performed using commercial media from IVF Bioscience and according to the protocol recommended by the company. Briefly, after grading, COC were transferred and washed three times in BO-Wash medium, washed once in BO-HEPES-IVM medium and transferred to 1.5 mL sonication tubes filled to 75% with BO-HEPES-IVM and kept at 38.8°C in a portable incubator for 22 hours for IVM (Studies I and II). *In vivo* matured oocytes from calves and heifers in Study I were given 6 hours of IVM in the same conditions as for the *in vitro* maturation. Immediately after IVM, COC

were washed once in pre-equilibrated BO-IVF medium and transferred to IVF wells and incubated at 38.8°C in 6% CO_2 balanced air humidified atmosphere. Two 0.25 mL frozen-straws of semen from a fertility-proven dairy bull were thawed, pooled and washed twice in BO-Semen Prep medium, and sperm were added to IVF wells at a concentration of 2 x 10^6/mL. After 18 hours of IVF, COC were transferred to warmed BO-Wash medium, denuded using a stripper pipette (135 µm inner diameter; Stripper®, ORIGIO Inc., Charlottesville, VA, USA), washed once in pre-equilibrated BO-IVC medium and transferred to BO-IVC wells under BO-Oil and incubated at 38.8°C in 5% CO_2, 5% O_2 balanced N_2 humidified atmosphere for IVC. Oocytes from each animal were handled and cultured separately. Cleavage rate was assessed at 48 hours after IVF (Day 2) and blastocyst rate at Days 7, and 9 of culture in Study I and Days 7, 8, and 9 in Study II.

7.3.4. Blood Samples and Ovarian Ultrasonography

Blood samples were collected by jugular venipuncture in a 10 mL heparinized tube (Becton Dickison Vacutainer® Systems; Franklyn Lakes, NJ, USA), centrifuged at 3000 x g for 15 minutes and the plasma was stored at -20°C until analyses. Cine-loops of ovarian ultrasound examinations were recorded, follicles were counted, measured and classified as small (3 to 5 mm), medium (6 to 8 mm) or large (≥ 9 mm) sizes.

In Study I, blood samples were collected, and ovarian ultrasound examinations were performed at the time of the first FSH treatment, at the time of LH treatment (for animals in the *in vivo* groups), at the time of COC collection and on Days 3, 7 and 11 after COC collection.

In Study II, blood samples were collected immediately before follicular ablation, at the time of the first FSH treatment and daily thereafter until COC collection. Ovarian ultrasound examinations were performed at the time of the first FSH injection (Day 0.5), Day 3, the day of the last FSH injection and immediately before COC collections. In order to compare follicle growth among treatments calves of the 6-day protocol were also examined on Day 4, while calves of the 7-day protocol were also examined on Days 4 and 6.

7.3.5. Hormone Analysis

In Study II, plasma LH concentrations were measured in duplicates using a validated double-antibody RIA [1]. The range of the standard curve was 0.06 to 8 ng/mL, and the intraassay

coefficients of variation were 3.3% and 6% for plasma with concentrations of 0.68 and 1.38 ng/mL, respectively.

In Study II, plasma estradiol concentrations were measured in duplicates using previously described radioimmunoassay [186]. The range of the standard curve was 0.5 to 20 pg/mL, and the intraassay coefficients of variation were below 15%.

Plasma progesterone concentrations in both studies were measured using a commercial radioimmunoassay kit (ImmunoChem™ Progesterone ^{125} RIA kit, MP Biomedicals, Costa Mesa, CA, USA). The range of the standard curve was 0.15 to 80 ng/mL, and the interassay coefficients of variation were 2.6%, 2.7% and 3.6% for low (0.79 ng/mL), medium (3.26 ng/mL) and high (6.94 ng/mL) reference sera, respectively, in Study I and 3%, 3.4% and 2.2% for low (1.0 ng/mL), medium (4.1 ng/mL) and high (9.3 ng/mL) reference sera, respectively, in Study II.

7.3.6. Statistical Analysis

All analyses were performed using Enterprise Guide 6.1 with SAS 9.4 (SAS Institute Inc., Cary, NY, USA). Glimmix procedure was used to analyze proportions data (COC classification, cleavage and blastocyst rates), GLM procedure for single timepoint measurements (follicles and COC numbers) and one-way or factorial repeated measures mixed linear model (Proc Mixed) for sequential data (plasma progesterone concentrations, LH concentrations, estradiol concentrations). Wherever appropriate, post-hoc multiple comparisons were made using the least squared differences. Log transformation was performed when data did not have equality of variances and normality of the residuals. Mean and the standard error of the mean (mean±SEM) were used in graphs and tables to describe response variables. Differences with $P \leq 0.05$ were considered significant, while P values between 0.05 and 0.10 were considered as tendencies.

7.3.6.1. Study I

The number of follicles ≥ 1 at wave emergence and ≥ 6 mm at COC collection, morphological classification of oocytes, cleavage and blastocyst rates were compared in calves using a 2x2 factorial analyzes between duration of FSH treatment (4 vs. 7 days) and oocyte maturation groups (*in vitro* vs. *in vivo*) and between calves and heifers using a 2x3 factorial analyzes for duration (4 vs. 7 days) and oocyte maturation grouped along with the animal category (*in vitro* calves vs. *in vivo* calves vs. *in vivo* heifers).

7.3.6.2. Study II

The number of follicles ≥ 3 mm, the number of COC collected and the COC grading were compared among treatment groups by one-way ANOVA. The number of follicles in size categories (small vs. medium vs. large size follicles) were compared among treatment groups (4-day vs. 6-day vs. 7-day) by factorial ANOVA using a General Linear Model (GLM). Plasma concentrations of progesterone, LH and estradiol from follicular ablation (-36 hours; wave emergence = '0' hour) until Day 4 (84 hours) and from -54 hours towards oocyte collection (time '0') were compared among treatment groups and time by factorial ANOVA using repeated measures mixed linear model. Regression analysis was used to address the relationship between plasma concentrations of progesterone and estradiol during the treatments on embryo development on Day 9 of culture.

7.4.Results

7.4.1. Study I

7.4.1.1. Ovarian Response

The total number of follicles ≥ 1 mm at wave emergence did not differ between treatment groups, nor among the oocyte maturation groups (Fig. 7.3A).

Ultrasound examinations at the time of LH treatment revealed the presence of corpora lutea in 3 calves (3/8) of the 7-day *in vivo* maturation group (considered spontaneous ovulations during the FSH treatment) and at the time of COC collection a decrease in the number of follicles ≥ 9 mm from previous examination (20 hours interval) in 5 calves of the *in vivo* maturation groups. At COC collection, the presence of corpora lutea was also recorded in one calf of the 7-day treatment/*in vitro* maturation group. Overall, at the time of COC collection, ovulations were recorded in 9 of 26 calves (visible corpus luteum and/or follicle disappearance), while no ovulations were observed in the heifer groups. Six of the 9 calves that ovulated were from the 7-day *in vivo* calf group, 2 from the 4-day *in vivo* calf group and 1 from the 7-day *in vitro* calf group. The number of ovulations did not differ between the 4-day vs. 7-day treatment groups (4.3±3.1 vs. 7.0±4.8; T-test P=0.7).

To consider the superstimulatory response at the time of COC collections, the number of follicles disappearing between LH treatment and COC collection and corpora lutea at COC collection were added to the number of follicles. At COC collection, the number of follicles ≥ 6

mm did not differ between the 4-day vs. 7-day groups (P=0.5; Fig. 7.3B), but was lower (P=0.01) in the *in vitro* calf groups (16.3±4.1; 4-day and 7-day groups combined) than in the *in vivo* calf (27.9±3.8) and heifer groups (29.1±3.4).

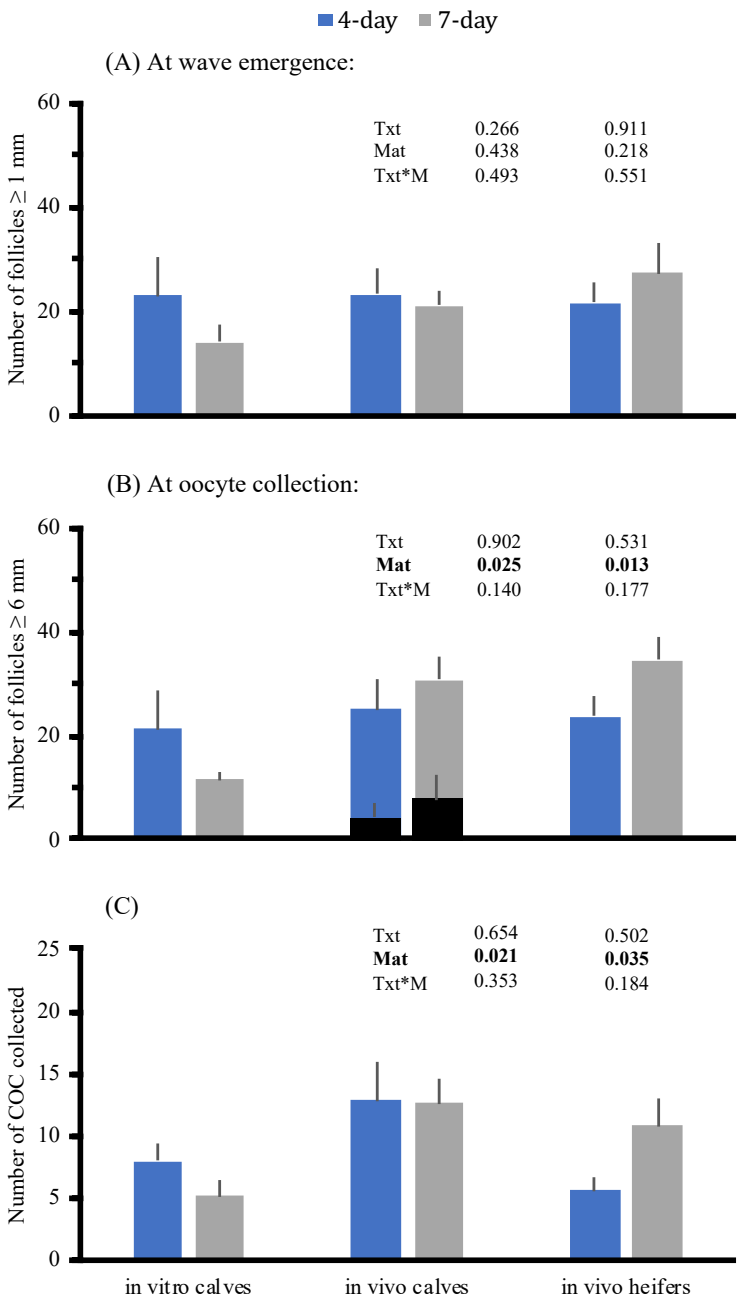

Fig. 7.3. Ovarian response Study I. The total number of (A) follicles ≥ 1 mm at wave emergence; (B) follicles ≥ 6 mm at COC collection, by including the number of ovulated follicles (i.e., the number of follicles that disappeared between LH treatment and COC collection and the number of

visible corpora lutea at COC collection; black bars); and (C) Cumulus oocyte complexes (COC) collected in calves and heifers (heifer) following 4 (4-day) or 7 (7-day) days of exogenous FSH treatment and *in vitro* or *in vivo* oocyte maturation.

7.4.1.2. COC Collection and Morphological Classification

The number of COC collected did not differ between the 4-day vs. 7-day groups (P=0.5; Fig. 7.3C) but was higher (P=0.03) in the *in vivo* calf groups (12.1±1.7) compared to the *in vitro* calf groups (6.8±1.0) while the *in vivo* heifer groups were intermediate (8.0±1.4).

Interactions (P ≤0.02) were discovered between treatment and oocyte maturation groups in the proportion of the recovered fully expanded, partially expanded, and compact COC (Table 7.1). The proportion of expanded COC in the 7-day *in vitro* and *in vivo* calf groups were lower than in the 7-day *in vivo* heifer group. The proportion of partially expanded recovered COC were higher in the 7-day than in the 4-day *in vivo* calf groups while the proportion of compact COC was higher in the 7-day *in vitro* calf group than in the 7-day *in vivo* heifer group.

Table 7.1. Cumulus oocyte complexes (COC) morphological classification* in Study I in calves and heifers (heifer) following 4 (4-day) or 7 (7-day) days of exogenous FSH treatment and *in vitro* (vitro) or *in vivo* (vivo) oocyte maturation.

	Expanded	P. Exp.	Compact	Denuded
4-day-vitro	23.1^{AB} (9/39)	15.4^{AB} (6/39)	43.6^{A} (17/39)	17.9 (7/39)
7-day-vitro	10.3^{A} (3/29)	6.9^{AB} (2/29)	82.8^{B} (24/29)	0
4-day-vivo	52.4^{BC} (44/84)	4.8^{B} (4/84)	22.6^{AC} (19/84)	20.2 (17/84)
7-day-vivo	19.4^{A} (19/98)	31.6^{A} (31/98)	36.7^{A} (36/98)	12.2 (12/98)
4-day-heifers	32.1^{ABC} (9/28)	7.1^{AB} (2/28)	39.3^{A} (11/28)	21.4 (6/28)
7-day-heifers	61.5^{C} (32/52)	9.6^{AB} (5/52)	5.8^{C} (3/52)	23.1 (12/52)
Txt	0.189	0.229	0.876	0.975
Mat/Cat	0.005	0.591	<.0001	0.496
Txt*M/C	**0.0004**	**0.012**	**0.0003**	0.617

*COC classification was based on the characteristics of the cumulus cells in expanded (fully expanded cumulus cells), partially expanded (outmost layers of cumulus cells expanded, and innermost layers compact), compact (compact cumulus cells) and denuded (absence of cumulus cells). Data analyzed by logistic regression (Glimmix) in a 2 (4-day vs. 7-day) x 3 (*in vitro* calves vs. *in vivo* calves vs. *in vivo* heifers) factorial design.

7.4.1.3. Embryo Development

A significant interaction between treatment and oocyte maturation groups was observed for cleavage and blastocyst rates (Table 7.2). Cleavage rate (number of cleaved embryos at Day 2 of *in vitro* culture/total number of COC collected) was lower in the 7-day *in vivo* calf group than in all other calf groups and the 7-day *in vivo* heifer group. Blastocyst rates on Days 7 and 9 were lower in the 7-day *in vivo* calf group than in the 4-day *in vitro* calf and 7-day *in vivo* heifer group.

Table 7.2. Embryo development Study I. Cleavage rate* 48 hours post *in vitro* fertilization (IVF) and blastocyst rate** after 7 (Blast D7) and 9 (Blast D9) days of culture in calves and heifers (heifer) following 4 (4-day) or 7 (7-day) days of exogenous FSH treatment and *in vitro* (vitro) or *in vivo* (vivo) oocyte maturation. Data analyzed by logistic regression (Glimmix) in a 2 (4-day vs. 7-day) x 3 (*in vitro* calves vs. *in vivo* calves vs. *in vivo* heifers) factorial design.

Endpoint (%)	4-day-vitro	7-day-vitro	4-day-vivo	7-day-vivo	4-day-heifers	7-day-heifers	P	
Cleavage	71.8^{A} (28/39)	72.4^{A} (21/29)	54.8^{A} (46/84)	24.5^{B} (24/98)	53.6^{AB} (15.28)	69.2^{A} (36/52)	Txt Mat/Cat **T*M/C**	0.444 0.0001 **0.004**
Blast D7	25.6^{A} (10/39)	13.8^{AB} (4/29)	10.7^{AB} (9/84)	2^{B} (2/98)	10.7^{AB} (3/28)	44.2^{A} (23/52)	Txt Mat/Cat **T*M/C**	0.612 0.003 **0.003**
Blast D9	33.3^{A} (13/39)	17.2^{AB} (5/29)	16.7^{AB} (14/84)	1^{B} (1/98)	14.3^{AB} (4/28)	48.1^{A} (25/52)	Txt Mat/Cat **T*M/C**	0.126 0.004 **0.0009**

* Total number of cleaved embryos/total number of recovered oocytes

** Total number of blastocysts/total number of recovered oocytes

7.4.1.4. Plasma Concentrations of Progesterone

At the time of COC collection, plasma progesterone concentrations were higher in the 7-day than in the 4-day treatment groups (data combined; 0.8±0.3 vs. 0.1±0.03; P=0.05) and were lower in the *in vitro* calf groups than in the *in vivo* calf and heifer groups (0.02±0.008 vs. 0.7±0.3 vs. 0.1±0.04; P=0.01). Plasma progesterone concentrations in all groups increased (P<.0001) from the time of COC collection until eleven days after COC collection (time-point that the last blood sample was collected; Fig.7.4). Overall, progesterone concentrations were higher in the 7-day than in the 4-day treatment groups (20.2±2.8 vs. 9.9±1.9; P=0.04) and in the *in vivo* groups (calves and heifers) than in the *in vitro* groups (16.6±2.5 vs. 16.6±3.2 vs. 6.0±2.2, respectively; P<.0001).

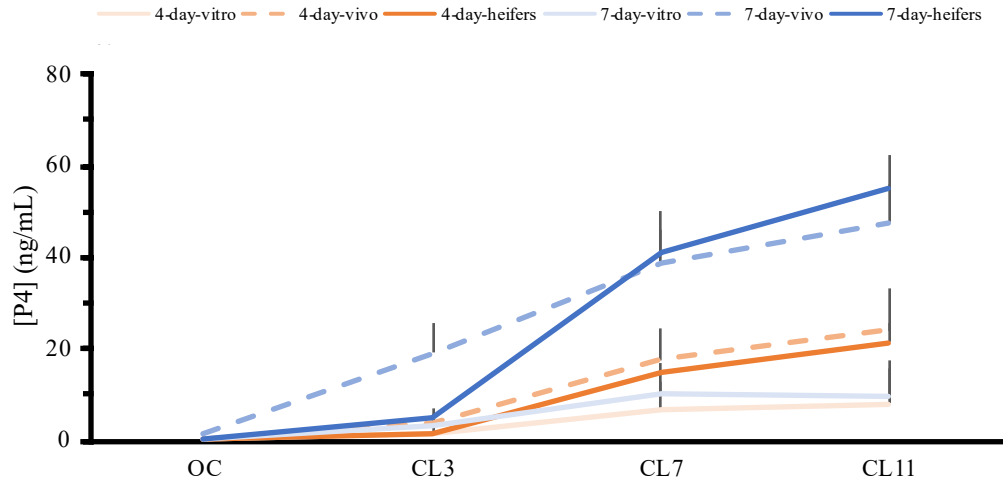

Fig. 7.4. Plasma progesterone concentrations from the time of oocyte collection (OC) to 3 (CL3), 7 (CL7) and 11 days (CL11) after oocyte collection in calves and heifers (heifer) following 4 (4-day) or 7 (7-day) days of exogenous FSH treatment and *in vitro* (vitro) or *in vivo* (vivo) oocyte maturation.

7.4.2. Study II

The objective of the study was to compare developmental competence after *in vitro* maturation of COC collected following exogenous FSH treatment for 4, 6 or 7 days in 4- to 5-month old prepubertal calves that were given 600 mg of progesterone in a sustained-release formulation to prevent spontaneous ovulations during treatment. The number of follicles ≥ 1 mm

or the numbers of 1-2 mm and 3-5 mm follicles at the time of first FSH injection (i.e., 36 hours after follicular ablation) did not differ among treatment groups (Fig. 7.5A).

7.4.2.1. Ovarian Response

All treatment groups were given the 8th FSH injection on Day 4 and as expected, the number of follicles ≥ 3 mm on Day 4 of FSH treatment did not differ among treatment groups (P=0.4). Averaged among groups, the number of medium size follicles (6 to 8 mm) tended to be higher than the number of large size follicles (≥ 9 mm) (11.2±2.0 vs. 6.6±1.2; P=0.06; Fig 7.5B).

At the time of the last FSH injection, the number of follicles ≥ 3 mm was higher in the 7-day than in the 4-day treatment group (42.1±7.3 vs. 18.0±2.9; P=0.04), while the number of follicles in the 6-day treatment group was intermediate (28.7±7.6) and did not differ from either the 4-day or 7-day groups (data not shown).

At the time of COC collection, an interaction between treatment and follicle size category (P=0.003) showed that the number of large-sized follicles was greater in the 7-day group (27.2±4.5) than in the 4-day group (7.0±1.2), while the 6-day group was intermediate (21.1±5.3; Fig 7.5C).

The number of follicles ≥ 6 mm at the time of COC collection was higher (P=0.03; Fig. 7.6A) in the 7-day treatment group (37.3±5.5) than in the 4-day (14.7±2.5) or 6-day (30.1±7.8) groups. The sustained release progesterone treatment was effective in controlling endogenous LH release, and no ovulations were detected during the treatments.

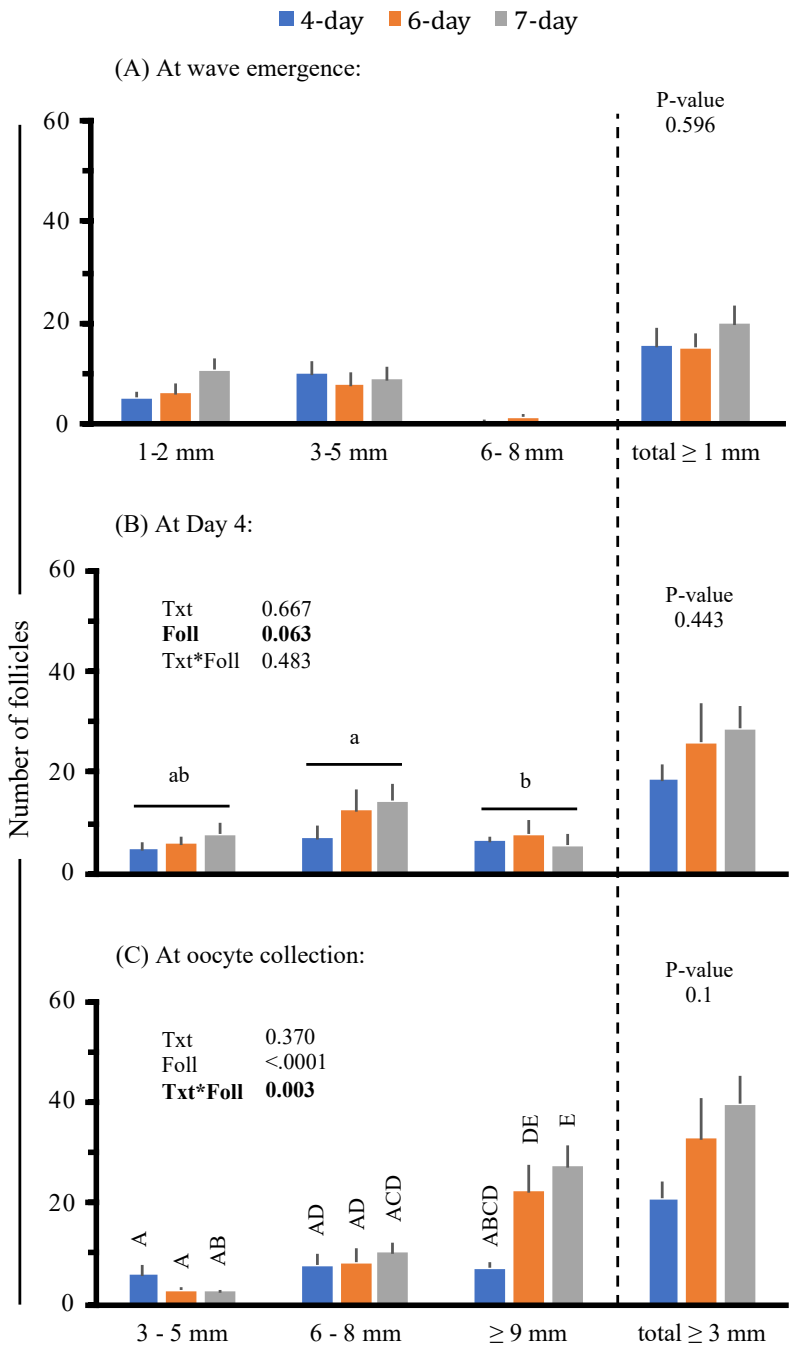

Fig. 7.5. Ovarian response in Study II. (A) Follicle category (1 to 2, 3 to 5 and 6 to 8 mm) and total number of ≥ 1 mm follicles at the time of wave emergence (i.e., 36 hours after follicular ablation); (B) Follicle category (3 to 5, 6 to 8 and ≥ 9 mm) and total number of ≥ 3 mm follicles on Day 4 of exogenous FSH treatment; and (C) Follicle category (3 to 5, 6 to 8 and ≥ 9 mm) and total number of ≥ 3 mm follicles at the time of oocyte collection in prepubertal calves given 4 (4-day; blue bars),

6 (6-day; orange bars) or 7 (7-day; grey bars) days of exogenous FSH treatment. Data were analyzed by 3 (duration of FSH treatment) x 3 (follicle category) factorial or one-way ANOVA (4 vs. 6 vs. 7 days of FSH treatment).

7.4.2.2. COC Collection and Morphological Classification

The number of COC recovered did not differ among treatment groups (overall 9.5±1.6 oocytes per calf; P=0.1; Fig. 7.6B). Recovery rate (i.e., number of COC recovered/number of follicles ≥ 6 mm) was also similar among treatment groups (overall 34.5±2.8%; P=0.7; data not shown).

The number of Grade 1 + Grade 2 COC (P=0.8) did not differ among treatment groups, while the number of Grade 3 + Grade 4 COC tended to be greater in the 7-day group than in the 4-day or 6-day groups (P=0.08; Fig. 7.6C). The proportion of higher quality COC (number of Grade1+2/total COC) was also similar among treatment groups (overall 55.3%, P=0.1).

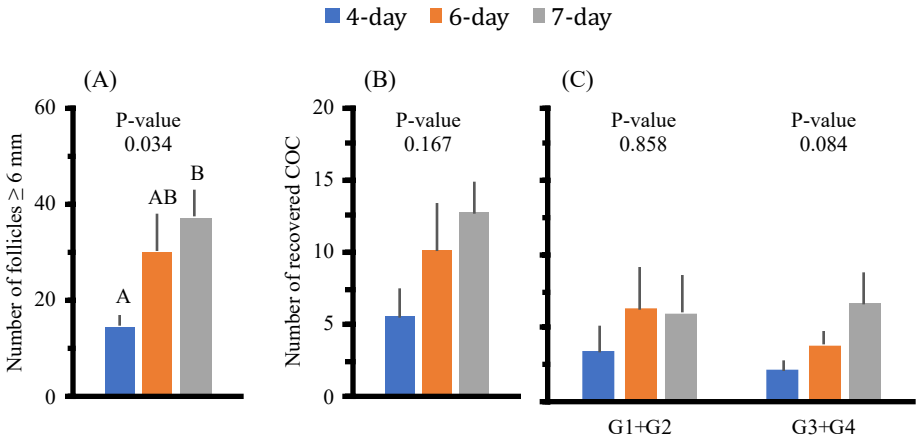

Fig. 7.6. At COC collection. (A) Number of follicles ≥ 6 mm; (B) Number of cumulus oocyte complexes (COC) collected; and (C) Number of COC in grades 1 and 2 (G1+G2) and grades 3 and 4 (G3+G4) combined in prepubertal calves given 4 (4-day; blue bars), 6 (6-day; orange bars) or 7 (7-day; grey bars) days of exogenous FSH treatment. COC were classified according to the characteristics of the cumulus cells and the cytoplasm in Grade 1 (≥ 3 layers of compact cumulus cells and homogeneous cytoplasm), Grade 2 (1 to 3 layers of compact cumulus cells and homogeneous cytoplasm), Grade 3 (absence of cumulus cells – denuded oocytes) and Grade 4 (expanded and/or partially expanded cumulus cells and/or heterogeneous cytoplasm).

7.4.2.3. Embryo Development

Cleavage rate on Day 2 of culture (Day 0 = *in vitro* fertilization) was higher (P=0.01) in the 6-day treatment group (73.2±5.3%) than in the 4-day (51.3±8.0%) or 7-day (47.2±5.3%) groups (Table 7.3). On Day 7 of culture, the proportion of oocytes that developed to morula and blastocyst stages was similar among treatment groups (20.9±4.6%; P=0.5). Blastocyst rate on Day 8 of culture tended to be higher (P=0.06) in the 6-day treatment group (38.0±5.8%) than in the 4- (20.5±6.5%) and 7-day (21.4±4.3%) groups, while on Day 9 of culture, blastocyst rate was higher (P=0.02) in the 6-day treatment group (40.9±5.8%) than in the 4-day (20.5±6.5%) or 7-day groups (20.2±4.3%).

Table 7.3. Recovery rate* and embryo development in Study II. Cleavage rate** 48 hours post *in vitro* fertilization (IVF), morula and blastocyst rate*** at day 7 of and blastocyst rate**** at days 8 and 9 of culture. Data analyzed by logistic regression (Glimmix).

Endpoint	4-day	6-day	7-day	P-value
Recovery	37.9±4.8 (39/103)	33.7±3.3 (71/211)	34.1±2.9 (89/261)	0.7
Cleavage	51.3 ± 8.0^{A} (30/39)	73.2 ± 5.3^{B} (52/71)	47.2 ± 5.3^{A} (42/89)	**0.01**
Morula and Blast D7	20.5±6.5 (8/39)	26.8±5.3 (19/71)	20.2±4.3 (18/89)	0.5
Blast D8	20.5±6.5 (8/39)	38.0±5.8 (27/71)	21.4±4.3 (19/89)	0.06
Blast D9	20.5 ± 6.5^{A} (8/39)	40.9 ± 5.8^{B} (29/71)	20.2 ± 4.3^{A} (18/89)	**0.02**

*Total number of recovered oocytes/total number of ≥ 6 mm follicles

**Total number of cleaved embryos/total number of recovered oocytes

***Total number of morula and blastocysts/total number of recovered oocytes

****Total number of blastocysts/total number of recovered oocytes

7.4.2.4. Plasma Hormone Concentrations

Plasma hormone concentrations are shown in Figure 7. A marked increased in progesterone (P4) concentrations in all groups (above 2 ng/mL) was observed from the time of P4 injection to the first FSH treatment (36 hours interval) and remained above 1 ng/mL in all groups until Day 4 (P<.0001), except for one calf in the 7-day treatment group (1/21; Fig. 7.7A). Plasma P4 concentrations were not different among treatment groups during the interval from P4 injection until Day 4 of FSH treatment (P=0.1). From -54 hours until COC collection (hour '0'), plasma P4 concentrations showed a steady decline in all groups (P<.0001), but were, overall, higher (P=0.0005) in the 4-day than in the 6-day or 7-day groups (2.1±0.2 vs. 1.3±0.1 vs. 0.9±0.1 ng/mL, respectively).

Plasma concentrations of LH showed a varied distribution over time (P=0.008) from follicular ablation (hour -36) until Day 4 of FSH treatment (Fig. 7.7B) and tended to be higher (P=0.07) in the 6-day group than in the 4-day or 7-day groups (0.09±0.01 vs. 0.05±0.01 vs. 0.05±0.01 ng/mL, respectively). From -54 hours until COC collection (hour '0'), plasma LH concentrations were higher (P=0.01) in the 6-day group than in the 7-day group (0.09±0.012 vs. 0.04±0.005 ng/mL) but were not different from the 4-day group (0.06±0.005 ng/mL).

Plasma estradiol (E2) concentrations from -54 hours until COC collection (hour '0') are shown in Figure 7.7C. Since E2 was not measured at -54 hour in the 4-day group, the analysis presented is between plasma E2 concentrations on the hour -6 and hour '0' (COC collection). There was a tendency for an interaction between time and plasma E2 concentrations (P=0.02), on the hour -6, E2 concentrations were lower in the 4-day group (3.6±0.5 pg/mL) than in the 6-day (8.1±2.0) or 7-day (7.2±0.8) groups. At COC collection, plasma E2 concentrations were higher in the 7-day group (13.4±2.3 pg/mL) than in the 4-day group (4.5±1.1) but were not different from the 6-day group (10.4±3.2).

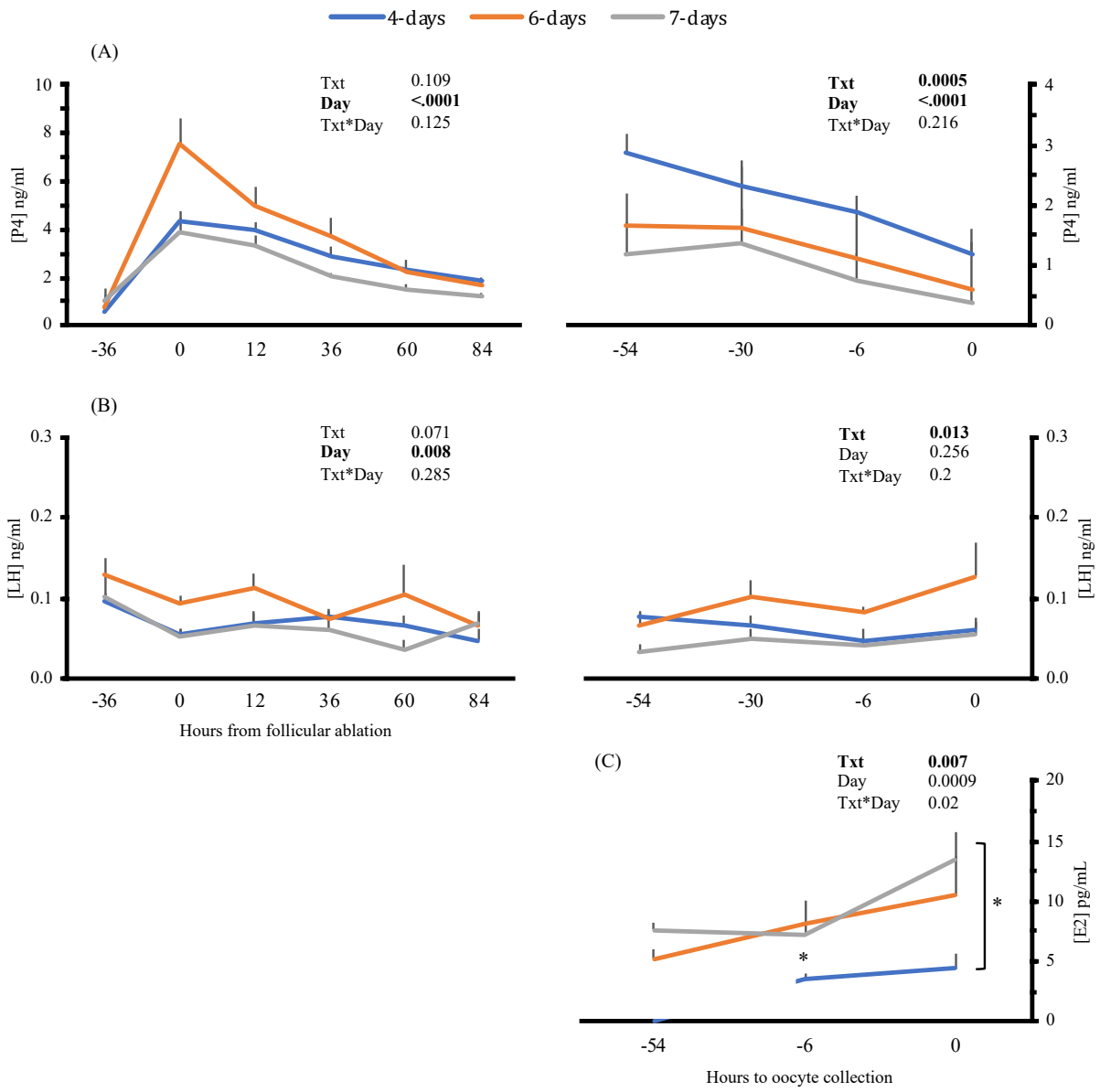

Fig. 7.7. Plasma concentrations of (A) Progesterone; and (B) LH from the time of follicular ablation (hour -36; wave emergence = 0) until Day 4 of FSH treatment (hour 84) (left panel) and from the last days of FSH treatment (hour -54, -30 and -6) until COC collection (hour 0); (C) Plasma concentrations of estradiol from the last days of FSH treatment (hour -54 and -6) until COC collection (hour 0) in prepubertal calves given 4 (4-day; blue lines), 6 (6-day; orange lines) or 7 (7-day; grey lines) days of exogenous FSH treatment.

7.4.2.5. The relationship between plasma concentrations of progesterone and estradiol and embryo development

The relationship of individual plasma progesterone and estradiol concentrations and the number of blastocysts produced at Day 9 of culture was evaluated through regression analysis (Table 7.4), to investigate the effects of different plasma levels of these hormones (independent variable) on embryo development (dependent variable). Plasma progesterone concentrations showed no relationship to blastocyst numbers. Plasma estradiol concentrations on Day 4 (0.3; P=0.004), at the time of the last FSH injection (0.5; P=0.0001) and at the time of oocyte collection (0.4; P=0.002) showed a positive relationship with blastocyst numbers at Day 9.

Table 7.4. The relationship between plasma progesterone ([P4]) and estradiol ([E2]) concentrations on Day 4 of FSH treatment (D4), at the time of last FSH injection (last FSH) and at the time of oocyte collection (OC) on the blastocyst production on Day 9. Regression analysis was used to investigate the effect of different plasma levels of these hormones (independent variable) on embryo development (dependent variable).

1st endpoint	2nd endpoint	Regression Analysis		
		r2	Adjusted r	P-value
[P4]D4	Number of blastocysts at Day 9 of culture	0.039	-0.011	0.390
[P4] last FSH		0.044	-0.0006	0.360
[P4] OC		0.043	-0.007	0.368
[E2] D4		0.360	0.326	0.004
[E2] last FSH		0.544	0.520	0.0001
[E2] OC		0.429	0.395	0.002

7.5.Discussion

In Study I, results showed that despite proportionally lower, calf oocytes exhibited similar developmental capacity as heifer oocytes after nine days of culture. Seven days of exogenous FSH treatment did not improve prepubertal calf oocyte developmental competence after *in vitro* maturation, but because of spontaneous ovulations, the hypothesis was not adequately tested with *in vivo* matured oocytes. Therefore, study II was designed to compare the developmental competence of *in vitro* matured oocytes following exogenous FSH treatment for 4, 6 or 7 days in 4- to 5-month old prepubertal calves that were given exogenous progesterone in a sustained-release formulation to prevent spontaneous ovulations during FSH treatment. A previous study showed that circulating progesterone concentrations resulting from the treatment were effective in suppressing the LH surge and ovulations during 7 days of exogenous FSH treatment. A greater number of follicles ≥ 6 mm resulted from the 7-day treatment, than from the 4- or 6-day treatment. However, the total number of recovered oocytes and the number of high-quality oocytes did not differ among treatment groups. Cleavage rate on Day 2 of culture and blastocyst rate on Day 9 were greater in the 6-day treatment group than in the 4- or 7-day treatment groups suggesting that duration of gonadotropin treatment influences follicular growth and oocyte developmental competence in prepubertal calves.

In sexually mature animals, higher rates of development to the blastocyst stage were associated with *in vivo* oocyte maturation [180], but surprisingly, in the present study, *in vitro* matured calf oocytes had similar rates of development than *in vivo* matured heifer oocytes. Comparable results were also observed in a previous study, in which similar rates of embryo development between calf oocytes matured *in vivo* and calf and cow oocytes matured *in vitro* were reported [135, 154]. Also, oocytes induced to mature *in vivo* had a varied degree of cumulus cell expansion, forming a highly heterogeneous population, which disagrees with the previous study in which *in vivo* maturation was associated with the recovery of a very homogenous oocyte population at the morphological and ultrastructural levels [202]. One possible explanation for this discrepancy could be that, an endogenous LH surge before pLH treatment may have induced premature oocyte maturation in this study, since the circulating progesterone concentrations resulting from the intravaginal device did not prevent LH release. Cumulus cell expansion is one of the first morphological indicators of successful oocyte maturation [156] and *in vitro* fertilization (IVF)

relies upon achieving proper oocyte maturation. Since *in vivo* matured oocytes were given only 6 hours of *in vitro* maturation after collection and before IVF, all oocytes may not have been at the metaphase II stage at the time of IVF. Although the low number of oocytes evaluated did not allow meaningful comparisons, previous results indicate that oocytes collected from calves given 4 or 7 days of exogenous FSH treatment and exogenous LH to induce *in vivo* oocyte maturation were all beyond metaphase I stage, but only a small proportion were at metaphase II at the time of collection (*Unpublished data, Chapter 4 – Supplementary information*). Cleavage rate as an indicator of fertilization following maturation was very low in the *in vivo* matured oocytes from calves given the 7-day treatment. Spontaneous ovulations may have prevented the recovery of matured oocytes, and indeed the majority of the oocytes recovered from this group were classified as compact. Therefore, *in vivo* maturation, fertilization, and developmental competence of oocytes collected from calves given 7 days of exogenous FSH treatment remain to be evaluated.

It has been demonstrated that the proportion of competent oocytes increases greatly in follicles > 8 mm in diameter in both FSH-treated and untreated animals and that the origin of the oocyte is the main factor affecting blastocyst rate [15, 180, 203]. Although calves that were given the 7-day treatment ovulated before oocyte collection, results showed that exogenous FSH treatment can indeed be beneficial to oocyte developmental competence in calves and that the duration of follicular growth under FSH support will exert direct effects on the competence of the enclosed oocyte, expressed by its ability to reach the blastocyst stage once fertilized. Improved rates of *in vitro* embryo production have been achieved in cattle when FSH-treated follicles undergo follicular differentiation through a rise and fall in FSH levels (coasting) before oocytes are recovered [86, 97]; however, no such improvements were observed in a study with calf oocytes [11]. Results in sexually mature cattle showed that *in vitro* embryo production of *in vivo* matured oocytes can be enhanced by 7 days of exogenous FSH treatment, in comparison with the traditional 4 days of treatment [13]. Prolonged follicular growth resulted in a higher number of large sized-follicles and follicular maturity in calves (*Unpublished data – Chapter 4*) and heifers [133], expressed by the higher proportion of fully expanded COC collected and lower intrafollicular estradiol:progesterone ratio. A longer duration of FSH treatment, that matches the interwave interval observed in 6-month-old calves [2] would appear to be optimal for follicular growth and ovulatory potential (*Unpublished, Chapters 3 and 4*); but perhaps follicle diameter may not be

associated linearly with oocyte competence in prepubertal calves, as has been suggested in sexually mature animals [97].

In the second study, a higher number of follicles ≥ 6 mm were observed in calves given the 7-day treatment, but neither the number of COC collected nor the number of high-quality COC differed among groups. However, the number of low-quality COC collected was higher in the 7-day treatment group, suggesting that the prolonged follicular growth in this group was not as beneficial to oocyte competence as expected since the COC recovered from calves in this group showed the morphological characteristics of COC recovered from follicles that had undergone prolonged dominance. It has been shown that bovine oocytes from prolonged dominant follicles undergo premature maturation *in vivo*, with expanded cumulus cells at the time of collection, condensed chromatin dispersed in the ooplasm and reduced fertility [204, 205]. Prolonged dominance is characterized by increased LH pulse frequency and suppression of the LH surge due to the maintenance of lower progesterone concentrations, which can lead to the loss of communication between cumulus cells and the oocyte, allowing the oocyte to resume meiosis while inside of the follicle.

In 6-month-old calves, the interwave interval has been reported to be on average 7 days, in which growth, static and regression phases lasted 3.5, 4.4, and 4.4 days, respectively [2]. Higher developmental capacity has been observed in oocytes recovered from follicles at the later stages of follicular growth, early atretic follicles, in which the oocytes remain within the follicular environment longer than usual [84]. Indeed, suppression of endogenous LH pulses and delay of the preovulatory LH surge by a GnRH antagonist resulted in higher rates of follicular atresia, without a negative effect on the oocyte [206]. In the current study, 6 days of exogenous FSH support resulted in higher rates of fertilization and blastocyst formation, supporting the hypothesis that manipulation of follicular growth under exogenous FSH support can be a key strategy to improve oocyte developmental competence in prepubertal calves. However, artificially extending or reducing follicle lifespan may have a direct impact on oocyte developmental competence and the ideal duration of follicular growth must be achieved to obtain positive effects.

The Long-acting P4 treatment was essential to control endogenous LH release in order to prevent ovulations during the FSH treatment, but different plasma levels of progesterone at the time of oocyte collection were observed among treatments. Although all calves were given the progesterone formulation from the same batch, differences in body mass and metabolic rate are

likely the cause. In a previous study (*Unpublished - Chapter 5*), plasma progesterone concentrations resulting from the progesterone treatment were observed to decrease slowly, and similar results were obtained in this study. Differences in progesterone concentrations were indeed anticipated due to different durations of FSH treatment. Earlier studies showed that progesterone supplementation during follicular growth resulted in the production of increased rates of transferable embryos [207]; however, a positive association between progesterone concentrations and the number of blastocysts produced was not found. On the contrary, there was a positive relationship between the plasma concentrations of estradiol and the number of blastocysts produced. In sexually mature cattle, decreased pregnancy rates have been associated with lower circulating concentrations of estradiol on the day of insemination [208].

In summary, despite proportionally lower, similar rates of embryo development were observed between *in vitro* matured calf oocytes and *in vivo* matured heifer oocytes following 4 or 7 days of exogenous FSH treatment. Results support the hypothesis that prolonged follicular growth results in a greater number of follicles reaching larger sizes (≥ 9 mm), but oocytes collected from those follicles did not have greater developmental competence. Six days of exogenous FSH treatment under controlled endogenous LH release was associated with the greatest developmental competence of *in vitro* matured oocytes collected from 5 months old calves, suggesting that duration of gonadotropin support influences follicular growth and oocyte developmental competence in prepubertal calves.

CHAPTER 8

8. GENERAL DISCUSSION

Overall, the presented studies aimed to compare the effects of different durations of exogenous FSH treatment on the ovarian response, follicle maturation, ovulation, and the oocyte developmental competence in prepubertal calves. Several studies were conducted to address this overarching objective in Hereford crossbreed prepubertal calves, ranging from 4 to 8 months of age. We tested the hypothesis that a longer duration of exogenous FSH treatment, that matches the seven days interwave interval observed in 6-month-old calves [2] would be optimal for follicular growth and oocyte developmental competence. The first study was designed to evaluate the effect of dose and duration of FSH treatment on the ovarian response of 6-month-old prepubertal calves (Chapter 3). Selection of calves for this study was based on the number of follicles ≥ 1 mm detected at the time of wave emergence (antral follicle counts, AFC) to minimize the variation among animals assigned to treatment groups. This study was followed by a second study (Chapter 4), in which we determined the repeatability of the AFC at wave emergence and the relationship between AFC and ovarian response following exogenous FSH treatment in prepubertal calves. Also, based on the results regarding FSH dose from the first study, we aimed to evaluate the effect of duration of FSH treatment on the ovarian response of 7-month-old prepubertal calves with low and high antral follicular counts (AFC) at the time of wave emergence. In the following study (Chapter 5), we compared the effect of duration of FSH treatment on the ovarian response, intrafollicular steroid concentrations, and number and quality of COC collected in prepubertal and pubertal cattle. We also investigated the relationship between the AFC and plasma concentrations of AMH and FSH at wave emergence in calves at 4 and 7 months of age. Subsequently, we aimed to compare *in vitro* versus *in vivo* oocyte maturation in calves following 4 or 7 days of gonadotropin treatment (Chapter 7, Study 1). Spontaneous ovulations during FSH treatment were observed, especially in calves given the 7 days treatment; this observation led us to evaluate three different methods of controlling endogenous LH release to prevent ovulation during 7 days of FSH treatment in 4 to 5-month-old calves (Chapter 6). Finally, based on the results of our previous studies combined, we compared the effect of 4, 6 or 7 days of exogenous FSH treatment on the ovarian response and developmental

competence of *in vitro* matured oocytes collected from 5-month-old prepubertal calves (Chapter 7, Study 2).

Gonadotropin treatment has been widely used in human and domestic animals as an assisted reproductive technique to increase the number of oocytes collected or embryos recovered after treatment by stimulating the growth of multiple competent antral follicles. In cattle, follicular development has been shown to occur in waves, and each wave is characterized by the synchronous growth of a group of 1 to 3 mm follicles [45], selection of a dominant follicle and regression of subordinates and this pattern was already observed in 2 week-old animals [1, 2, 16, 91, 127]. It was demonstrated in sexually mature [134, 149] and immature cattle [91], that the characteristics of an induced wave were not different from a spontaneous wave, in which an FSH peak precedes the emergence of each wave and that the ovarian response was enhanced when the FSH treatment was initiated at the time of wave emergence (before dominant follicle selection). These principles were used to guide the experimental design used for our research in prepubertal calves.

Prepubertal calf oocytes offer the potential to decrease the generation interval and increase the rate of genetic gain by the production of embryos *in vitro*. However, attempts of benefitting from the immature female germ cell have revealed that the sexual immaturity of the animal was also reflected in their gametes since prepubertal calf oocytes showed a reduced developmental capacity when compared to adult oocytes [6, 9, 11, 94]. In adult cattle, it was demonstrated that the developmental competence of oocytes *in vitro* was higher in oocytes recovered from follicles > 6 mm in diameter than from smaller follicles [15] and that the proportion of competent oocytes increased significantly in follicles > 8 mm in both treated and untreated animals [203]. Results from our lab showed that follicular growth under FSH support for 7 days resulted in a greater number of large follicles at the end of the treatment and in 2.5 more transferable embryos produced *in vitro* than for 4 days [13]. These durations of treatment were based on the length of the growing phase of the dominant follicle of 3 or 2 wave cycles in adult cattle. The number of waves per cycle cannot be determined in prepubertal calves, since their cycles are characterized for anovulatory waves of follicle development [43]; but in 6-month-old calves follicular growing, static and regression phases were observed to last 3.5, 4.4 and 4.4 days, respectively, resulting in an interwave interval of approximately 7 days [2].

In our first study (Chapter 3), dose and duration of FSH treatment were evaluated in a group of calves with similar ovarian condition; therefore, differences in ovarian response would be indeed

attributed to treatment. After 4 days of exogenous FSH treatment the majority of follicles observed were in the small and medium size categories and by evaluating the dose-rate of FSH per treatment, we observed that more follicles were rescued for growing with a dose of 25 mg of FSH than with a dose of 14 mg, but not with 44 mg, suggesting that exogenous FSH support may be dose-dependent until an upper plateau is reached. Similar to the observations in sexually mature cattle, a greater number of follicles ≥ 9 mm were observed in calves given 7 than 4 days of exogenous FSH treatment with a dose rate of 25 mg of FSH per treatment, resulting in a cumulative dose of 350 mg of FSH. However, when cows were given the 4- or 7-days treatment with a cumulative dose of 400 mg of FSH, the lower FSH dose rate given to cows in the 7 days treatment resulted in slower growth-rate of follicles and similar number of large size follicles at the end of the treatment; although, the reduced dose rate was sufficient to maintain the growth and viability of follicles within the wave, since a higher number of ovulations were observed in cows given the 7-days treatment [131, 132]. In cattle, the superstimulatory effect of exogenous FSH treatment was attributed to the rescue of small antral follicles present at the time of wave emergence, since there was no evidence for continuous recruitment of new follicles during 4 or 7 days of treatment [132]. During our first studies, spontaneous ovulations were not anticipated but were observed during FSH treatment, indicating a possibly positive feedback of estradiol eliciting an endogenous LH surge; therefore, superstimulation in prepubertal calves requires control of endogenous LH release to prevent ovulation, as in sexually mature animals. In Study I, 60% of calves (14/23) ovulated before exogenous LH administration, i.e., due to endogenous LH surge.

Earlier studies in superstimulated calves demonstrated that the plasma level of LH after superstimulation was similar to that observed in mature cows [79, 80]. It was also noted that while plasma LH levels were similar between calves and heifers the mean LH concentration of pituitary tissue in calves was much higher than in sexually mature heifers (5.0 versus 1.1 mg LH/g of pituitary) and that the pituitary LH levels decreased markedly after superovulation, reaching the values found in mature heifers [79]. In unstimulated prepubertal calves, LH secretion was shown to be more sensitive to the negative feedback of estradiol than in sexually mature animals [67]. Indeed, the sexual immaturity of prepubertal calves is characterized by a lower pulse frequency of LH secretion, anovulatory follicular waves and the absence of corpus luteum. However, earlier studies showed that GnRH treatment could induce a preovulatory LH surge in prepubertal calves, suggesting that the prepubertal period is characterized by lack of pituitary stimulation, rather than

an inability to respond to GnRH [61]. While ovulatory capacity in adult cattle was shown to be acquired when a follicle reaches a diameter of about 10 mm, a 10 mm follicle required a higher LH dose to ovulate than a larger follicle [140]; however, ovulatory size and the attainment of ovulatory capacity during the prepubertal period has not been fully investigated. We observed in our study that calves did not ovulated during the first 4 days of exogenous FSH treatment, but the majority of calves given the 7 days treatment ovulated due to endogenous LH surge. Since follicles in the 7-day treatment grow for a longer duration, they attain larger sizes, which suggests that in prepubertal calves as well as in adult animals, a given follicular diameter must be achieved before ovulation occurs. Secretion of LH is critical to maintain dominant follicle growth and viability, and it was demonstrated that the number of LH receptors in granulosa cells increased between Days 2 and 4 of the follicular wave [138]. The expression of LH receptors by granulosa cells seems to be the mechanism responsible for the acquisition of ovulatory capacity and an increase in LH receptor expression was associated with follicular growth beyond 10 mm and a decrease in the LH dose necessary to cause ovulation [140]. In the normal estrous cycle of sexually mature animals, estradiol production is enhanced at the time of dominant follicle selection, when the future dominant follicle reaches 8 to 9 mm in diameter [52, 209, 210], and interestingly intrafollicular concentrations of estradiol were shown to be similar between single dominant follicle and FSH-treated follicles [185].

In adult cattle, the number of follicles within a wave, i.e., the antral follicle counts (AFC), were shown to be highly variable among individuals, but highly repeatable within animal [128, 129]. Based on that, an ovarian ultrasound examination could reliably be used to predict the ovarian response to gonadotropin treatments [129]. Although in 2-month-old calves, a high correlation between the number of antral follicles observed before FSH treatment and the number of oocytes collected after treatment was observed [93], no information regarding repeatability and predictive relationships of the AFC were reported in prepubertal calves. The results from Chapter 4 showed that the number of follicles recruited in a wave was predictive of the number recruited into subsequent waves and the ovarian response to gonadotropin treatment, confirming in prepubertal calves the findings reported for sexually mature animals. Classification of calves according to the AFC also confirmed that AFC pattern is maintained over time, suggesting in agreement with others, that AFC phenotype may be established at the prepubertal period [150]. Calves with a high AFC at wave emergence had a greater number of follicles at the end of the FSH treatment and a higher

number of COC collected than calves with a low AFC (Chapter 4). In adult cattle, low AFC has been associated with not only a poor response to gonadotropin treatment but also with a reduced number of morphologically health follicles and oocytes in the ovaries [147]. *In vitro* studies showed that granulosa cells recovered from follicles of animals with low AFC had minimal responsiveness to FSH than granulosa cells from animals with high AFC, resulting in lower FSH-induced estradiol and AMH production [152], but interestingly, the 7 days treatment resulted in a greater number of larger follicles at the end of the treatment than the 4 days in calves with low AFC, but not in calves with high AFC, suggesting that perhaps animals with low AFC may benefit from prolonged follicular growth during superstimulation since there are fewer responsive follicles disputing for FSH.

Together with the AFC, measurements of circulating anti-Müllerian hormone (AMH) concentrations have been used as predictive of the size of the ovarian reserve [151]. A positive correlation was observed among the AFC, the AMH concentrations, and the total number of morphologically healthy follicles (from primordial to antral) and oocytes in the ovaries in cattle [151]. AMH is expressed in granulosa cells of all growing follicles, with the highest expression observed in healthy small antral follicles [211]; therefore, AMH is a reliable endocrine marker of the population of gonadotropin responsive follicles. Biweekly measurements from birth until puberty revealed that plasma AMH concentrations exhibited a characteristic profile in cattle, increasing gradually after birth until reach a peak at 10 weeks of age, before gradually declining to a steady pattern at 6 weeks before puberty [170]. Interesting, the peak of AMH levels observed at 10 weeks of age was preceded by surges in plasma FSH concentrations and previous studies have reported similar observations regarding circulating plasma FSH concentrations during the first weeks of age in cattle [2, 4]. In these studies, an increase in gonadotropin concentrations between 8 and 20 weeks of age was observed and was followed by a peak in the number of ovarian antral follicles. Visual examination of bovine ovaries at different ages [125, 126] and frequently ovarian ultrasound examinations of the same animals showed that the number of antral follicles increases from birth to 4 months of age, decreases from 5 to 8 months of age and remains constant thereafter [2]. Although the reasons of the effect of age on the number of follicles during the prepubertal period is not clear, it is plausible to assume that it is related to the development and maturation of the hypothalamus-pituitary-gonadal axis. In Chapter 5, a higher AFC and plasma AMH

concentrations were observed in calves at 4 months of age than at 7 months, indeed, follicle counts at wave emergence were almost 2 times higher at 4 months than at 7 months of age.

Interesting, plasma AMH concentrations at 16 weeks of age were shown to correlate with post-pubertal AMH levels positively [170], and differently from the prepubertal period, plasma AMH levels were shown to be stable and to vary minimally during the estrous cycle after puberty [144, 161]. In 2 to 4-month-old calves, plasma AMH concentrations were positively correlated with the AFC and the number of COC collected by laparoscopy; moreover, AMH levels were positively correlated with the number of COC suitable for cultured and the number of blastocysts produced, suggesting that AMH and AFC can be used reliably to select oocyte donors [88]. Differently from the last study, that measured plasma AMH concentrations at the time of COC collection, we measured AMH levels at the time of wave emergence; we found a positive correlation between AMH levels and the AFC at wave emergence and the ovarian response after FSH treatment at 4 and 7 months of age. The results from both studies confirmed that plasma AMH levels are a useful marker of the AFC in prepubertal calves and that both AMH and AFC were predictive of the ovarian response to exogenous FSH treatment.

The AFC and the ovarian response to FSH treatment were markedly higher at 4 than at 7 months of age, what is in agreement with previous observations [2, 43, 125, 126]. Seven days of FSH treatment almost tripled the number of follicles ≥ 9 mm at the end of the treatment when compared to the 4 days treatment in calves at 4 months of age, but not at 7 months of age or in sexually mature heifers. Prolonged follicular growth under exogenous FSH support resulted in lower intrafollicular levels of estradiol, lower estradiol (E2) by progesterone (P4) ratio and higher rates of fully expanded COC collected, which altogether indicates a higher level of follicular maturation. The intrafollicular ratio of E2:P4 can be used to distinguish growing from atretic or preovulatory follicles [54, 55, 173], since atretic follicles showed a reduced capacity to produce estradiol due to a decrease in the number of healthy granulosa cells and consequently gonadotropin receptors [55, 138]. It is important to note that the follicular fluid in our study was aspirated from follicles 24 hours after an exogenous LH stimulus and therefore follicles were expected to be at the preovulatory stage. Preovulatory follicles are characterized by high intrafollicular steroid levels, both E2 and P4, despite a marked reduction in the expression of key enzymes of androgen production in theca cells (P450C17) and estrogen production in granulosa cells (P450 aromatase) [174].

Cumulus cells expansion is the morphological indicator of oocyte maturation [156]; transzonal projections between cumulus cells and the oocyte are responsible for the exchange of metabolites, nutrients and signaling molecules during growing and maturation of the oocyte and these communicating channels were shown to be disrupted at 9 to 12 hours after LH surge/stimulus [178]. In previous studies from our lab, the number of oocytes in metaphase II stage collected after an exogenous LH stimulus was 3 times higher in the 7 days treatment than in the 4 days. Bovine oocytes have been shown to reach metaphase II stage around 19 to 20 hours after LH stimulus [178]. In Chapter 5 (Supplementary Information), oocytes collected from 4-month old calves and sexually mature heifers were submitted to *in vitro* fertilization and culture; higher rates of embryo development after 9 days of culture were observed in oocytes collected from heifers that were given the 7 than the 4 days treatment and from oocytes collected from calves, regardless of treatment duration. No differences were observed on the blastocyst rates between the 4- and 7-days treatment among calf oocytes and calf oocytes of both groups and heifer oocytes given the 4 days treatment. A third of the oocytes collected from the 7-month-old calves were evaluated for nuclear maturation and the results showed that the majority of the oocytes were beyond metaphase I stage, but an accurate estimation of oocytes in metaphase II was not possible, due to a higher number of cumulus cells that remained attached to the oocytes (Supplementary Information, Chapter 5). Based on these data, Chapter 7 (Study I) aimed to compare oocyte developmental competence between *in vivo* versus *in vitro* oocyte maturation following the 4 or 7 days of exogenous FSH treatment in prepubertal calves. Although the blastocyst rates achieved from calf oocytes were lower when compared to blastocyst production from slaughterhouse ovaries (around 35% in our laboratory), the blastocyst rate was not different among calves of both groups and heifers that were given the 4 days treatment. Previous reports showed that the developmental competence of calf oocytes induced to mature *in vivo* was not different from calf and adult cow oocytes matured *in vitro* [135]. However, based on the characteristics of cumulus cells, collection of oocytes following FSH treatment and GnRH administration to induce *in vivo* oocyte maturation resulted in a highly heterogeneous pool of oocyte collected from 5-month-old calves, but apparently no differences in blastocyst rate between *in vivo* or *in vitro* (9 vs. 8%) matured oocytes [90]. Contrarily, *in vivo* oocyte maturation was associated with higher rates of blastocyst production in sexually mature cattle [180] and developmental competence was higher when the oocyte was collected from

follicles > 8 mm in diameter than from follicles between 3 and 7 mm, but the competence of oocytes from follicles > 8 mm was higher after *in vivo* than after *in vitro* maturation [203].

Current *in vitro* maturation conditions of bovine oocytes have resulted in 80% of oocytes reaching metaphase II, and it has been shown that although nuclear maturation follows the same pattern *in vivo* and *in vitro*, variation and delay in cortical granule spread in *in vitro* matured oocytes were noticed in ultrastructural studies [178], which may decrease the probability of normal fertilization, since cortical granules spread is essential for proper oocyte maturation. Higher rates of polyspermy have been observed in *in vitro* matured calf [87] and lamb [12] oocytes, but a steadily decrease in the polyspermy rate was observed with age in calves, decreasing from 45% in calves younger than 100 days of age to 12% in calves with more than a 130 days of age [87]. Maturation environment has been shown to influence the transcription pattern of both the matured oocyte and the future embryo [212-214] and culture conditions during oocyte maturation and early embryo development has been shown to affect the metabolic activity and the ability of embryos to adapt to different environments [214], therefore exerting significant influences on the capacity of oocytes to further develop into blastocysts. In Chapter 7, similar rates of blastocyst production after 9 days of culture between calf oocytes matured *in vitro* and heifer oocytes matured *in vivo* following the 4 and 7 days of FSH treatment, respectively was observed. Among calves given 4 days of FSH treatment, no difference in the blastocyst rate was observed between oocytes matured *in vivo* or *in vitro*, but spontaneous ovulations during FSH treatment prevented comparisons among calves given the 7 days treatment. Therefore, fertilization and developmental competence of *in vivo* matured calf oocytes following the 7 days treatment remain to be evaluated. Calf oocytes matured *in vitro* following the 7 days treatment developed at similar rates than calf oocytes matured *in vivo* following the 4 days treatment. Based on the findings of Chapters 5, 6, and 7 (Study I), it is possible to conclude that a greater ovarian response can be induced under prolonged follicular growth, with a higher number of follicles reaching larger sizes. However, since the developmental competence of oocytes collected from calves given 7 days of treatment was not greater than from calves given 4 days of treatment as expected (based on studies in sexually mature animals [13]), we can infer that the relationship between follicle size and oocyte competence in prepubertal calves may not be linear, similar to the observations in sexually mature animals, in which follicular size was not correlated with increased oocyte competence after follicles reach 10 mm in diameter [97].

Indeed, it was showed in Chapter 7 (Study II) that in oocytes collected from 5-month-old calves given 4, 6 or 7 days of FSH treatment and submitted to *in vitro* maturation, the greatest developmental competence was observed in oocytes from calves given 6 days of treatment. Interestingly, the number of good quality oocytes was not different among treatment groups, but a higher number of low-quality oocytes was recovered following the 7 days treatment, in which a greater number of oocytes showed expansion of cumulus cells. Morphologically, the low-quality oocytes collected from calves given the 7 days treatment resembled oocytes collected from follicles that underwent prolonged dominance, in which oocytes exhibited expanded cumulus cells and condensed chromatin dispersed in the ooplasm, suggesting premature germinal vesicle breakdown and resumption of meiosis *in vivo* [204, 205] and similar characteristics were observed in oocytes collected from subordinate follicles and were associated with the arrest of follicular growth observed in these follicles [99]. Although bovine oocytes were shown to achieve competence to resume meiosis and sustain fertilization and early embryonic development inside a 2 to 3 mm follicle, ultrastructural changes observed in oocytes of dominant follicles before the LH surge are believed to be a prerequisite for the oocyte to acquire full competence. These changes were associated with an increase in intrafollicular concentrations of E2 and P4 and were characterized by a spatial rearrangement of mitochondria, decrease in the size of the Golgi compartment, increase in the number of lipid droplets, enlargement and vacuolization of the nucleolus and nuclear envelope undulation [99]. Therefore, it appears that oocyte competence to become a viable embryo is acquired during the last days of follicular growth before ovulation, a crucial period in which LH drives the final stages of differentiation of the dominant follicle [215]. It would be interesting to evaluate the ultrastructural changes resulting from the different durations of FSH treatment in oocytes collected from calves in the presented studies, to better understand the effect of follicular growth under FSH support on the acquisition of developmental competence in these oocytes. Dominant follicle selection in cattle is characterized by the transition from FSH to LH dependency but maintaining elevated the circulating concentrations of FSH by exogenous administration allows several follicles to be rescued from regression and atresia, continuing growth [72, 127, 215]. In sexually mature cows, the number, distribution, and size of lipid droplets did not differ between *in vivo* matured oocytes collected following the 4 days or the 7 days treatment; however; individual mitochondria were more active and tended to produce more ATP after the 7 days treatment than

the 4 days [216], and higher ATP content were associated with higher developmental potential of bovine oocytes [217].

In contrast to our studies, in which FSH support was maintained for a given number of days, another research group has focused in FSH withdrawal (or coasting) after a period of FSH treatment to generate immature germinal vesicle oocytes with maximum competence [215]; initially FSH is used to prevent single follicle dominance and later FSH is removed during a defined period to mimic dominant follicle growth and differentiation under basal endogenous LH support. With this procedure, great improvements on blastocyst rates (from 30 to 70%) have been reported in oocytes collected from sexually mature cows [86, 97], but such improvements were not observed in oocytes collected from prepubertal animals [11]. The treatment of GnRH-immunized anestrus heifers with exogenous FSH and LH showed that LH alone failed to stimulate the follicular growth beyond 5 mm, FSH alone stimulated the growth of medium sized follicles (5 to 9.5 mm) that were estrogen inactive, while treatment with both gonadotropins stimulated the growth of medium sized follicles and E2-active large follicles (≥ 10 mm) resulting in a 10 to 14-fold increase in circulating concentrations of estradiol [218]. In our study, a regression analysis showed a positive relationship between plasma concentrations of estradiol and the number of blastocysts produced; plasma estradiol concentrations were higher in the 7 days than in the 4 days treatment but were not different from the 6 days treatment. Such differences in plasma concentrations of estradiol among treatment groups may be attributed to differences in follicle size resulting from different durations of follicular growth. Contrarily, the gradual decrease in plasma progesterone concentrations following the P4 treatment resulted in different concentrations of progesterone at COC collection among treatment groups, but plasma P4 concentrations were not associated with blastocyst numbers but may have affected LH pulse frequency. Despite all calves were given the P4 mixed into the slow-releasing formulation from the same batch, a greater individual variation in the plasma levels of progesterone achieved after treatment was observed among calves; however, it is important to note that at the time of COC collection, calves of all groups had sub-luteal levels of progesterone, ranging from 0.29 to 1.68 ng/mL. Since elevated concentrations of estradiol and progesterone are not observed during the prepubertal period, the effects of artificially increasing plasma E2 and P4 concentrations on the hypothalamus-pituitary-gonadal axis and oocyte developmental competence are not known. In sexually mature cattle, pregnancy rates were not different when the dominant follicle was submitted to luteal or sub-luteal circulating concentrations of progesterone during its

growth phase; however, a progesterone free period during the final stages of dominant follicle growth was shown to be required, i.e., normal proestrus, since shorter proestrus resulted in lower pregnancy rates [219]. In this last study, duration of follicular growth was shown to play a major role, since prolonged follicular growth under sub-luteal levels of progesterone resulted in poor fertility, due to premature oocyte maturation [204, 205]. Interesting, the dominant follicles submitted to prolonged dominance retained ovulatory capacity, resulting in the ovulation of aged oocytes [189, 205].

Progesterone exerts negative feedback on LH pulse frequency, therefore preventing ovulation during the luteal phase in adult cattle, but low circulating concentrations of progesterone resulting from intravaginal devices or implants have resulted in a high frequency of LH pulses [220]. We observed that in 5-month-old prepubertal calves, circulating concentrations of progesterone resulting from an intravaginal device containing 0.3 grams of progesterone did not control endogenous LH surge and ovulations were observed during FSH treatment (Chapter 6). In this study, we compared different methods to prevent endogenous LH release during 7 days of gonadotropin treatment; calves in the control group (no prevention of LH release) and calves receiving the intravaginal (0.3 grams progesterone) device showed increases in plasma LH concentrations that resembled an LH surge (a rise from nadir). Ovulations during the 7 days of FSH treatment were prevented by the circulating progesterone resulting from P4 mixed into a slow-releasing formulation treatment and by treatment with the GnRH receptor antagonist Cetrorelix. Cetrorelix has been shown to block both LH pulse and basal levels of LH secretion [199]. Interesting, when a GnRH antagonist treatment was used to maintain a preovulatory state in follicles from FSH treated heifers, follicular atresia and a decrease in intrafollicular levels of steroids (E2 and P4) were observed, without affecting oocyte developmental competence [206]. It has been demonstrated in cattle that oocytes of morphologically low quality (beginning of expansion in outer cumulus layers and slightly granulated ooplasm) collected from follicles showing first signs of atresia developed significantly better *in vitro* than good-quality oocytes [84]. These findings were the base for the coasting regimen adopted in cattle, which manipulates follicular development to produce developmentally competent oocytes [86, 97]. Interesting, a GnRH antagonist treatment used to suppress LH secretion during follicular differentiation (coasting period) did not affect the quality of oocytes collected, since blastocyst rates were not different between animals receiving a GnRH antagonist or no-treatment during coasting; however,

treatment with the GnRH antagonist affected the abundance of specific mRNAs known to have crucial role in translation and chromosome segregation [221].

In summary, the duration of exogenous FSH treatment was shown to affect follicle development, therefore influencing ovarian response and oocyte developmental in prepubertal calves. Calves of all ages used were responsive to FSH treatments and despite a highly individual variation in the ovarian response, classification of calves according to AFC at wave emergence was effective in predicting the ovarian response and the number of oocytes recovered. Longer duration of FSH treatment resulted in prolonged follicular growth and a greater number of large size follicles at the end of treatment but was not associated with an improved developmental capacity of oocytes. Based on our results, the greatest developmental competence of oocytes was obtained in calves given 6 days of exogenous FSH treatment than 4 or 7 days. In 6-month-old calves, 6 days of follicle development comprises 3.5 days of growth and half of 4.4 days of the static phase. Four days of exogenous FSH treatment was not enough for follicles to reach larger sizes and resulted in a similar number of embryos produced than the 7 days treatment. Further studies aiming to better understand the effects of exogenous FSH treatment on the hypothalamus-pituitary-gonadal axis and oocyte ultrastructure are advised.

CHAPTER 9

9. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Based on the studies in prepubertal calves and the results presented in Chapters 3 to 7, it can be concluded that:

9.1. Ovarian response to gonadotropin treatment based on cumulative dose (200 vs. 350 mg) and duration (4 vs. 7 days) of treatment

a. The greatest ovarian response was observed in calves given 7 days of exogenous FSH treatment with a cumulative dose of 350 mg FSH;

b. The total number of ovulations (spontaneous and induced) was higher in calves given 7 days of FSH treatment than 4 days.

9.2. Repeatability and predictive relationships of AFC at wave emergence

a. The number of follicles ≥ 1 mm at wave emergence was predictive of the number of follicles in subsequent waves and ovarian response following superstimulation.

9.3. The effect of AFC, plasma AMH concentrations and age on the ovarian response to exogenous FSH treatment

a. High AFC at wave emergence resulted in a higher number of follicles at the end of the FSH treatment and COC collected than low AFC;

b. Plasma AMH concentrations were useful as a marker of the AFC, and both AMH and AFC were predictive of the ovarian response to exogenous FSH treatment;

c. A greater number of ≥ 1 mm follicles at wave emergence and ovarian response to exogenous FSH treatment was observed in calves at 4 months of age than at 7 months of age.

9.4. Ovarian response and oocyte developmental competence following different durations of gonadotropin treatment and *in vitro* vs. *in vivo* oocyte maturation

a. Seven days of exogenous FSH treatment followed by an exogenous LH stimulus resulted in evidence of greater follicular maturation than 4 days, that was characterized by lower intrafollicular estradiol:progesterone ratio and higher proportion of fully expanded COC collected;

b. Similar rates of embryo development were observed among *in vitro* matured calf oocytes and *in vivo* matured heifer oocytes following 4 or 7 days of exogenous FSH treatment, respectively;

c. Six days of exogenous FSH treatment was associated with the greatest developmental competence of oocytes to the blastocyst stage in culture when compared to 4 or 7 days of treatment in 5-month-old calves.

9.5. Controlling endogenous LH release to prevent spontaneous ovulations during exogenous FSH treatment

a. Maintaining the circulating concentrations of progesterone ≥ 1 ng/mL during 7 days of exogenous FSH treatment prevented endogenous LH surge and ovulations; ovulations were observed after FSH treatment, when plasma progesterone concentrations reached sub-luteal levels;

b. Treatment with the GnRH antagonist Cetrorelix during exogenous FSH treatment prevented ovulations during and after exogenous FSH treatment.

The presented conclusions can be used to guide future studies, aiming to evaluate:

- The hormonal control of ovulation in prepubertal calves;
- The effects of exogenous FSH treatment on the hypothalamus-pituitary-gonadal axis;
- The effects of different durations of exogenous FSH treatment on the ultrastructure of calf oocytes, the quality of produced embryos and embryo viability after transfer.

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