USE OF AN AROMATASE INHIBITOR FOR OVARIAN SYNCHRONIZATION IN CATTLE

A Thesis Submitted to the College of

Graduate Studies and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Department of Veterinary Biomedical Science

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

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ABSTRACT

Letrozole is a third-generation aromatase inhibitor approved for use in post-menopausal women to treat hormone-responsive breast cancer. Letrozole is used off-label in women for the induction of ovulation and for use in ovarian stimulation. The overall objective of this thesis was to determine the effect of letrozole on ovarian function using the bovine model.

In the first study, letrozole was formulated into a silicone intravaginal device consisting of a small or a large surface area compared to a wax-based device or a control intravaginal device given for 8 days in pubertal heifers. The large-surface area device resulted in the greatest plasma letrozole concentration throughout the treatment period. The biological effect on the ovaries was also most profound in the large surface area group where the dominant follicle was largest at the time of device removal and plasma estradiol concentrations decreased the most during device insertion.

In the second study, letrozole residues in milk were characterized in lactating dairy cattle. A silicone letrozole-releasing device (LRD) was inserted for 4 days, blood and milk sampling were performed at 12-hour intervals, and a partial validation for letrozole in milk was done using tandem mass spectrometry. Although letrozole was detected in milk, maximum concentration of letrozole was significantly lower in milk than in plasma. There were no differences in the elimination half-life or time to maximum concentration for letrozole in milk or plasma.

The objective of the third study was to determine if letrozole would increase the superovulatory response in cattle. Non-lactating cows were given an LRD or a sham device at the time of transvaginal follicular ablation to induce wave emergence. At wave emergence, cows were given 8 doses of follicle-stimulating hormone at 12-hour intervals and were artificially inseminated 48 hours after prostaglandin (PGF). The LRD group exhibited estrus significantly

later and estrus was more synchronous than the sham group. However, the LRD group had a lower proportion of quality one embryos and transferable embryos than the sham group.

The objective of the fourth study was to characterize the effect of letrozole on follicular function and oocyte competence in cattle in three experiments. In the first experiment, transvaginal ablation was done to induce wave emergence and an LRD or sham device was given for 4 days followed by follicular aspiration to obtain granulosa cells for gene expression. In the second experiment following a 4-day LRD or sham device, frequent blood collections were done for analysis of plasma luteinizing hormone (LH) concentrations. Oocytes were collected that were either immediately denuded and stained or underwent 24 hours of *in vitro* maturation followed by staining. In the last experiment, following aspiration, oocytes were subjected to *in vitro* maturation, fertilization, and culture. There was no difference between treatments for gene expression from granulosa cells. Heifers in the LRD group had a significantly higher LH area under the curve and pulse amplitude than the sham group. The oocytes from the LRD group *in vitro* matured for 24 hours had the highest proportion of oocytes in the MII stage than the other groups. The LRD group following *in vitro* fertilization had a greater proportion of blastocysts and transferable embryos than the sham group.

The objectives of the fifth study were to determine the interval to ovulation following LRD treatment, compare an LRD-based synchronization to commercially used protocols for fixed-time artificial insemination (FTAI), and to develop a new LRD designed to reduce vaginitis and increase letrozole release. In the first experiment, a 4-day LRD was given at random stages of the estrous cycle followed by PGF at device removal. Cattle (n=10/group; 6 groups) were given GnRH at 48- or 60-hours after PGF, or no GnRH. In the second experiment, the 48-hour interval from PGF to GnRH following a 4-day LRD treatment was chosen for a

FTAI trial to compare pregnancy per AI (P/AI). In the third experiment, heifers (n=10/group) were given a newly designed LRD or an LRD designed in study one and frequent blood sampling was performed along with assessment of vaginitis. The 48-hour GnRH cow group ovulated more synchronous than the other groups. Following FTAI, the LRD group had significantly lower P/AI than the other two protocols. The newly designed LRD significantly increased plasma letrozole concentrations and numerically lowered the vaginitis score at device removal.

The objectives of the last study were to determine the: interval to estrus following a 4-day LRD or sham device and PGF at device removal, effect of estrous cycle stage when letrozole treatment was initiated on P/AI, and to compare a newly developed LRD protocol to a progesterone-based protocol. Following PGF, no difference was detected in the interval to estrus between the LRD and sham groups. When LRD treatment was initiated during the proestrus period, it did not prevent ovulation. However, treatment during the metestrus period resulted in the highest P/AI. There was no difference in P/AI between a newly developed LRD-based protocol compared to a progesterone-based protocol.

In summary, we have determined that intravaginal letrozole treatment can control ovarian function in cattle. Letrozole is not deleterious on the ability of the oocyte to undergo fertilization and can be used for FTAI protocols in cattle. Future studies should address the effect of letrozole during the proestrus period.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Gregg Adams for his guidance and assistance throughout my journey. I am thankful that he took a chance on a farm kid from Wisconsin when others didn't. I consider myself fortunate to be accepted as his student with a non-veterinarian background and I am thankful for the love of research that he instilled in me. I would like to thank my committee members, Drs. Al Chicoine, Marcos Colazo, Reuben Mapletoft, Jaswant Singh, and my committee chairs Drs. Ali Honaramooz and Suraj Unniappan. You all pushed me to become a better researcher and reproductive physiologist and for that I am extremely grateful.

I want to especially thank my father for raising me on a dairy farm, which from an early age developed my interest in cattle and agriculture. Special thanks to my mother for all her love and support she has provided throughout my childhood and educational endeavors. I would like to thank my mentor and former employer, Dr. Eugene Buchner for introducing me to embryo transfer and therefore sparking my interest in cattle reproduction.

I want to thank my wife and love of my life, Miranda, for joining me in Canada to pursue graduate school. You believed in me when I had little faith in myself. I will forever cherish our time working together on research. Finally, I want to thank the graduate, summer, and visiting students in our lab: Ana Rita, Awang, Brittany, Candy, Carlos, Chris, Fahrid, Manuel, Miriam, Moveed, Rodrigo, Sayuri, Steve, and Will. I am forever grateful for all of your help at the farm, engaging discussions, and our time in and out of the office spent together. Thank you to Cindy Pollard for all of her help scheduling committee meetings and going beyond her secretarial duties. I also take this opportunity to acknowledge funding from Alberta Livestock and Forestry, Venco Animal Health, and the University of Saskatchewan Devolved Scholarship.

Finally, I want to thank my family and friends for all their support they have provided during my time in Canada. I am grateful for their understanding when I have missed important holidays and life events because of my graduate school journey.

DEDICATION

I would like to dedicate this dissertation to my wife, Miranda. I cannot thank you enough for the love and support you have given me during my journey as a graduate student. Thank you for joining me in Canada for our pursuit of graduate degrees. I am excited to continue our life together, wherever it may take us.

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LIST OF ABBREVIATIONS

AETA	American Embryo Transfer Association
AI	Artificial insemination
AMH	Anti-müllerian hormone
ANOVA	Analysis of variance
AR	Androgen receptor
AUC	Area under the curve
BCS	Body condition score
BO	Brackett Oliphant
BP	Base pairs
BS	Blood sample
CAD	Canadian Dollar
cAMP	Cyclic adenosine monophosphate
CC	Clomiphene Citrate
CIDR	Controlled internal drug release
CL	Corpus luteum or corpora lutea
cm	Centimeter
COC	Cumulus-oocyte complex
CV	Coefficient of variation
CYP	Cytochrome P450
DAPI	Diamidinophenylindole
DHEA	Dihydroepiandrosterone
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
E2	Estradiol
ER	Estrogen receptor
ET	Embryo transfer
EU	European Union
FDA	Food and Drug Administration
FGA	Fluorogestone acetate
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
FTAI	Fixed-time artificial insemination
FTET	Fixed-time embryo transfer
g	Gram
x g	Times gravity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown

hCG	Human chorionic gonadotropin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
HPO	Hypothalamic-pituitary-ovarian
HSD	Hydroxysteroid dehydrogenase
ICSI	Intracellular sperm injection
IETS	International Embryo Technology Society
im	Intramuscular
INHBA	Inhibin A
IU	International units
IV	intravenously
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
kg	Kilogram
L	Liter
LC-MS/MS	Liquid chromatography-mass spectrometry
LH	Luteinizing hormone
LHCGR	Luteinizing hormone + human chorionic gonadotropin receptor
LLOD	Lower limit of detection
LRD	Letrozole-releasing device
LSA	Large surface area
m	Meter
mg	Milligram
MI	Metaphase I
MII	Metaphase II
mL	Milliliter
mm	Millimeter
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
OHSS	Ovarian hyperstimulation syndrome
P4	Progesterone
PBS	Phosphate buffered solution
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PGF	Prostaglandin F2a
PRID	Progesterone releasing intravaginal device

PVA	Polyvinyl acetate
P/AI	Pregnancy per artificial insemination
QC	Quality control
RNA	Ribonucleic acid
RSD	Residual standard deviation
SAS	Statistical Analysis System
SD	Standard deviation
SEM	Standard error of mean
SPE	Solid phase extraction
SSA	Standard surface area
STAR	Steroidogenic acute regulatory protein
TGF	Transforming growth factor
µg	microgram
µL	microliter
µm	micrometer
UPLC	Ultra-performance liquid chromatography
US	Transrectal ultrasonography
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
VEGF	Vascular epithelial growth factor

1. CHAPTER 1: GENERAL INTRODUCTION, HYPOTHESES, AND OBJECTIVES

Controlling ovarian function is vital to implement advanced reproductive technologies in cattle. According to the National Animal Health Monitoring System in the United States, artificial insemination (AI) is only used in 8% of beef operations in comparison to 89% of dairy operations (Johnson and Dahlke, 2015; USDA, 2018). The low percentage of beef operations utilizing AI was credited to its time, labor, and difficulty in implementation. In the United States, synchronization protocols involving estradiol are not approved for use in cattle and in Canada estradiol-based protocols can only be used following veterinarian prescription. Therefore, producers continue to search for alternatives that are effective yet require minimal handling.

The aromatase inhibitor, letrozole, is approved for use in postmenopausal women for the treatment of hormone-dependent breast cancer. It functions by blocking the conversion of androgen into estrogen, depleting the body of estrogens and suppressing tumor growth (Chumsri et al., 2011). However, letrozole has been used off-label for ovulation induction and ovarian stimulation in premenopausal women. The use of letrozole for the control of ovarian function in cattle has been recently investigated (reviewed in Yapura et al., 2018). It was discovered that by releasing the negative feedback of estradiol on the hypothalamus, luteinizing hormone (LH) secretion (but not follicle-stimulating hormone FSH) increased, which prolonged the length of dominance of the dominant follicle. An increased diameter of the corpus luteum (CL) was also attributed to a luteotropic effect of LH. However, initial fertility trials involving fixed-time artificial insemination after letrozole treatment resulted in a low pregnancy per AI (P/AI) (Yapura et al., 2016). The reasoning behind this low P/AI was unknown, but most likely due to an effect of low estradiol at the time of artificial insemination.

In the following section, current knowledge of bovine female reproduction and synchronization strategies implemented for advanced reproductive programs in cattle, along with the use of aromatase inhibitors in women will be reviewed.

1.1. Bovine reproductive physiology

1.1.1. Ovarian follicular dynamics

Before the utilization of transrectal ultrasonography, ovarian follicular dynamics in cattle was a challenging concept. Individual assessment of ovaries after euthanasia on specific days after estrus led to the initial hypothesis that follicles ≥5 mm show two waves of growth during the estrous cycle (Rajakoski, 1960). Others refuted this study and claimed that follicular development is continuous and independent of the cycle (Choudary et al., 1968; Donaldson and Hansel, 1968). Following the advent of real-time ultrasonography, it has become accepted that wave emergence occurs when 8 to 41 small follicles (3 to 4 mm in diameter) are detected following a surge in FSH (Pierson and Ginther, 1987a; Sirois and Fortune, 1988; Adams et al., 1992b). The majority of bovine estrous cycles are composed of 2 or 3 follicular waves with the first wave occurring at the time of ovulation (Day 0). The emergence of the second wave occurs on Days 9 or 10 and Day 8 or 9 in two- and three-wave cycles, respectively. The third wave occurs on Day 15 or 16 in three-wave cycles (Ginther et al., 1989). Luteolysis commences on Day 16 and Day 19 in two- and three-wave cycles, respectively, resulting in a shorter inter-ovulatory interval in two- vs three-wave cycles (i.e. 19-20 vs 22-23 days).

Follicular waves result from the negative feedback of estradiol and inhibin (produced from granulosa cells of follicles in the wave, and especially the dominant follicle) on FSH release (Kaneko et al., 1995; Mihm et al., 1997). When the dominant follicle ovulates or undergoes atresia, a surge of FSH occurs initiating wave emergence approximately 12 to 24

hours later (Adams et al., 1992b). The dominant follicle of the first wave exhibits a larger diameter in comparison to the subsequent dominant follicle(s) in the cycle, which is attributed to lower levels of circulating progesterone during its growth (Adams et al., 1992a). More recent research has indicated that progesterone plays an inhibitory role on LH release from the pituitary (Bergfeld et al., 1996). As the dominant follicle grows, it acquires more LH receptors on the granulosa cells than the subordinate follicles and is able to shift its dependence from FSH to LH during the time of follicular deviation (Adams et al., 2008). The increase in understanding of ovarian follicle wave dynamics has led to the development of estrus synchronization protocols to maximize pregnancy success in cattle.

1.2. Exogenous control of the estrous cycle in cattle

1.2.1. Prostaglandin- and gonadotropin-releasing hormone-based protocols

The the ability of the uterine horn ipsilateral to the CL to respond to oxytocin and shorten the estrous cycle in cattle was first identified in the late 1960's (Ginther et al., 1967). Prostaglandin F_{2a} (herein referred to as PGF), a product from oxytocin receptors in the endometrium, was later identified as the luteolytic agent and, when given exogenously, could induce luteolysis so that AI could be done (Hafs et al., 1974; Thatcher and Chenault, 1976). However, the CL is refractory to a single administration of PGF for 5 to 6 days after ovulation (Levy et al., 2000). Discovery of the ability of the gonadotropin-releasing hormone (GnRH) agonist (Buserelin) to elicit both LH and FSH release and subsequent ovulations showed promise to initiate fixed-time artificial insemination (FTAI) (Macmillan and Thatcher, 1991).
Taken together, the luteolytic properties of PGF, understanding of follicular waves, and the ability of dominant follicles to ovulate following the administration of exogenous GnRH laid the

groundwork for the first ovulation synchrony protocol coined “Ovsynch” (Pursley et al., 1995). At random stages of the estrous cycle, lactating dairy cows were given GnRH, followed by PGF 7 days later. Forty-eight hours later, cattle were given GnRH again, and FTAI was done 24 hours later with a P/AI of 55%. This FTAI protocol eliminated the need for estrus detection and significantly increased the number of pregnant cows at 100 days in milk (Pursley et al., 1997). Unfortunately, ovulation synchrony around the time of AI was dependent upon ovulation following the first GnRH given at random stages of the cycle. Subsequent studies showed that GnRH given at random stages of the estrous cycle results in 44% to 54% of ovulations in dairy cows (Bello et al., 2006; Colazo et al., 2009), but 95% when given 5 to 8 days after estrus (Vasconcelos et al., 1999). Using this knowledge, Pre-synch, in which PGF was given twice 14 days apart, before initiating Ovsynch 12 to 14 days after the second PGF was developed (Moreira et al., 2001). Although this approach increased the P/AI following AI, anovular cows did not respond to the Pre-synch. To address this problem, an additional Ovsynch protocol, Double-Ovsynch, was developed (Souza et al., 2008). This protocol resulted in higher P/AI, especially in primiparous cows, in comparison to Pre-synch/Ovsynch. Utilization of Ovsynch protocols has greatly reduced the number of days not pregnant in lactating Holsteins (reviewed in Wiltbank and Pursley, 2014), but the adaptation of these protocols for beef cattle is not feasible due to handling requirements.

Cosynch (performing AI and giving GnRH concurrently) was developed for use in beef and dairy cattle to reduce the number of handlings needed (Geary et al., 1998). Many adaptations of Cosynch exist including protocols with or without an intravaginal progesterone device (Lamb et al., 2001), 5- or 7-day interval from GnRH to PGF (Bridges et al., 2008), or no GnRH given at the time of a progesterone device insertion (Colazo and Ambrose, 2011). By lengthening

proestrus (i.e., the interval from progesterone device removal and PGF to AI and GnRH) some studies have shown an increase in P/AI in dairy cattle (Santos et al., 2010; Lima et al., 2011). However, as seen with Ovsynch protocols, synchronized wave emergence is only achieved when ovulation occurs following the first GnRH; therefore following device removal and PGF, variable intervals to estrus exist between cattle following Cosynch protocols (Larson et al., 2006).

1.2.2. Progesterone and estradiol-based protocols

The role of exogenous progesterone and estradiol for the control of follicular wave emergence has led to the increased use of advanced reproductive technologies in cattle worldwide. Estradiol-17β, given exogenously induced atresia of the dominant follicle in rhesus monkeys (Hutz et al., 1988). The impact of estradiol on follicular dynamics in cattle was first observed in the early 1990s. In an effort to increase the superstimulatory response, cows were treated with a progesterone ear implant and given estradiol valerate on Day 2 or 7 (Day 0 = estrus) with superstimulation induced on Day 9. Treatment with estradiol induced atresia of the dominant follicle approximately two days after administration and the Day 2 group resulted in a greater number of CL and embryos following collection compared to the Day 7 group (Bo et al., 1991). Subsequent studies showed that estradiol valerate caused atresia of the dominant follicle in early- and mid-growing phases, and following atresia, resulted in an early surge of FSH and the emergence of a new follicular wave. In that study, LH surged following the administration of estradiol valerate, in the early- and mid- growing stages but not in the late growing stage (Bo et al., 1993). The elicitation of the LH surge was understood in the following study in which heifers were given either a progesterone ear implant or not at the time of ovulation (Day 0) followed by 5 mg of estradiol-17β on Day 1 (Bo et al., 1994). Although no difference in plasma estradiol

following treatment, progesterone plus estradiol prevented the LH surge, caused atresia of the dominant follicle, and elicitation of an FSH surge 3 days later. Subsequently, the second follicular wave emerged early and synchronously. A single dose of 5 mg of estradiol-17β was as effective as increased dosages or repeated dose regimens in causing synchronous wave emergence 4 to 5 days later (Bo et al., 1994).

The ability to exogenously control wave emergence with estradiol and progesterone was soon adapted for superstimulatory protocols. Treatment with estradiol plus progesterone at random stages of the estrous cycle followed by superstimulation 4 days later resulted in a comparable number of ova/embryos but a higher percentage of fertilizable ova recovered compared to superstimulation initiated 8 to 12 days after estrus (Bo et al., 1995, 1996). This allowed donors to be managed as a group and eliminated the need for a reference estrus before superstimulation. Initial pilot studies using E-17β plus progesterone for estrous synchronization protocols showed that P/AI were high (80%) following progesterone device removal and PGF-induced luteolysis followed by artificial insemination 12 hours after estrus (Martinez et al., 2000). No difference was detected for conception rates in beef heifers treated with E-17β and then treated with an intravaginal progesterone device or melengestrol acetate prior to synchronization of ovulation with estradiol benzoate and FTAI (Martinez et al., 2002). When additional progesterone (100 mg in oil) was given im at the time of estradiol treatment plus a new or used intravaginal progesterone device, there was no effect on P/AI following FTAI (Colazo et al., 2004). Some studies have shown that the dominant follicle did not regress in cattle given estradiol cypionate plus progesterone treatments giving around the time of spontaneous luteolysis and an aged (persistent) follicle ovulated following removal of the intravaginal device (Colazo et al., 2003).

Due to the development of estradiol- and progesterone-based protocols, the number of FTAI, fixed-time embryo transfer, and superstimulatory treatments in cattle have increased, particularly in South American countries. A total of 11 million cattle underwent FTAI in Brazil in 2016 with a majority utilizing estradiol- and progesterone-based protocols (Mapletoft et al., 2018). Utilization of these protocols in the United States, Europe, Australia (dairy) and New Zealand (dairy) has been banned due to the public's concern of steroid use in food-producing animals (Lane et al., 2008), but can still be used in Canada following veterinary prescription.

1.3. Estradiol production in cattle

1.3.1. Steroidogenic pathway of estradiol production

The synthesis of estradiol (Figure 1.1) begins with the parent molecule, cholesterol, which is derived from low-density lipoproteins from dietary cholesterol (Gwynne and Strauss, 1982). Cholesterol is transported into the outer mitochondrial membrane during the first stage of steroidogenesis (Chang et al., 2006). Subsequently, the steroidal acute regulatory protein (StAR) facilitates the transport of cholesterol into the inner mitochondrial membrane. Conversion of 1 mole of cholesterol to pregnenolone requires 3 moles of NADPH (Miller and Auchus, 2011). This reaction, occurring in the mitochondria, in a rate-limiting and hormonally regulated step utilizing the P450 side-chain cleavage (P450scc; CYP11A1 encoding gene) enzyme (Stone and Hechter, 1955) and occurs in three successive steps: 1) the hydroxylation on C22 of cholesterol, the hydroxylation of C20 of the intermediate, followed by oxidation of the C20-22 bond (i.e. the side-chain cleavage event, which yields pregnenolone and isocaproaldehyde (Miller and Auchus, 2011). At this point, pregnenolone can either be converted to 17α-hydroxypregnenolone or progesterone, by the enzymes 17α-hydroxylase (CYP17A1 gene encoding) and 3-beta-

hydroxysteroid dehydrogenase (3β-HSD), respectively. Progesterone and 17α-hydroxypregnenolone can be converted further into 17α-hydroxyprogesterone by the enzymes 17α-hydroxylase and 3β-HSD, respectively. Similarly, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone yield dehydroepiandrosterone (DHEA) and androstenedione, respectively, with the use of the enzyme 17,20 lyase. At this point, DHEA can either be synthesized into androstenediol via 17β-HSD or synthesized into androstenedione via 3β-HSD. Both androstenediol and androstenedione can be transformed into testosterone by 3β-HSD and 17β-HSD, respectively. Estrogens are subsequently synthesized with the use of the aromatase enzyme ($P450_{arom}$; gene encoding CYP19A1) of androstenedione and testosterone into estrone and estradiol, respectively. Finally, estrone can be converted to estradiol by 17β-HSD (Stone and Hechter, 1955; Chang et al., 2006; Miller and Auchus, 2011).

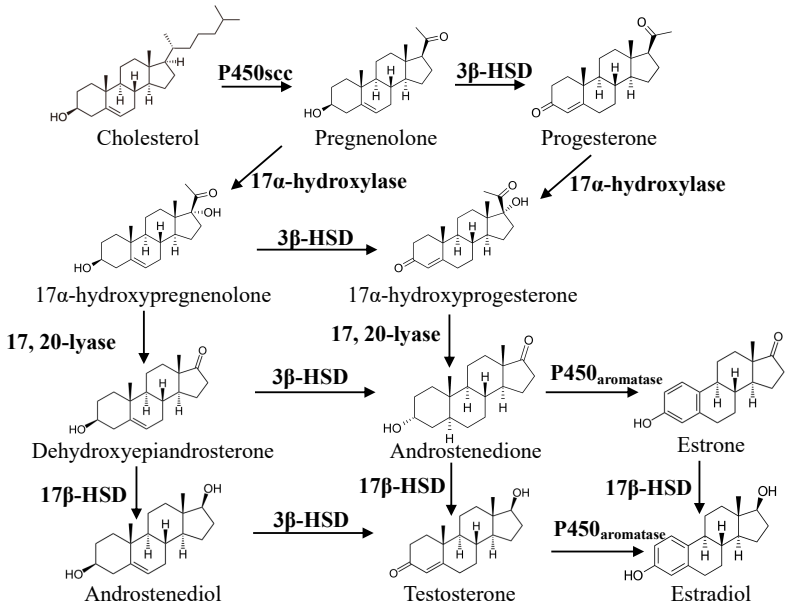

Figure 1. 1**.** Steroidogenic synthesis of estrogens beginning with cholesterol utilizing the enzymes (bolded): P450 side cleavage chain (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase, 17, 20-lyase, 17β-hydroxysteroid dehydrogenase (17β-HSD), and P450 aromatase.

1.3.2. Hypothalamus-pituitary-ovarian axis

Neurological control of the female bovine reproductive system encompasses both the brain and ovaries, which play a vital role in the secretion of GnRH from the mediobasal hypothalamic region of the diencephalon (Dees and McArthur, 1981; Leshin et al., 1988). Following its release into the hypothalamic-hypophyseal portal vein, GnRH reaches the adenohypophysis, binds to the GnRH-receptors, and subsequently discharges LH and FSH into the systemic circulation. These gonadotropins bind to receptors on the granulosal (FSH) and thecal (LH) cells of individual follicles. The original "two-cell, two-gonadotropin" model was proposed in 1977 (Armstrong and Dorrington, 1977). The two-cell, two-gonadotropin model states that increased circulating LH stimulates the CYP 17, 20 lyase enzyme activity found exclusively in theca cells and subsequently increases androgen production. Estrogens are then synthesized in the granulosa cells where the CYP19 (aromatase) enzyme is stimulated by FSH from the locally produced androgens (Hillier et al., 1994). Therefore, androgens exert a paracrine effect within the ovary, while estrogens (primarily estradiol) exert an endocrine feedback effect on gonadotropin secretion.

Both estradiol and progesterone exert positive and negative feedback on the hypothalamus. Progesterone inhibits the frequency of LH release, which is hypothesized to be mediated by opioid peptides in the hypothalamus (Bergfeld et al., 1996; Goodman et al., 2002). The negative feedback of estradiol on LH is more complex and appears to be regulated by progesterone as well. During the luteal phase, estradiol enhances the progesterone inhibition of LH pulsatility (Skinner et al., 1998), but when concentrations high enough to induce an LH surge occur, estradiol reduces the frequency of LH release (Kesner et al., 1981; Evans et al., 1994). During luteolysis, as progesterone drops, estradiol begins to rise and LH frequency increases.

The positive feedback of estradiol is achieved by the activation of estradiol receptor neurons in the hypothalamus. The estradiol signal is then transmitted to GnRH neurons, which stimulate the surge release of GnRH at the median eminence resulting in the preovulatory LH and FSH surge from the anterior pituitary (Evans et al., 2002). The regulation of FSH secretion from the pituitary (non-GnRH mediated) remains unclear. The follicle-produced hormones, estradiol and inhibin act as endocrine factors regulating FSH release from the anterior pituitary. It appears that negative feedback of estradiol on the anterior pituitary is due to the inhibition of FSH-β expression. Similarly, heifers that were immunized against inhibin had increased FSH concentrations and a higher number of small follicles in the ovaries (Medan et al., 2004). Moreover, inhibin does not affect LH release and does not act on the hypothalamus for control of GnRH secretion (Sharma et al., 2012). Using an ovariectomized animal model, a combination of estradiol and inhibin was shown to be needed for the full inhibition of FSH release (Martin et al., 1988). Although a great understanding of the HPO axis has been achieved in the past 30 years, it is clear that increased comprehension of neurological control of the HPO axis is still needed.

1.3.3. Estrogen receptors

In cattle, estrogen receptors consist of 2 subunits: estrogen receptor α and β (ERα and ERβ) and their respective encoding genes: ESR1 and ESR2. In ovarian tissues, ERα is localized mainly in theca cells, stromal cells, interstitial glands, and germinal epithelium while ERβ is localized mainly in granulosa cells of growing follicles (Rosenfeld et al., 2001; Berisha et al., 2002; Van Den Broeck et al., 2002; Amrozi et al., 2004). Estrogen has been shown to downregulate granulosa cell expression in ERβ (Sharma et al., 1999). In cattle given an ovulatory dose of LH, ovarian ERβ does not change, although it decreases in rats (O'Brien et al., 1999; Rosenfeld et al., 2001). Following studies with the intrafollicular administration of an ER-

antagonist, it was concluded that both ERs are needed for the development of a dominant follicle in cattle (Rovani et al., 2014). An increase in intrafollicular levels of estrogens increases granulosa cell sensitivity to LH and FSH due to the increase of LH- and FSH-receptors. Similarly, *in vitro* models indicate that estradiol increases steroidogenesis and preantral follicle growth (Rosenfeld et al., 2001). The ESR1 knockout mouse model indicates that females are infertile and non-receptive to males, indicating the effect of ESR1 in the central nervous system (Lubahn et al., 1993). It was concluded, therefore, that ERα controls the estrogen feedback in the hypothalamus (Glidewell-Kenney et al., 2007). Similarly, ESR2 knockout mice have a lower number of growing follicles and decreased litter size indicating a role in follicular development (Krege et al., 1998).

1.3.4. Role of estradiol during proestrus and estrus

The expression of estrus is associated with improved pregnancy success following FTAI. However, many preceeding endocrinological events must transpire before estrus can occur. Estradiol from the dominant follicle is needed to stimulate PGF secretion from the endometrium, leading to induction of luteolysis (Araujo et al., 2009). As progesterone decreases, pulse frequency of LH increases, further fueling the growth of the dominant follicle. This continuous cascade leads to both increased androgen production from the theca cells as well as upregulation of FSH-receptors, increasing the aromatization of androgens to estrogens in the granulosa cells (Weil et al., 1999). Estrous behavior is then prompted by peak plasma estradiol levels (Henricks et al., 1971). Similarly, once peak estradiol levels are achieved, a surge of GnRH is produced in the hypothalamus, and subsequent surges of LH and FSH are released from the anterior pituitary gland. The LH surge results in disruption of the cumulus-oocyte complexes (COC) from the granulosa cells, inducing meiotic maturation of the COC, depleting estradiol production in the

granulosa cells, followed by subsequent ovulation of the dominant follicle 24 to 30 hours later (Hyttel et al., 1997; Komar et al., 2001).

1.3.4.1. Sperm transport

Not only does estradiol contribute to endocrinological control of estrus and ovulation, but it is also important in sperm and oocyte transport through the female tubular genitalia. Transportation of sperm is hypothesized to occur in two phases: 1) a rapid initial phase where a small fraction of sperm reach the uterine tubes within minutes, and 2) a slower movement of an increased number of sperm through the uterus and uterine tubes (Hawk, 1983). Following copulation in sheep, semen is deposited in the anterior portion of the vagina, followed by a large number of sperm found in the uterine tubes within 5- or 10-minutes (Mattner and Braden, 1963). Propulsion from the sperm itself is not a limiting factor, as non-motile, dead ram sperm have been found in the uterine tubes within minutes of insemination. The prolonged period is characterized by an ever-increasing number of sperm in the uterine tubes maximized approximately 22 to 24 hours following copulation (Hunter, 1975). When estradiol was given exogenously to sheep within 18 hours after natural mating, there was an eightfold increase in the number of sperm recovered from the uterus and uterine tubes compared to non-treated control animals (Hawk and Cooper, 1975). Therefore, the effect of estradiol on the contractile movement of the uterus is necessary for sperm transport to the uterine tubes for *in vivo* fertilization.

1.3.4.2. Uterine environment

Estradiol also has a profound effect on the uterine lumen pH. A lower pH in the uterine lumen has been shown to affect motility and longevity of bull sperm (Jones and Bavister, 2000). Interestingly in cattle, the uterine pH was negatively correlated to estradiol, and the pH reached a nadir at the time of estrus (Perry and Perry, 2008). Therefore, it was hypothesized that a low pH

might extend the longevity of sperm due to the prolonged period from estrus to ovulation (i.e., 24 to 30 hours; (Komar et al., 2001)). To mimic this environment, exogenous estradiol was given 12 hours after PGF, which increased the proportion of cows in estrus and decreased uterine pH (Perry and Perry, 2008). Similarly, exogenous estradiol given 24 hours before GnRH/AI increased the P/AI of beef cows that ovulated follicles <12.2 mm, which was attributed to the change in pH of the uterine environment (Jinks et al., 2012).

1.3.4.3. Effect on short lifespan CL

In cattle, the first spontaneous pubertal or postpartum ovulation results in a short estrous cycle of 7 to 10 days (Garverick et al., 1992). Originally, it was proposed that progesterone before ovulation was needed for normal luteal function in cattle (Copelin et al., 1987). Similarly, it was proposed that only circulating estradiol levels preceding ovulation would also affect the lifespan of the resultant CL. Using the ovariectomized bovine model, cows were given low, medium, or high doses of estradiol at 8-hour intervals for 40 hours, followed by increasing amounts of injectable progesterone to simulate CL progesterone production. Subsequently, cows were given intramuscular oxytocin 6 days after the last estradiol treatment, which resulted in an increase in PGF metabolite in both the low and medium estradiol groups (Mann and Lamming, 2000). It was concluded that high levels of estradiol (with or without pretreatment of progesterone) inhibit endometrial oxytocin receptor concentrations in a dose-dependent manner. Therefore, following the first postpartum or pubertal ovulation, the CL lifespan is shortened due to estradiol not fully inhibiting oxytocin receptors on the endometrium. Consequently, oxytocin can bind to the remaining oxytocin receptors, which releases PGF and leads to early luteolysis (Mann and Lamming, 2000). This decrease in oxytocin receptors may have been a contributing factor for the observed increase in P/AI in dairy cows given exogenous estradiol 2 days before

AI (Cerri et al., 2004). Many extensive field-trial studies do not incorporate ultrasonography or blood sampling 7 to 10 days after AI, so the incidences of short lifespan CL is unknown.

1.4. Aromatase enzyme

1.4.1. Location and classification

The aromatase enzyme is a member of the cytochrome P450 family. It catalyzes the rate-limiting step of estrogen biosynthesis: aromatization of testosterone and androstenedione into estradiol and estrone, respectively (Chumsri et al., 2011). The preferred substrate, androstenedione, is aromatized in three successive oxidation steps: hydroxylation of the angular C-19 methyl group followed by aromatization of the A ring of androstenedione and loss of the C-19 carbon atom which in turn cleaves the C_{10}-C_{19} bond (Colozza et al., 2014). The first two of these steps are common in all P450 cytochrome proteins, but the third step is unique to aromatase (Akhtar et al., 2011). Aromatase has been found in a variety of tissues, including the ovary, placenta, testis, skin, brain, fat and bone (Thompson and Siiteri, 1974; Valladares and Payne, 1979; Durham et al., 1985; Simpson et al., 1994). In breast cancer cells, aromatase has been shown to exhibit higher activity than non-cancer cells, and this is the impetus for breast cancer treatment with aromatase inhibitors (Harada, 1997). In fetal bovine ovaries, immunohistochemical studies have found aromatase expression between 45 to 250 days of gestation (Burkhart et al., 2010). In the adult bovine ovary, the presence of aromatase is most profound in mural granulosa cells of the follicle, but it has also been found in the CL, which has been hypothesized to play a role in luteolysis (Okuda et al., 2001). In cattle, although detected in follicles as small as 4 to 6 mm, aromatase mRNA expression continues to increase throughout follicle selection and is maximal during the preovulatory stage in association with follicular fluid estradiol concentrations (Bao et al., 1997; Bao and Garverick, 1998). The aromatase enzyme is

encoded by the CYP19A1 gene, which has been used to understand aromatase mRNA expression.

1.4.2. CYP19A1 gene

The CYP19A1 gene is located on chromosomes 10 and 15 in cattle and humans, respectively (Chumsri et al., 2011; National Center for Biotechnology Information, 2019). The upregulation of the CYP19A1 mRNA expression is achieved mainly by FSH (Stocco, 2009). In an autocrine manner, estradiol acts to enhance FSH actions, and this effect is believed to play a role in follicle dominance in the rat (Fitzpatrick and Richards, 1991). Similarly, androgens can enhance FSH's effect on CYP19A1 mRNA expression. Testosterone was more effective than estradiol in enhancing the effect of FSH on CYP19A1 (Hillier and Tetsuka, 1997). Therefore, it was hypothesized that androgens derived from theca cells are not only used as a substrate for estradiol synthesis, but also control the action of FSH on CYP19A1 (Fitzpatrick and Richards, 1991).

1.5. Aromatase inhibitors

Aromatase inhibitors were initially investigated in the 1970s as a means of treating metastatic breast cancer in women (Chumsri et al., 2011). The first aromatase inhibitor, aminoglutethimide, inhibited multiple cytochrome P450 enzymes, including CYP19 (aromatase) as well as cortisol production (from CYP11). This required patients to undergo cortisol replacement treatment, and therefore aminoglutethimide was abandoned as a breast cancer treatment (Fisher et al., 1994).

1.5.1. Mechanism of action

The modern age of aromatase inhibitors can be classified into two categories: steroidal (i.e. formestane and exemestane) or non-steroidal (i.e. anastrozole and letrozole). Steroidal aromatase inhibitors (Type I) irreversibly bind to the substrate-binding spot and are referred to as "suicide inhibition" or noncompetitive inhibition (Brodie et al., 1981; Santen et al., 2009). Following suicide inhibition, the aromatase enzyme initiates hydroxylation that produces a covalent bond between the inhibitor and enzyme that is unbreakable. Therefore, enzyme activity is permanently blocked and can only be restored by synthesis of new enzymes (Buzdar, 2003). For this reason, steroidal aromatase inhibitors are generally long-term and specific, so the long-term presence of the drug is not needed. Non-steroidal (competitive) aromatase inhibitors (Type II) have higher specificity and less long-term, toxic effects (Chumsri et al., 2011). Non-steroidal aromatase inhibitors bind in a non-covalent manner to the heme group and saturate the binding sites (i.e., prevent the binding of androgens). Due to the competitive binding nature, this inhibition can be reversed by an increase in circulating androgens. Therefore, the effectiveness of non-steroidal aromatase inhibitors is based on the concentrations of the drug in the circulation (Buzdar, 2003). Currently, there are 3 aromatase inhibitors approved by the U.S. Food and Drug Administration for breast cancer treatment: anastrozole and letrozole (non-steroidal) and exemestane (steroidal; Chumsri et al., 2011).

1.5.2. Use of aromatase inhibitors for breast cancer treatment

Approximately 70% of human breast cancers are estrogen-receptor positive (Lumachi et al., 2013). Therefore, aromatase inhibitors are one of the first treatments chosen for postmenopausal women (Smith and Dowsett, 2003). Tamoxifen, a selective estrogen receptor modulator, is also used to block tumor growth by competitively antagonizing estrogen at its

receptor. Unfortunately, treatment with tamoxifen has been linked to thromboembolism and uterine cancer (Fisher et al., 1998). Although most aromatases are found in gonadal tissues, they have also been located in adipose, liver, brain, breast and breast cancer tissues (Miller et al., 1982). Mean plasma estradiol concentrations fall from 110 to 7 pg/mL following menopause in women, but the levels in carcinoma tissue have been reported as 10x the concentrations in plasma due to aromatase activity in tumors (Thijssen and Blankenstein, 1989). Treatment with third-generation aromatase inhibitors (letrozole and anastrozole) reduces plasma concentrations of estradiol, estrone, and estrone sulfate by 81% to 98% (Geisler et al., 1996, 2002). For metastatic breast cancer, treatment with letrozole has been shown to be superior to tamoxifen in time to disease progression (42 vs 23 weeks, respectively; Mouridsen et al., 2001). In neoadjuvant therapy (i.e. treatment given before surgery), treatment with letrozole led to a significantly higher rate of tumor suppression in comparison to tamoxifen (Eiermann et al., 2001; Ellis et al., 2001). In premenopausal women, treatment with exemestane (steroidal aromatase inhibitor) was just as effective as tamoxifen but required ovarian suppression by GnRH agonist or ovariectomy (Francis et al., 2014). For this reason, tamoxifen is the preferred treatment for premenopausal breast cancer. Unfortunately, after 5 years, treatment with tamoxifen is not beneficial, and during this period, breast cancer relapse occurs (Fisher et al., 1996). Studies have shown that a 5-year regimen of letrozole following 5 years of tamoxifen significantly increased the survival rate compared to placebo-treated premenopausal women (Goss et al., 2003).

1.5.3. Use of aromatase inhibitors for induced ovulation and stimulation in women

The most commonly used ovulation-inducing treatment in women from the 1960s to the early 2000s was clomiphene citrate (CC; Casper and Mitwally, 2006). Clomiphene citrate acts as both an estrogen agonist and antagonist by binding to estrogen receptors throughout the body.

Treatment with CC can lead to depletion of estrogen receptors in the hypothalamus and an incorrect interpretation of estrogens in circulation. When estrogens are thought to be low, an increase in gonadotropins (FSH and LH) occurs, which can lead to an ovulation rate of 60% to 80% following CC treatment (Garcia et al., 1977). Although ovulation rates are elevated, pregnancy rates are low, possibly due to the antiestrogen effect of CC on both the endometrium and cervical mucus because of its extended terminal half-life (5 days to 3 weeks; Casper and Mitwally, 2006). The terminal half-life of the commonly used aromatase inhibitor, letrozole is 45 hours in women (Winer et al., 2002). Theoretically then, by decreasing circulating estrogens via aromatase inhibitors, the release of hypothalamic negative feedback could be achieved without adverse long-term effects on the uterus and cervix.

Although aromatase inhibitors are not approved for use for any other condition than hormone-dependent breast cancer in postmenopausal women (Health Canada, 2005), the first study involving aromatase inhibitors for ovulation induction in women with polycystic ovarian syndrome (PCOS) was done in 2001. These women, who failed to respond to CC, had ovulation and pregnancy rates of 75% and 25%, respectively following aromatase inhibitor treatment (Mitwally and Casper, 2001). Another benefit of letrozole treatment was a significant reduction in the number of twin pregnancies in women undergoing infertility treatment in comparison to CC (Mitwally et al., 2005). Treatment with letrozole also resulted in the highest pregnancy rate per cycle in comparison to CC, FSH, or untreated controls (Casper and Mitwally, 2006). Due to the inability to perform frequent blood sampling in women, understanding the effect of letrozole on LH and FSH release is contradicting in the literature. Following oral letrozole administration, circulating estradiol begins to decrease and in several studies an increase in circulating LH occurred (Verpoest et al., 2006; Bedaiwy et al., 2011; Lee et al., 2011; Allaway et al., 2017), and

circulating FSH was reported to either increase (Allaway et al., 2017), decrease (Lee et al., 2011) or remain unchanged (Verpoest et al., 2006). The reasons for this discrepancy may be due to the menstrual day that treatment was initiated. Comparisons to the bovine model are reviewed in Section 1.4.4.3 below.

Aromatase inhibitors have also been used recently to increase the ovarian stimulatory response in women in combination with FSH (reviewed by Garcia-Velasco, 2012). The reasoning behind this stems from two hypotheses: 1) release of the estradiol negative feedback on the hypothalamus increases FSH release from the pituitary (Requena et al., 2008), or 2) increased intraovarian androgens upregulating FSH-receptors in the granulosa cells requiring less FSH to stimulate follicular growth (Weil et al., 1998). In recent years, the latter has been mainly accepted due to conflicting literature regarding the first hypothesis. Also, treatment with aromatase inhibitors combats the adverse reactions seen with supraphysiological estrogen levels in the endometrium following stimulation and embryo transfer (Forman et al., 1988; Simon et al., 1998). The original trial comparing FSH stimulation with the addition of letrozole to FSH alone resulted in a significant reduction in the amount of recombinant FSH required in the letrozole group to produce an increase in the number of mature follicles counted on the day of administration of human chorionic gonadotropin (Mitwally and Casper, 2002). Three years later, the first study showing an increase in follicular fluid and serum testosterone following administration of letrozole was shown to increase the number of oocytes retrieved (Garcia-Velasco et al., 2005). As FSH constitutes one of the highest costs of ovarian stimulation, co-treatment with letrozole may also allow assisted reproductive technologies to be an affordable option for a larger group of couples struggling with infertility (Casper and Mitwally, 2006).

1.5.4. Letrozole

1.5.4.1. Classification

Letrozole (Figure 1.2; molecular weight: 285.3 g/mol) is a 3rd generation, reversible, non-steroidal aromatase inhibitor approved for the treatment of hormone-dependent breast cancer. Letrozole is marketed by Novartis under the trade name Femara and was approved by the U.S. Food and Drug Administration in 2001 (Cohen et al., 2002). In comparison to first- and second-generation aromatase inhibitors, letrozole is highly specific to the $P450_{arom}$ enzyme and does not interfere with progesterone on corticosteroid synthesis (Attar and Bulun, 2006; Hong and Chen, 2006).

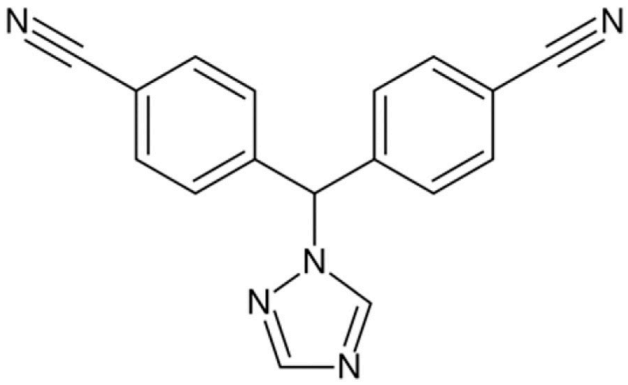

Figure 1. 2**.** Molecular structure of letrozole which contains a triazole group that selectively binds to the heme group of the aromatase enzyme.

1.5.4.2. Pharmacokinetics and metabolism

In women, letrozole is taken orally (0.1 to 5 mg/day) and is rapidly absorbed from the gastrointestinal tract. In healthy postmenopausal women, reported bioavailability of a single oral dosage is 99.9%, and the time to maximum plasma concentration and terminal half-life was 1 and 42 hours, respectively. Following an intravenous bolus, letrozole plasma levels rapidly

decreased, suggesting a fast distribution into tissues (Sioufi et al., 1997a), although no differences were detected in letrozole pharmacokinetic parameters between age groups (Pfister et al., 2001). Letrozole suppressed estradiol, estrone and estrone sulfate by 75% to 95% within 2 to 3 days (Cohen et al., 2002) and inhibited aromatization (as analyzed by isotopic analysis) by 98 to > 99% (Dowsett et al., 1995; Geisler et al., 2002). Elimination of letrozole is mainly by P450 isozymes (CYP3A4 and CYP2A6), which metabolize letrozole into the primary inactive carbinol metabolite, 4,4'-methanol-bisbenzonitrile (Sioufi et al., 1997b). A total of 90% of radiolabeled letrozole is recovered in urine consisting of 75% of the carbinol metabolite, 9% unidentifiable metabolites, and 6% unchanged letrozole (U.S. Food and Drug Administration, 2007).

1.5.4.3. Use of letrozole in cattle

The hypothesis proposed was that letrozole treatment in cattle would terminate the growth of the dominant follicle, and that the emergence of a new follicular wave would subsequently occur. On the contrary, letrozole given iv 2.5 days after wave emergence increased the size and period of dominance of the largest follicle in the wave, presumably due to increased circulating LH (Yapura et al., 2012). Further investigation revealed that regardless of the day in which a 3-day iv regimen of letrozole was given (Days 1 to 3, 3 to 5, or 5 to 7 post-ovulation), letrozole treatment extended the interwave interval in comparison to non-treated control heifers (Yapura et al., 2011). Although release of the inhibitory effects of estradiol on the hypothalamus resulted in an increase in circulating LH profiles, letrozole treatment failed to elicit increased FSH release (Yapura et al., 2011, 2012). Therefore, it was hypothesized that administration of increased concentrations of letrozole might elicit FSH release. To test this hypothesis and to compare the route and vehicle of administration, letrozole (1 mg/kg) was given either iv (in benzyl alcohol) or im (in oil) 3 days after ovulation. Following treatment, circulating estradiol

concentrations dropped most rapidly in the iv group, but estradiol suppression was sustained in the im group for up to 6 days after treatment. Plasma FSH and LH concentrations at post-treatment wave emergence were higher in letrozole than in control heifers, but no differences in FSH concentrations were detected immediately following letrozole treatment. This unchanged plasma FSH supported the notion that the hormone responsible for the extended interwave interval in cattle following letrozole treatment is LH rather than FSH (Yapura et al., 2014). To mitigate the issue of giving letrozole im or iv at multiple intervals, an intravaginal device was developed in subsequent studies (Yapura et al., 2013, 2015). The first intravaginal device was developed with wax used in humans with a melting point of 37°C, and therefore much of the wax/letrozole formulation was lost through the vulvar opening (Yapura et al., 2013). This study showed that intravaginal letrozole administration was feasible, and that the terminal half-life of letrozole was approximately 33 hours in beef heifers. Three different aromatase inhibitors (letrozole, anastrozole and fenbendazole) were cultured with bovine granulosa cells in a subsequent study (Yapura et al., 2015) and letrozole was shown to be the most potent inhibitor. Further investigation revealed that a letrozole-containing gel covering a wax-vehicle intravaginal device resulted in a more rapid increase of plasma letrozole. This device yielded a prolonged suppression of estradiol and increased follicular growth and dominant follicle lifespan (Yapura et al., 2015). A study was then done to investigate the effect of a 4-day letrozole intravaginal device at known stages of the estrous cycle on fertility. Following ovulation (Day 0), heifers were assigned randomly to one of five treatment groups and a 4-day letrozole was initiated on Days 0, 4, 8, 12, or 16. Heifers were given PGF at the time of device removal, and GnRH was given and AI was done 24 hours later. Ultrasonography was done daily from device insertion until ovulation was detected. The percentage of heifers that ovulated and ovulation synchrony

following letrozole treatment was greater compared to the control group, which were given PGF at a random stage of the estrous cycle followed by GnRH 24 hours later (Yapura et al., 2016). The P/AI was 4% in the letrozole group, indicating a disruption in oocyte health or due to a short-lived CL from premature release of PGF from the endometrium.

1.6. Intravaginal drug delivery

The use of intravaginal drug delivery is a convenient and attractive method that provides sustained drug release to control the estrous cycle in farm animals. As early as the 1960s, sponges and silicones devices have been used to deliver pharmaceuticals (predominantly progestins) intravaginally in farm animals (Abecia et al., 2011).

1.6.1. Sponges

Following the identification of progestins that could be used to control the estrous cycle in sheep, the use of intravaginal sponges was first reported in 1965 (Robinson, 1965). The sponge was prepared from a dense polyurethane foam in which progestins (50 to 800 mg) were dissolved in ethanol and pipetted onto each sponge. Following evaporation of ethanol, sponges were inserted into the vagina of ewes for 10 to 30 days. Following treatment, 30/36 sponges were retained, and following removal, 29/30 ewes showed signs of estrus within 3 days. Following natural breeding, 22/29 ewes became pregnant and produced lambs 151 to 157 days later. One of the negative aspects of intravaginal sponges was that copious discharge of cloudy mucus was observed, but this did not appear to affect fertility. Sponges were subsequently used for estrous control in cattle in the mid-1960s. In the initial study, sponges containing flugestone acetate (FGA) were inserted at random days of the estrous cycle for 18 or 19 days, and following removal, cows were observed for estrus and artificially inseminated. Neither estrus nor

ovulations were detected during treatment, but following removal, 20/36 cows came into estrus (Carrick and Shelton, 1967). The use of sponges in cattle has varied considerably due in part to their retention rates (~62%; Scanlon et al., 1971). In recent years, most sponges have utilized the potent progestins, FGA (20-40 mg/sponge; Synchropart; Ceva Santé Animale, France) and medroxyprogesterone acetate (MPA; Veramix; Pharmacia and Upjohn Animal Health, Canada) for ovarian control in ewes in Europe and Australia, but are not currently approved in North America (Abecia et al., 2011; Bartlewski et al., 2015; Swelum et al., 2015). To date, only silicone intravaginal devices impregnated with progesterone are permitted for use in ruminants in the USA and Canada (Eazi-Breed CIDR [sheep and cattle; USA and Canada], Zoetis Inc. and PRID Delta [cattle; Canada], Ceva Animal Health, Inc.) (Health Canada, 2019; U.S. Food and Drug Administration, 2019).

1.6.2. Silicone

The use of silicone-impregnated progesterone releasing intravaginal devices (PRID) for estrous control in cattle were first reported in the 1970s (Mauer et al., 1975). This intravaginal device consisted of a stainless-steel spiral that was coated with progesterone-impregnated silicone rubber. It showed efficacy by elevating plasma progesterone to ~6 ng/mL within 2 hours of insertion. Today's intravaginal progesterone devices consist of 3 parts: the silicone portion homogeneously impregnated with ~10% wt/wt of progesterone, a plastic or stainless steel spine to sustain rigidity in the vagina, and a tail protruding from the vulva to facilitate removal (Rathbone and Burke, 2013). Initial intravaginal devices were formulated to quickly elevate and maintain plasma progesterone ≥ 2 ng/mL for a treatment period of 12 to 14 days, but were later manufactured to sustain release of progesterone for 5 to 8 days (de Graaff and Grimard, 2018). For example, the controlled intravaginal drug release (CIDR) was reengineered in the early

2000s to reduce the amount of progesterone from 1.9 g (CIDR-B 1900) to 1.38 g (CIDR 1380) per device while maintaining the same circulating progesterone concentrations. To achieve this, the thickness of the silicone covering the plastic spine was reduced to 1 mm, as progesterone is released in a surface area-dependent manner from the outermost 1 mm (Rathbone et al., 2002). The decreased diameter resulted in less residual progesterone after use. However, recent studies suggest a CIDR can be autoclaved and reused, resulting in elevated initial plasma progesterone (Zuluaga and Williams, 2008; Melo et al., 2018).

1.6.2.1. Molecular structure of silicone

Silicones, commonly referred to as polysiloxanes, are chemically stable, unreactive, and hydrophobic. They contain silicon and oxygen atoms as their backbone, and by varying the length of the polymer chain, silicones can range in consistency ranging from oil to solid plastic resins (Gunatillake and Adhikari, 2016). The molecular makeup of all silicone can be seen in Figure 1.3 below.

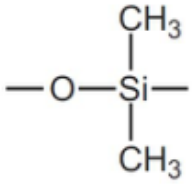

Figure 1. 3**.** Molecular structure of all silicones, including the silicon and oxygen backbone.

The silicone used in manufacturing the CIDR (and in this thesis) is supplied as a viscous, 2-part liquid. One part contains a platinum portion, and when the 2 portions are combined, the platinum catalyzes the reaction, which initiates the curing (hardening) process (Figure 1.4) at room temperature (Mashak and Rahimi, 2009).

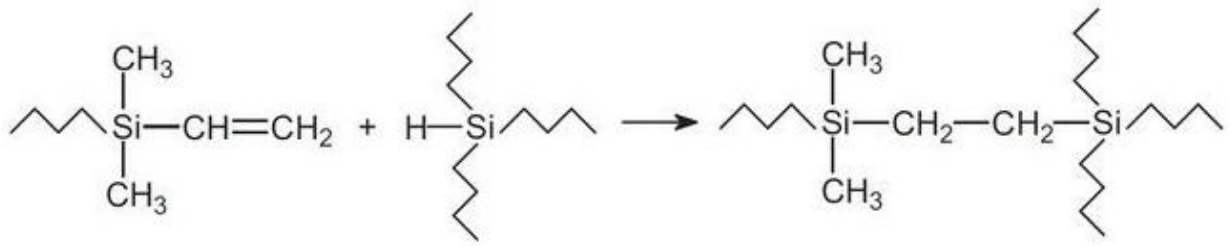

Figure 1. 4**.** The platinum addition cure reaction to form solid silicone.

1.6.2.2. Characteristics of silicone

Due to its unique structure, silicone can be incorporated into the matrix, membrane and coating of a drug delivery system. Prior to curing, hydrophobic molecules (i.e. steroids) may be homogenously incorporated into the mix due to their high solubility in silicone (Malcolm et al., 2003). The silicone matrix can then be injection molded into any shape and subsequently heated to hasten the curing process. *In vivo* drug delivery from the silicone matrix is achieved by diffusion and is dependent on several factors, including molecular size, surface area, solubility in silicone, and thickness of tissues (Fu and Kao, 2010). The negative aspect of using silicones for drug delivery is that silicone rubbers are non-biodegradable, and they will not breakdown in a landfill setting.

1.7. General Hypothesis

Our working hypothesis is that a letrozole treatment protocol will be effective for controlling and synchronizing ovarian function in cattle.

1.8. General Objectives

The overall objectives of the studies reported herein were:

- To determine if letrozole can be impregnated into a silicone intravaginal device
- To determine if letrozole is secreted into milk and to understand the pharmacokinetics of letrozole in lactating dairy cows
- To test the hypothesis that letrozole increases the superstimulatory response in follicle-stimulating hormone-treated cattle
- To determine the effect of letrozole on the ovarian-oocyte environment in cattle
- To determine the interval to estrus and ovulation following letrozole treatment in cattle
- To develop a letrozole-based protocol for fixed-time artificial insemination in cattle

The following six manuscripts in this dissertation are all first-authored by EM Zwiefelhofer and have not been published previously.

CHAPTER 2:

2. RESEARCH AND DEVELOPMENT OF A SILICONE LETROZOLE-RELEASING DEVICE TO CONTROL REPRODUCTION IN CATTLE

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Relationship of this study to the dissertation

Previous letrozole intravaginal device formulations incorporated a wax-based delivery system which was difficult to manufacture and resulted in varying letrozole drug loads and drug release. In our first study, we determined that letrozole could be released from a silicone matrix both *in vitro* and *in vivo*. Further, we tested the hypothesis that plasma concentrations of letrozole released from the silicone matrix will have a biological effect on the ovaries in beef heifers.

Authors' Contributions

As first author, EMZ participated in designing the experiment, manufacturing of intravaginal devices, collecting and analyzing data, and in writing and revising the manuscript. BMD contributed assistance in manufacturing intravaginal devices and collection and interpretation of data. As senior author, GPA provided the overall intellectual knowledge, experimental design, analysis and interpretation of data, as well as revising the manuscript.

2.1. Abstract

Two experiments were conducted to test the effectiveness of a silicone matrix as an intravaginal drug delivery device for letrozole, an aromatase inhibitor used for synchronization protocols in cattle. A wax dip-coat formulation of the intravaginal device used in previous studies was effective in releasing letrozole but was cumbersome to manufacture and deploy, resulting in unwanted variation in drug delivery and circulating concentrations of letrozole. In Experiment 1, a 3 x 3 design was used to test the release kinetics of letrozole from silicone *in vitro*. Silicone was mixed with 3 different letrozole drug loads (5%, 10%, 15%) and 3 different mineral oil loads (5%, 10%, 15%), and letrozole release into 62.5% ethanol was compared with the wax dip-coat formulation (positive control) by UV spectrophotometry. Letrozole was released from silicone in a dose-dependent manner, with no effect of mineral oil. Release kinetics were then examined *in vivo* (Experiment 2) in nulliparous beef heifers assigned randomly to six groups (n=6/group) and given a large surface area (LSA) or standard surface area (SSA) intravaginal silicone device impregnated with 5%, 10%, or 15% drug load, or a wax dip-coat device (positive control) or blank device (negative control). Devices were inserted on Day 3 (Day 0=ovulation) until Day 11. Blood samples were collected at 0, 30 mins, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h, and twice daily until Day 11 to determine letrozole plasma concentrations by LC-MS/MS. The ovaries were examined once daily from Day 3 to 13 by ultrasonography to determine follicular and luteal responses to treatment. Letrozole plasma concentrations were higher in heifers given a device with an LSA vs SSA ($P<0.05$) and those given a device with 15% vs 5% or 10% drug-load ($P<0.05$). Plasma concentrations of estradiol decreased the most in heifers given the 15% LSA device ($P=0.05$). The interval between emergence of successive follicular waves was longest ($P<0.05$) in the positive control and the 15% LSA groups. As well,

the diameter profiles of the dominant follicle and the corpus luteum were largest ($P<0.01$) in the positive control and 15% LSA groups. In conclusion, letrozole was released from a silicone matrix in a dose-dependent manner, and the 15% LSA devices achieved target effects on ovarian function. Results may be used to manufacture a silicone intravaginal device for delivering aromatase inhibitors in a novel synchronization protocol for cattle.

2.2. Introduction

The intravaginal drug-delivery method has become widely accepted by the cattle production industry. Beginning in the late 1960s, polyurethane sponges containing progesterone were used for synchronization of estrus in cattle, but due to low vaginal retention rates, they were not adopted for commercial use (Scanlon et al., 1972). Silicone technology for intravaginal drug release began in the early 1970s with the development of the progesterone releasing intravaginal device (PRID) consisting of a stainless-steel spiral coated in progesterone-impregnated silicone rubber (Mauer et al., 1975).

Development of synchronization methods for fixed-time artificial insemination (FTAI) began with the ability to control follicular wave emergence by follicular ablation or treatment with a combination of estradiol and progesterone (Adams, 1994; Bo et al., 1995). Use of the steroid combination of estradiol and progesterone for synchronization has become commonplace throughout South America, but due to increasing regulation of the use of estrogens in food-producing animals, its use is restricted in North America, Australia, New Zealand, and the European Union (reviewed in Yapura et al., 2018). The development of gonadotropin-releasing hormone (GnRH)-based synchronization protocols began in the mid 1990s (Pursley et al., 1995), but the efficacy was affected by a wide range in interval to ovulation (Wiltbank and Pursley,

2014). Many variations of steroid-based and GnRH-based protocols have been devised in the last 20 years (Martinez et al., 2002; Fricke et al., 2003; Souza et al., 2008; Colazo and Ambrose, 2011), and although P/AI have increased, costs of multiple hormone treatments have also increased. An alternative to current steroid- or GnRH-based synchronization protocols is the use of non-steroidal aromatase inhibitors, such as letrozole (Yapura et al., 2014).

Letrozole, a 3rd generation non-steroidal aromatase inhibitor, prevents the body from synthesizing its own estrogen by actively binding to aromatase, the P450 enzyme that is solely responsible for converting androgens to estrogens (Buzdar, 2003). Currently, letrozole is licensed for use in humans for the treatment of breast cancer in post-menopausal women (Demers, 1994) but has been used in women experiencing infertility (Casper and Mitwally, 2012). The putative mechanism by which letrozole affects ovarian function was tested using the bovine model (Yapura et al., 2013, 2014), and the effectiveness of letrozole for controlling the estrous cycle in cattle has subsequently been documented in a series of recent studies (Yapura et al., 2013, 2014, 2015, 2016). A letrozole-releasing intravaginal device was developed to enable continuous treatment over an extended period (Yapura et al., 2015). By effectively suppressing estradiol production (over a period of days), circulating LH concentrations increased, resulting in prolongation of the growing phase of the extant dominant follicle and delaying emergence of the next follicular wave (Yapura et al., 2014, 2015). Treatment with a vaginal device coated with a letrozole-in-wax formulation resulted in synchronous ovulation in over 90% of heifers regardless of the stage of the estrous cycle at which the protocol was initiated (Yapura et al., 2016). Further, results suggest that letrozole treatment is luteotrophic i.e., results in a corpus luteum (CL) that is larger and produces more progesterone (Yapura et al., 2013, 2014, 2015).

Coating a vaginal device with a letrozole wax formulation, however, does not lend itself to automated manufacture, and is subject to a lack of uniformity and consistency. Silicones can be effective as a drug delivery system for many reasons and are currently used in intravaginal steroid-releasing devices in women (NuvaRing, Merck, Kenilworth, NJ, USA) and cattle (CIDR, Zoetis, Kirkland, QC, Canada; PRID-Delta, Ceva Animal Health, Cambridge, ON, Canada). Liquid silicone can be molded into any shape and can set or cure quickly. When placed in the vagina, silicone will not deteriorate over time and nothing will adhere to it i.e., tissues will not grow on silicone. Many lipophilic drugs are soluble and stable in silicone due to their ability to permeate the hydrophobic matrix (Kajihara et al., 2003).

With the intent of developing a device that will provide more uniformity in drug delivery compared to the wax dip-coat intravaginal devices used previously (Yapura et al., 2015) the objective of the present study was to determine if letrozole-impregnated silicone will provide an effective drug-delivery matrix for intravaginal treatment in cattle. Specifically, the release kinetics of letrozole-impregnated silicone *in vitro* (Experiment 1) and *in vivo* (Experiment 2) was characterized to determine if biologically effective drug levels can be achieved for the purposes of controlling ovarian function in cattle.

2.3. Materials and methods

2.3.1. Experiment 1 – In vitro testing of letrozole-release from silicone

The silicone intravaginal devices produced for the purposes of this experiment contained letrozole, mineral oil, silicone thinner (Smooth-On, Macungi, PA, USA), and medical-grade silicone (Silastic Q7-4840, Dow Corning Corp., Auburn, MI, USA). Using a 3 x 3 design, 9 different silicone formulations were prepared using 5, 10, or 15% (wt/wt) letrozole and 5, 10, or 15% (wt/wt) mineral oil. Addition-cured silicones require the addition of platinum as a catalyst

to facilitate the curing reaction. Biomedical grade liquid silicone rubber (Silastic Q7-4840, Dow Corning Corp., Auburn, MI, USA) was supplied as two-component kits (Parts A and B) of platinum-catalyzed silicone elastomers which must be mixed in equal portions, by weight. In the following order, mineral oil, silicone thinner, and letrozole were weighed and added to the silicone mixture. The ingredients were mixed for 5 minutes with a spatula until homogeneous. After giving time for letrozole to permeate the hydrophobic silicone matrix (approximately 30 minutes), the mixture was loaded into 60 mL catheter-tipped syringes and injected into a two-part aluminum mold (RMD Engineering Inc., Saskatoon, Saskatchewan, Canada) containing a centrally placed nylon spine to provide rigidity. The mold was then heated to 200°C for 5 minutes. The cured device was removed from the mold and a plastic tail was added to facilitate device removal (Fig. 2.1).

An Erlenmeyer flask containing 250 mL of 62.5% ethanol and a magnetic stirrer was maintained at 37ºC on a multi-plate stirrer (RT 10, IKA Works, Inc., Wilmington, NC, USA). Sink conditions, defined as maintaining a volume of dissolution media that is 5 to 10 times greater than the saturation point of the drug in the drug delivery system (saturation of letrozole in 62.5% ethanol = 6.11mg/mL), were achieved by placing 1.5 g portion of each silicone device and 0.248 g of the wax positive control into the volume of ethanol. Each silicone and wax portion was suspended centrally in the ethanol-filled flask, and a 10 μL sample was taken from each flask at 0, 5 min, 2, 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 hours to characterize the letrozole-release kinetics. Samples were placed in Eppendorf tubes and letrozole concentrations were determined with UV-Vis-Spectrophotometry (Nano-Drop, Thermo Scientific, Waltham, MA, USA). The letrozole concentration of each sample (10 μL) was measured 3 separate times.

2.3.2. Experiment 2 – In vivo testing of letrozole-release from silicone intravaginal devices

Hereford-cross beef heifers, 12 to 17 months of age and weighing between 355 and 534 kg (422 ± 5.71 kg) were used for the study and were maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52° N and 106° W). Heifers were fed a mixture of barley silage and free-choice hay to gain approximately 1.1 kg/day, and water was provided ad libitum during the experimental period from April to July. Heifers were chosen from a group of 42 after initial examination by transrectal ultrasonography (7.5 MHz linear array probe; MyLab Alpha, Esaote NA, Indianapolis, IN, USA) to detect the presence of a CL (i.e., to confirm puberty; Pierson and Ginther, 1987b). Animal procedures were done in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

2.3.2.1. Treatment and examinations

Based on the results of Experiment 1, 4 formulations of the silicone intravaginal device were developed to compare the kinetics of letrozole release : 10% and 15% letrozole drug-load in a device with standard surface area (121.8 cm^2, Fig. 2.1A), and 5% and 15% letrozole drug-load in a device with a larger surface area (490 cm^2; Fig. 2.1B). Heifers in which a CL was detected during the initial examination were given two doses of cloprostenol intramuscularly 12 hours apart (500 µg; Estrumate, Merck, Kenilworth, NJ, USA) to induce luteolysis and synchronize ovulation (Gorewit et al., 1977). On Day 3 (Day 0 = ovulation), heifers were assigned randomly to one of six groups and given one of the four letrozole-releasing devices described above, or the original waxed-based dip-coat device (Yapura et al., 2015) positive control), or a blank (drug-

free) silicone device (negative control). The intravaginal device was left in place for 8 days. The experiment was done in two replicates; n=6 heifers per group.

The ovaries were examined daily from Day 3 to 13 by transrectal ultrasonography and a sketch was made during each examination to record the size, number and relative position of ovarian structures (CL and follicles ≥ 4 mm; Knopf et al., 1989). Ovulation was defined as the disappearance of a follicle ≥ 10 mm between consecutive examinations and was confirmed by subsequent detection of a CL (Pierson and Ginther, 1987b). Follicular wave emergence was defined as the day of ovulation or determined retrospectively as the day when the dominant follicle was first identified at a diameter of 4 or 5 mm (Adams et al., 1993; Ginther et al., 1997). If the dominant follicle was not identified until it reached 6 or 7 mm, the previous day was considered day of wave emergence. The dominant follicle of a wave was defined as the largest follicle of the wave.

2.3.2.2. Blood sampling and hormone assays

To determine the pharmacokinetics of letrozole from the newly developed silicone devices, blood samples were taken 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 hours after device insertion, and twice daily until Day 11. Blood samples were collected by jugular venipuncture into 10 mL heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Blood samples were centrifuged at 1500 x g for 20 min, and plasma was separated and stored in polypropylene tubes at -20°C.

Plasma estradiol concentrations were measured by radioimmunoassay from samples taken every 2 hours for the first 12 hours from device placement (Day 3) and then daily until Day 11 at Texas A&M University as previously described (Kirby et al., 1997). Assay sensitivity was 0.5

pg/mL and intra- and interassay coefficients of variation for estradiol were 3.6 and 12.6%, respectively.

Plasma letrozole concentrations were determine by LC-MS/MS using a simple and quick protein precipitation method that was partially validated (Zarghi et al., 2007). Letrozole-d4 (Toronto Research Chemicals, Toronto, ON, Canada), a derivative of letrozole, was selected to serve as the internal standard. A standard stock solution of letrozole was prepared by dissolving 50 mg of letrozole in 50 mL of methanol (HPLC Grade Methanol, Fisher Chemical, Ottawa, ON, Canada) for a final concentration of 1 mg/mL (stock solution #1). Stock solution #1 was further diluted to a concentration of 51.2 µg/mL (stock solution #2). A volume of 995 µL of blank bovine plasma was spiked with 5 µL of stock solution #2 for a final concentration of 256 ng/mL (Standard #1). A total of 9 standards underwent serial dilution and ranged from 1 to 256 ng/mL. Quality controls were spiked independently with stock solution #2 to obtain concentrations of 3, 10, and 100 ng/mL. A total volume of 100 µL of the standard or quality control was placed into a 1.5 mL Eppendorf tube and 50 µL of the internal standard solution (letrozole-d4; 200 ng/mL) was added. The tube was vortexed for 5 seconds and 250 µL of acetonitrile were added. Each standard or quality control was vortexed for 5 seconds and centrifuged at 15,000 x g for 15 minutes at 4°C. Following centrifugation, the supernatant was transferred to a 2 mL vial for auto-injection into the LC-MS/MS. Double blank (- letrozole, - letrozole-d4) and blank (- letrozole, +letrozole-d4) samples were prepared in the same manner as described previously. Each lower limit of quantification (LLOQ) and quality control were prepared 6 times per day on 3 separate days; all other standards, double blanks, and blanks were prepared once per day for 3 days. LC-MS/MS conditions are described elsewhere (Joshi et al., 2011).

Bovine plasma samples were thawed at room temperature, and 100 µL of plasma was spiked with 50 µL of internal standard solution (200 ng/mL). A volume of 250 µL acetonitrile was added to each bovine plasma sample and vortexed, centrifuged, and transferred to a 2 mL vial, as described above. Linearity was tested by running 3 standard curves independently of each other. Regression coefficients were calculated and ratio counts vs. concentration were plotted. A regression coefficient (r^2) ≥ 0.99 was necessary for acceptance of the linearity curve. The mean (± SEM) r^2 obtained was 0.9996 ± 0.0003. The acceptance criterion for each calculated standard concentration was 15% deviation from the actual value except for the lower limit of quantification, where it did not deviate by > 20%. Precision was defined as residual standard deviation (RSD) and calculated using the following formula: RSD = SD x 100/mean. Accuracy was calculated using the concentration recovered divided by the concentration added. The standard curve is shown in Table 2.1.

2.3.3. Statistical analyses

Statistical analyses were done using the Statistical Analysis System software package (SAS Learning Edition 9.4, 2013; SAS Institute Inc., Cary, NC, USA). Serial data (letrozole and hormone concentrations, follicle and CL diameter) were compared among groups by analysis of variance for repeated measures using the PROC MIXED procedure to determine the effects of device formulation, time, and their interactions. If an interaction was detected, individual comparisons among groups and days were made using the method of least significant difference. Pearson correlations between follicle and CL growth and letrozole concentrations were made by PROC CORR. The inter-wave interval was compared among groups by analysis of variance.

Significance was defined as P≤0.05. Data are presented as mean ± SEM, unless otherwise specified.

2.4. Results

2.4.1. Experiment 1 – In vitro testing of letrozole-release from silicone

Release rates of letrozole from impregnated silicone are sumarized in Figure 2.2. Data document that letrozole was released from the silicone matrix in a dose-related manner. The previously designed wax dip-coat formulation released letrozole at a faster rate, but was exhausted by 60 hours. Data were deemed sufficient, without further replication, to proceed to Experiment 2; hence, no statistical analyses were done. Based on the results of the *in vitro* study, the silicone matrix formulated with the lowest, medium, and highest letrozole loads (i.e., 5, 10 and 15%) was selected for the following study.

2.4.2. Experiment 2 – testing of letrozole-release from silicone intravaginal devices

Following development of the LC-MS/MS assay for plasma letrozole, all points on the standard curve passed for precision and accuracy except for the sample at 256 ng/mL; therefore, 128 ng/mL was considered the maximum standard (Table 2.1). All quality control samples passed for precision and accuracy and were accepted (Table 2.2).

The vaginal device remained in place for the full 8 days in all heifers. Circulating concentrations of letrozole were higher (P<0.05) in heifers with the wax dip-coat device and those with 15% letrozole-impregnated silicone in the large surface area device than in the other groups (Fig. 2.3A). The 15% large surface area device exhibited greater release than the wax dip-coat device through the first 12 hours (P<0.05), and maintained release throughout the 8-day period of deployment. Plasma letrozole concentrations continued to rise for 5 or 6 days in all

letrozole-treated groups, but a treatment-by-time interaction ($P<0.0001$) was attributed to a rapid drop thereafter in the wax dip-coat group, unlike the silicone groups in which high letrozole concentrations were maintained (Fig. 2.3A). Among groups with an impregnated silicone device, those with a higher drug-load and those with a larger surface area had significantly higher plasma letrozole concentrations than their counterpart (Fig. 2.3A).

Follicle growth over the experimental period was positively correlated with letrozole concentrations ($r=0.41$; $n=257$, $P<0.0001$). At the time of device insertion (Day 3), there was no difference among groups in the diameter of the dominant follicle (Table 2.3). Similar to the pattern observed with plasma letrozole concentrations, the diameter of the dominant follicle was greatest in heifers with the wax dip-coat device and in those with 15% letrozole-impregnated silicone in the large surface area device than in the other groups, and smallest in the negative control group (Fig. 2.3B). Correspondingly, the interwave interval was greatest in the groups with a wax dip-coat device and 15% letrozole large surface area device ($P<0.05$; Table 2.3). By Day 13, the mean diameter of the dominant follicle had begun to decline in all groups except in the 15% letrozole large surface area group (Fig. 2.3B).

The diameter of the CL was positively correlated with plasma letrozole concentrations ($r=0.30$, $n=257$; $P<0.0001$). The CL diameter profile was greatest in groups with a large surface area device and smallest in the negative control group (Fig. 2.3C). Heifers with a large surface area device (5% and 15% groups combined) had a greater CL diameter profile ($P=0.05$) than that of heifers with a standard surface area device (10% and 15% groups combined). Maximum CL diameter was greatest in the 15% large surface area group, and occurred on Day 7 after ovulation (Fig. 2.3C).

A treatment effect on plasma estradiol concentration was attributed to a decrease in the 15% large surface area group during the sampling period (P=0.05; Fig. 2.4).

Table 2. 1. Precision and accuracy data from LC-MS/MS derived standard curve for letrozole in bovine plasma (n=3 per sample except lower limit of quantification ((LLOQ) n=18; Experiment 2).

Concentration added (ng/mL)	Concentration found (mean ± SEM; ng/mL)	Precision (%)	Accuracy (%)
1 (LLOQ)	0.99 ± 0.03	10.8%	98.6%
2	2.2 ± 0.19	14.8%	109.5%
4	3.91 ± 0.17	7.5%	97.7%
8	8.05 ± 0.04	0.8%	100.6%
16	17.2 ± 0.76	7.6%	107.5%
32	32.4 ± 0.55	2.9%	101.4%
64	64.6 ± 0.40	1.1%	100.9%
128	127 ± 0.58	0.8%	99.2%
256	214 ± 41.0	33.2%	83.6%

Table 2. 2**.** Precision and accuracy data of the LC-MS/MS method for quality control samples of letrozole in bovine plasma (n=18 per quality control sample; Experiment 2).

Concentration added (ng/mL)	Concentration found (mean ± SEM; ng/mL)	Precision (%)	Accuracy (%)
3	2.80 ± 0.04	6.1%	93.3%
10	9.73 ± 0.13	5.5%	97.3%
100	98.55 ± 1.30	5.6%	98.6%

Table 2. 3 The effects of treatment with intravaginal letrozole-releasing devices on follicular dominance in cattle (mean ± SEM). Devices were composed of silicone impregnated with varying percentages of letrozole in a standard- or large-surface area format, a previously designed wax dip-coat formulation, or a device containing no letrozole (control; Experiment 2).

Standard surface area device (121 cm^2)		**Large surface area device (490 cm^2)**		**Wax**	**Control**
10%	15%	5%	15%	dip-coat	device
Number of heifers					
6	6	6	6	6	6
Dominant follicle diameter (mm) at device insertion (Day 3)					
9.3 ± 0.3	9.3 ± 0.4	10.3 ± 0.4	10.0 ± 0.5	9.8 ± 0.6	10.1 ± 0.6
Follicular interwave interval (days)					
8.0 ± 0.4^{ab}	10.0 ± 0.7^{bc}	9.2 ± 0.4^{abc}	10.0 ± 0.4^{bc}	10.5 ± 0.4^{c}	7.5 ± 0.5^{a}

^{abc} Values with no common subscript across row are different ($P<0.05$)

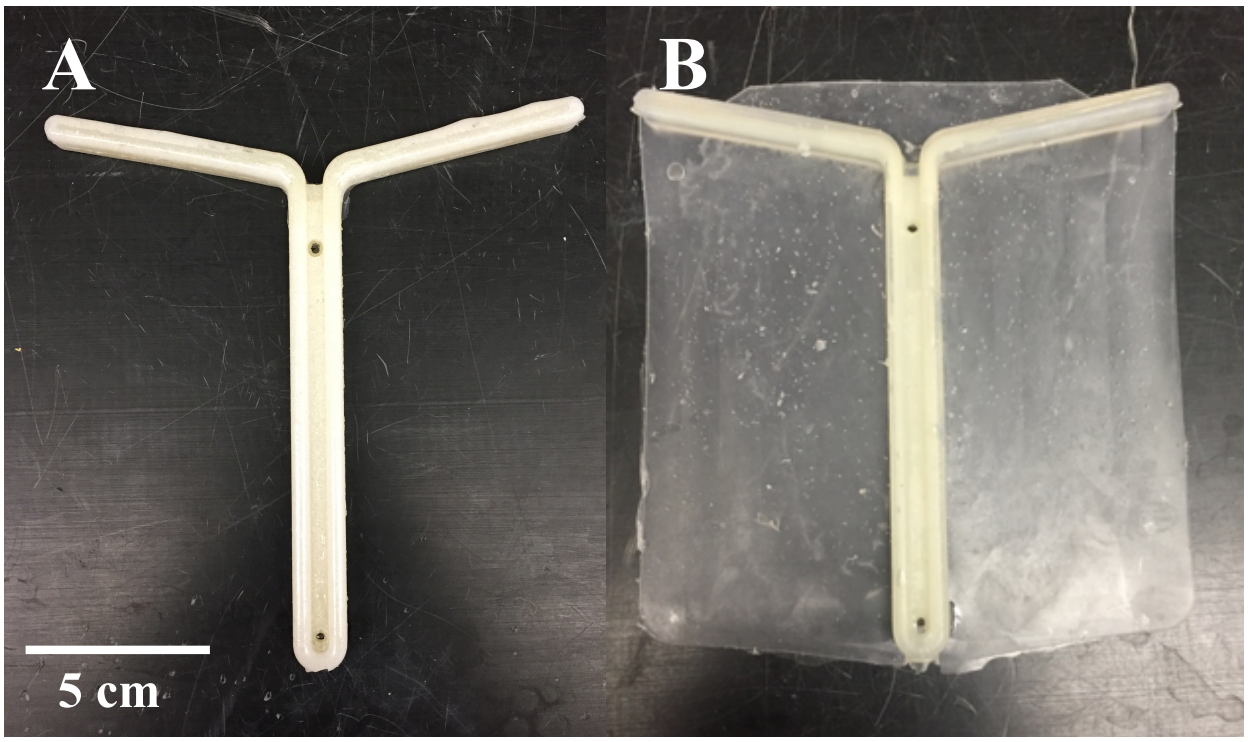

Figure 2. 1**.** Intravaginal silicone letrozole releasing devices (LRD) with a standard surface area of 121 cm^2 (A) and a large surface area of 490 cm^2 (B; Experiment 2).

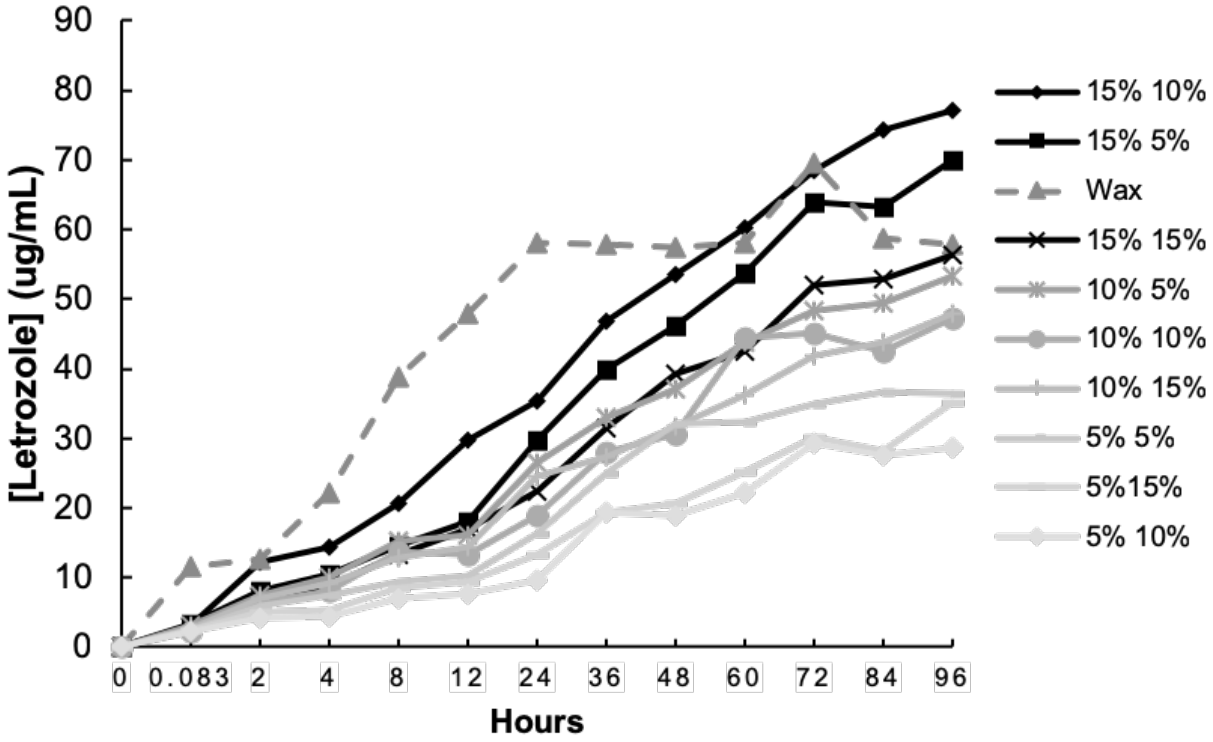

Figure 2. 2**.** *In vitro* release of letrozole from portions of vaginal devices composed of the original wax dip-coat formulation vs. impregnated silicone formulations (% letrozole : % mineral oil, respectively in the legend). Portions were placed in a volume 62.5% ethanol at 37°C, and 10 µL samples were taken over a 4-day period (Experiment 1).

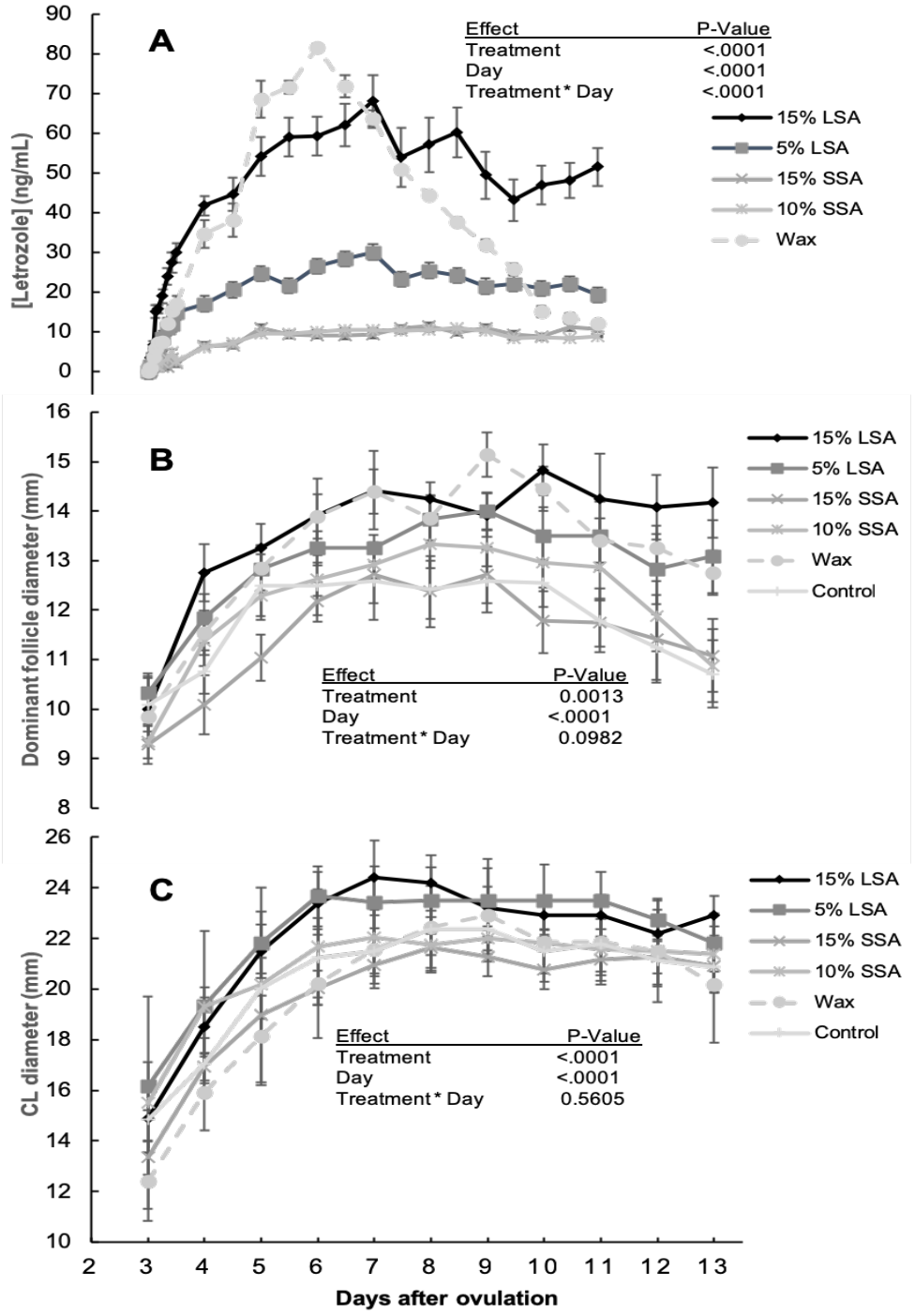

Figure 2. 3**.** Effects of treatment (mean ± SEM) with intravaginal letrozole-releasing devices in beef heifers on circulating concentrations of letrozole (A), the dominant follicle diameter profile (B), and the CL diameter profile (C). Devices were composed of the original wax dip-coat formulation, or silicone impregnated formulations with 5%, 10%, or 15% letrozole in a standard surface area (SSA) or large surface area (LSA) format, or a silicone device containing no letrozole (control). Devices were inserted on Day 3 and removed on Day 11 (Day 0=ovulation; Experiment 2).

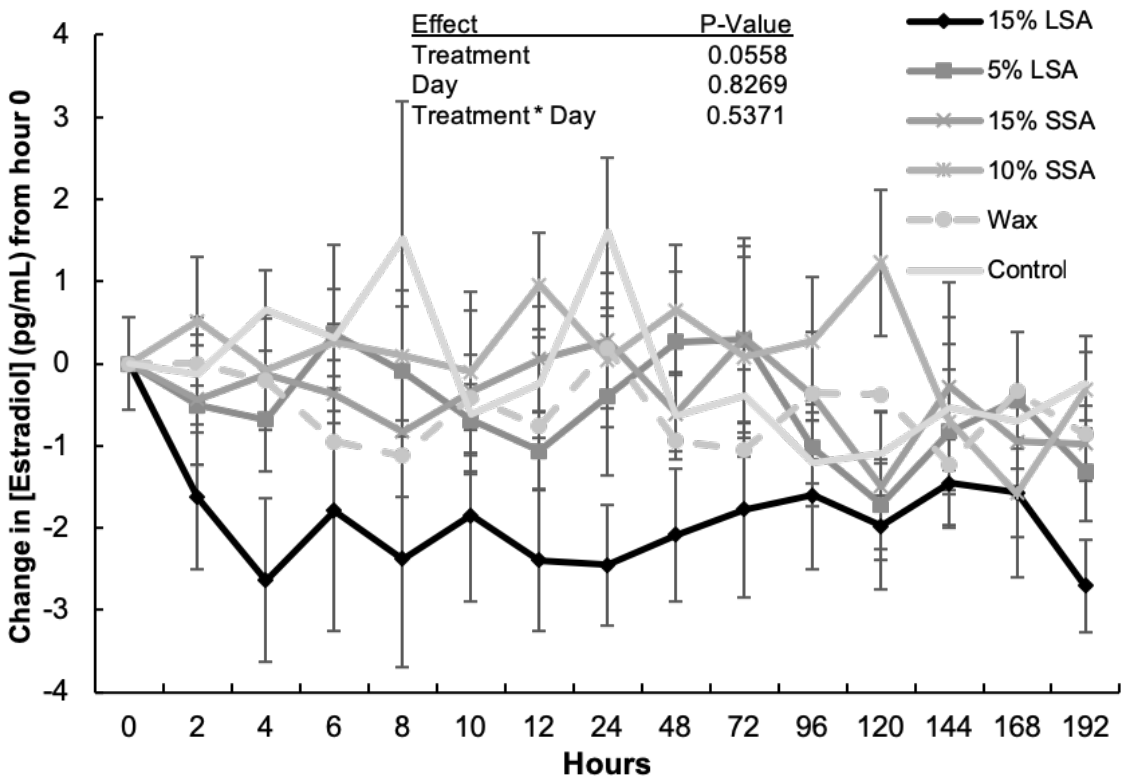

Figure 2. 4**.** Effects of treatment (mean ± SEM) with intravaginal letrozole-releasing devices in beef heifers on circulating concentrations of estradiol. Devices were composed of the original wax dip-coat formulation, or silicone impregnated formulations with 5%, 10% or 15% letrozole in a standard surface area (SSA) or large surface area (LSA) format, or a silicone device containing no letrozole (control). Devices were inserted on Hour 0 and removed at Hour 192 (Hour 0 = 3 days after ovulation; Experiment 2).

2.5. Discussion

The impetus for the study was the need for a device with more uniformity in drug delivery compared to the wax dip-coat intravaginal devices used previously. Beginning with an *in vitro* approach, we tested the drug-release from 9 silicone matrices of differing letrozole and mineral oil compositions. Results document that letrozole is released from a silicone matrix into a ethanol environment, similar to previous reports regarding the development of progesterone devices (Ogle, 1999). Further, release from the silicone matrix was dose-dependent; i.e., higher letrozole drug-loads into the device resulted in greater total release. In a study of progesterone release from silicone, release was positively correlated with the surface area of the device (van Werven et al., 2013), and was found to come from only the most superficial layer of silicone (≤1 mm thickness; Rathbone and McDowell, 2013). Initial silicone CIDR devices were loaded with 1.9 g of progesterone, but were later reduced to 1.38 g of progesterone by minimizing the thickness of silicone while maintaining the same surface area (Rathbone et al., 2002). In ovariectomized Holstein cows, a progesterone-releasing device with a surface area of 155 cm^2 (PRID-Delta, Ceva Animal Health Ltd, Amersham, Buckinghamshire, United Kingdom) resulted in significantly greater circulating progesterone concentrations during the first 4 days of insertion than a device with a surface area of 120 cm^2 (CIDR, Zoetis, Kirkland, QC, Canada; van Werven et al., 2013).

Results of the *in vitro* study permitted the selection of the most effective silicone formulation for use *in vivo*. In the first replicate, however, using a silicone device with a surface area of 121 cm^2, drug delivery was insufficient to raise circulating letrozole concentrations sufficiently to elicit a biological effect on the ovaries. Using past evidence that drug release from silicone is related to surface area, we developed a device with a surface area that was 4 times

greater than the original device (490 vs. 121 cm^2) by adding wing-like structures on the sides of the silicone device with a thickness of 2 mm. In the second replicate, utilizing the larger surface area device, resulted in higher circulating letrozole concentrations while still maintaining continuous release, unlike the original wax dip-coat device. The circulating half-life of letrozole was estimated to be 33 hours in nulliparous beef heifers (Yapura et al., 2013). Hence, the decline in plasma letrozole concentrations after 3 days of deployment of the wax dip-coat device was indicative of drug depletion in the device.

Letrozole treatment in cycling beef heifers prolonged the growing phase of the dominant follicle resulting in a larger follicle and a prolonged interwave interval. However, the maximum diameter of the dominant follicle (and by association, the duration of follicular dominance) was not sustained throughout the experimental period using the wax dip-coat device. By comparison, the 15% large surface area device used in the present study extended the period of follicle dominance, in association with a sustained elevation of plasma letrozole and suppression of plasma estradiol, which contributed to increased plasma LH concentrations (Yapura et al., 2013, 2014, 2015). On Day 13 (2 days after device removal), the 15% large surface area group yielded the largest dominant follicle. The wax dip-coat device provided a maximum plasma letrozole concentration of ~200 ng/mL in a previous study (Yapura et al., 2015), but only ~82 ng/mL in the present study. Correspondingly, the suppressive effect of letrozole treatment on plasma estradiol concentrations was apparent in the previous study using the wax dip-coat device (Yapura et al., 2015), but not in the present study. Higher letrozole concentrations and greater suppression of estradiol observed in the previous study were also reflected in the larger dominant follicle and a longer interwave interval than in the wax dip-coat group in the present study. The

differences between studies in the effect of the dip-coat device were unexpected and reflect the difficulty in consistent manufacture of this type of device.

As past trials have shown (Yapura et al., 2013, 2014, 2015) letrozole treatment was associated with the development of a larger CL and higher circulating concentrations of progesterone. In the present study, larger CL were also observed due to the luteotrophic effects of letrozole. Of the letrozole-treated animals, those in the 15% large surface area group had the largest CL diameter profile. Form and function of the CL has important implications for embryo development and maternal recognition of pregnancy (Mann and Lamming, 2001; Lonergan et al., 2007). In embryo transfer, recipients with a CL > 20 mm^2 6 days after ovulation had significantly higher circulating progesterone and significantly higher pregnancy per embryo transfer after transfer compared to recipients with CL <20 mm^2 (Baruselli et al., 2003). Further, in a FTAI system, increasing progesterone during the synchronization period by using 2 CIDR vs 1 CIDR/cow tended to increase P/AI at 32 days post-insemination in lactating dairy cows (52.6% vs 42.8%; Pereira et al., 2017).

In summary, letrozole impregnated silicone provided continuous release throughout the experimental period. Of the differing silicone surface area devices, the larger surface area provided the highest circulating letrozole concentrations, the largest dominant follicle and longest interwave interval, and the largest CL 7 days post-ovulation. Results demonstrate that letrozole is released from a silicone matrix in a manner related to drug loading and device surface area, and provide impetus for commercialization and manufacturing of a letrozole impregnated silicone intravaginal device.

2.6. Acknowledgements

We thank Brad Blackmore and the staff at the Livestock and Forage Centre of Excellence for assistance with handling and management the animals. We would also like to thank Deborah Michel for her technical assistance and revisions of the manuscript pertaining to LC-MS/MS. We thank Merck for providing Estrumate. This research was supported by grants from Alberta Agriculture and Forestry, Venco Animal Health, a subsidiary of Dechra Pharmaceuticals PLC, and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 3:

3. ANALYSIS OF LETROZOLE MILK RESIDUES FOLLOWING INTRAVAGINAL LETROZOLE TREATMENT IN LACTATING DAIRY CATTLE

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Relationship of this study to the dissertation

Results of previous studies have used nulliparous beef heifers to understand the pharmacokinetics of letrozole. Before any trials with letrozole can be completed in lactating dairy cattle, the milk residues of letrozole must be understood. Using the letrozole-releasing device developed in Chapter 2, we tested the hypothesis that letrozole is secreted into the milk of lactating dairy cattle.

Authors' Contributions

As first author, EMZ participated in designing the experiment, manufacturing of intravaginal devices, collecting and analyzing data, performing mass spectrometry and in writing and revising the manuscript. ALC contributed assistance in analyzing pharmacokinetics, interpreting data, and revising the manuscript. As senior author, GPA provided the overall intellectual knowledge, experimental design, analysis and interpretation of data, as well as revising the manuscript

3.1. Abstract

The aromatase inhibitor letrozole has been used in non-lactating cattle to control ovarian function. To date, letrozole has not been tested in lactating dairy cattle and due to its lipophilic nature, letrozole may be excreted into milk and enter the food supply. The primary objective of this experiment was to characterize and quantify concentrations of letrozole in blood plasma and milk during and after treatment with an intravaginal letrozole-releasing device (LRD) and the secondary objective was to compare ovarian effects in lactating dairy cattle. Non-pregnant, lactating Holsteins cows (n=12) at random stages of the estrous cycle were assigned and given either a silicone LRD or a blank (control) intravaginal device (Day 0). On Day 4, the intravaginal devices were removed, and cows were given a luteolytic dose of prostaglandin on Days 4 and 4.5 followed by GnRH on Day 6.5 to induce ovulation. Milk samples were collected from the LRD-treated cows twice daily from Day 0 to 14 to determine milk letrozole concentrations by a partially validated LC-MS/MS assay. Blood samples were collected twice daily from Day 0 to 14 in both groups. Transrectal ultrasonography was done daily from Day 0 to 6, thereafter twice daily until ovulation was detected, and again daily for 5 days post-ovulation to characterize the ovarian response to treatment. Single point measurements were compared by t-test, and serial data were compared by analysis of variance for repeated measures. Data are presented as mean ± SEM. A standard curve was developed utilizing LC-MS/MS, which achieved an r^2 value of 0.999 ± 0.0001 analyzed over three independent days. The lower limit of quantification (LLOQ) and lower limit of detection (LLOD) for milk letrozole were 5.0 and 0.8 ng/mL, respectively. All points on the standard curve and quality controls were accepted, and were within 8% and 5.5% for accuracy and precision, respectively. Milk letrozole concentrations fell below the LLOQ within 2 days of LRD removal. Letrozole concentrations tended to be higher in plasma than milk

throughout the treatment period (P=0.09). Milk letrozole concentrations and milk production were negatively correlated (R= -0.46; P<0.0001). No difference was detected for the time to maximum concentration of letrozole (2.3 ± 0.4 vs. 2.7 ± 0.3 days; P=0.52) or elimination half-life (14.6 ± 1.6 vs. 18.2 ± 1.4 hours) in plasma or milk, respectively. The variation in interval to ovulation following device insertion (residuals) was less in the LRD vs. control group (0 ± 0 vs. 42.7 ± 9.2 hours, respectively; P<0.001). In summary, a method for quantifying letrozole in milk was partially validated. These data may be used for the recommendation of milk withdrawal periods following estrus synchronization with letrozole in lactating dairy cows.

3.2. Introduction

Understanding how a new drug is metabolized and excreted in food-producing animals is an essential step in the veterinary drug approval process. For a drug intended for use in lactating dairy cows, milk residue levels of the drug must be quantified to establish a maximum residue level (i.e., residue that is legally permitted in food commodities) for human consumption by government agencies (Canadian Animal Health Institute, 2019).

Recent research in our laboratory has focused on the development of an aromatase inhibitor-based protocol for ovarian synchronization in beef cattle (reviewed in Yapura et al., 2018). The efficacy of the protocol is attributed to release of the negative feedback of estradiol on the hypothalamus, resulting in increased circulating concentrations of luteinizing hormone (LH; Yapura et al., 2013). Increased LH release resulted in a prolonged period of dominant follicle growth, allowing a 4-day letrozole treatment protocol to be initiated at random stages of the estrous cycle. Followed by prostaglandin F_{2a} induced luteolysis and ovulation induced by gonadotropin-releasing hormone (GnRH), >90% beef heifers ovulated in a synchronous fashion (Yapura et al., 2016). Recently, we have developed a silicone intravaginal letrozole-releasing

device (LRD) that continuously releases letrozole over a period of 8 days in beef heifers (Chapter 2). However, characterization of letrozole in plasma or milk in lactating dairy cows has yet to be done, which is a necessary step for the approval of a new veterinary drug. Moreover, a milk residue study of letrozole may provide insight for its use in lactating women undergoing breast cancer treatment.

The molecular characteristics of a drug (i.e., lipophilic vs. hydrophilic) may impact its accumulation in milk. Hydrophilic drugs concentrate in the skim portion while lipophilic drugs tend to concentrate in the fat portion of milk (Ozdemir et al., 2018). Penicillin, a hydrophilic drug, is found in serum at concentrations approximately 6x higher than in whole milk (3.42 IU/mL vs. 0.55 IU/mL; Dubreuil et al., 2001) in lactating dairy cows. In contrast, the highly lipophilic steroid, progesterone, is found in similar concentrations in serum and milk (Chenault et al., 2003). The use of tandem mass spectrometry (LC-MS/MS) has been employed, in recent years, to quantify pharmaceuticals found in milk due to its high molecular specificity and sensitivity.

The primary objective of this study was to characterize and quantify concentrations of letrozole in blood plasma and milk during and after treatment with an LRD in lactating dairy cows. The study also provided an opportunity to compare the ovarian effects of LRD synchronization treatment in lactating dairy cows.

3.3. Materials and methods

Procedures were approved by the University of Saskatchewan's Animal Research Ethics Board in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.1. Treatments and sampling

Non-pregnant, lactating Holstein cows (n=6; weight: 680 ± 4.1 kg; body condition score: 2.6 ± 0.1 [1 to 5 scale]; Ferguson et al., 1994) at various stages of lactation were purchased from local Saskatchewan dairy farmers. Cows were maintained outdoor pens at the Western College of Veterinary Medicine, University of Saskatchewan and fed alfalfa hay with free access to water during October. For comparison of ovarian effects, age-matched lactating Holsteins (n=6), 69.5 ±5.0 days in milk, housed at the Rayner Research Dairy Farm, University of Saskatchewan were selected to serve as controls. At random stages of the estrous cycle, test cows were given a silicone intravaginal device (Day 0) containing 5.6 grams letrozole (Chapter 2), and control cows were given a blank device (progesterone-free Cue-Mate; Bayer Australia, Pymble, NSW, Australia). On Day 4, the devices were removed, and prostaglandin F_{2a} (500 μg cloprostenol; Estrumate; Merck, Kirkland, QC, Canada) was given im on Day 4 and 4.5 to induce luteolysis. An analog of GnRH (100 μg gonadorelin; Fertiline; Vetoquinol N.A.; Lavaltrie, QC, Canada) was given im on Day 6.5 to induce ovulation. Transrectal ultrasonography was done daily from Day 0 to 6 and twice daily thereafter until ovulation was detected using a 7.5 MHz linear-array probe (MyLab5; Esaote North America, Fischers, IN, USA). Ovulation was defined as the disappearance of a follicle ≥10 mm in diameter from one examination to the next. Following ovulation, the resultant corpus luteum (CL) was measured by transrectal ultrasonography daily for 5 days. Follicular and luteal structures were measured and recorded on ovarian sketch sheets for each individual cow (Knopf et al., 1989).

Blood samples were collected twice daily from Day 0 to 14 via coccygeal venipuncture into heparinized vacutainer tubes (BD, Mississauga, ON, Canada) and centrifuged 1500 x *g* for 15 minutes within 2 hours of collection. Plasma was aliquoted into polypropylene tubes and

stored frozen at -20 ºC until analysis was done within 1 month. Plasma letrozole taken twice daily from Day 0 to 6 was quantified by tandem mass spectrometry (LC-MS/MS), as previously described (Chapter 2).

Letrozole-treated cows were milked twice daily at 12-hour intervals while restrained in a chute system using a portable milking system. Milk sampling began immediately before device insertion (Day 0) and continued twice daily for 14 days for letrozole analysis. Following complete milk-out, the milk container was weighed, and a 50 mL sample of milk was taken and subsequently frozen at -20°C until analysis was done within 1 month. Between each cow, the bucket was cleaned to prevent cross-contamination of samples. Cows selected as controls were milked 3x per day at 8-hour intervals at the Rayner Research Dairy Farm and produced 43.1 ± 3.2 kg of milk daily.

3.3.2. LC-MS/MS for letrozole milk residue

Whole milk (Dairyland, Saskatoon, SK, Canada) was purchased from the grocery store and brought to room temperature and briefly vortexed for homogeneity. A working solution of 1 mg/mL letrozole in methanol was created by dissolving 50 mg of letrozole in 50 mL of methanol. A total of 100 µL of the 1 mg/mL solution was diluted in 900 µL of methanol for a final concentration of 100 µg/mL. The 100 ug/mL solution was further diluted to make eight working solutions ranging from 500 to 10,000 ng/mL in methanol. A total of 4.950 mL of whole blank milk was added to a 15 mL Falcon tube and 50 µL of each working solution was added to the respective tube to yield true concentrations ranging from 5 (lower limit of quantification; LLOQ) to 100 ng/mL (n=8 total standards; Table 2.1). Each standard was spiked with 250 µL of the internal standard, letrozole-d4 (200 ng/mL in methanol; Toronto Research Company,

Toronto, ON, Canada) to quantify against peak analyte area and gently inverted to mix. A volume of 5 mL of acetonitrile was added to each tube, and 2 g of $MgSO_4$ and 0.5 g of NaCl was subsequently added within 10 seconds. The tube was vigorously shaken by hand for 1 minute to prevent coagulation followed by centrifugation at 3700 x *g* for 5 minutes. A volume of 1 mL of the organic layer (top layer) was added to a 2 mL QuEChERS tube containing 25 mg C_{18} sorbent and 150 mg $MgSO_4$ (QuEChERS Dispersive Kit for Meat Residues; Agilent Technologies, Santa Clara, CA, USA). The tube was vortexed for 1 minute, centrifuged at 3000 x *g* for 3 minutes, and 500 µL of the organic layer was added to a 2 mL vial for auto-injection into the LC-MS/MS. Quality control (QC) samples were spiked independently from the standards in a similar manner at concentrations of 15, 25, and 80 ng/mL. Double blank (- letrozole, - letrozole-d4) and blank (- letrozole, + letrozole-d4) were prepared in the same manner. Standards, quality controls, and blanks were prepared once per day on three separate days.

Previously frozen milk samples derived from letrozole-treated cows were thawed at room temperature. For homogeneity, samples were vortexed and inverted for 1 minute. A total of 5 mL of milk was pipetted into a 15 mL tube, and 250 µL of letrozole-d4 (200 ng/mL) was added to each sample. Thereafter, samples were prepared in the same manner as the standards and quality controls as described above.

Separation by high-pressure liquid chromatography (HPLC; Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) was done with an analytical column (50 x 2.1 mm, 3 µm particle size, Thermo Scientific Betasil C18; Thermo Scientific, Waltham, MA, USA) at 35°C. The HPLC flow rate, solvent conditions and LC-MS/MS conditions were done as previously described (Joshi et al., 2011).

Linearity was tested by running three standard curves independently of each other on separate days. Regression coefficients were calculated, and ratio counts vs. concentration were plotted. To accept the linear curve, a regression coefficient (r^2) ≥0.99 was required. The mean (±SEM) r^2 obtained was 0.999 ± 0.0001. The acceptance criterion for each calculated standard concentration was 15% deviation from the expected value except for the LLOQ (5 ng/mL) where it did not deviate by >20%. The following formula was used for precision: RSD= Standard deviation x 100/mean. Accuracy was calculated by using the concentration found (from the standard curve) divided by the expected concentration. All points on the standard curve and quality control samples were accepted (Table 3.1 and 3.2). The limit of detection for letrozole in milk was 0.8 ng/mL, based on a signal of the analyte to background noise ratio of 3:1.

The terminal elimination half-life ($t_{1/2}$), maximum concentration (C_{max}), time to maximum concentration (T_{max}) and area under the curve (AUC_{last}) were determined for plasma and milk letrozole concentrations using a commercial pharmacokinetic software program (WinNonlin, Version 2.1; Pharsight Corporation, Mountain View, CA, USA). The terminal elimination half-life ($t_{1/2}$) was extrapolated using the formula: $\ln 2/\lambda$, where λ was derived based on the quantifiable concentrations taken *after* the LRD was removed on Day 4.

3.3.3. Radioimmunoassay

Plasma progesterone concentrations were measured by a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA, USA). The minimum detection limit was 0.1 ng/mL and the intra-assay coefficients of variations for low and high samples were 14.0 and 8.6%, respectively.

3.3.4. Statistical analyses

Data were analyzed using SAS Enterprise Guide (Version 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Serial data (concentration profiles in plasma and milk, dominant follicle and CL diameters) were compared by analysis of variance for repeated measures. Single-point measurements (interval to ovulation following device removal, ovulation synchrony (residuals) and ovulatory follicle diameter) were compared by t-test. The proportion of ovulations before GnRH on Day 6.5 was analyzed by chi-square. Correlation between milk letrozole concentrations and daily milk production was done by PROC CORR. Data are presented as mean ± SEM unless otherwise stated, and significance was defined as $P \leq 0.05$.

3.4. Results

Average milk production, fat and protein percentages, and letrozole milk concentrations from letrozole treated cows are shown in Table 3.3. The concentration of letrozole in milk fell below the LLOQ (5 ng/mL) within two days following LRD removal (Fig. 3.1). The concentration of letrozole in plasma tended (P=0.09) to be higher than in milk during treatment (Fig. 3.1). There was a negative correlation (r= -0.46; P<0.0001) between total milk produced and concentration of letrozole in milk. Plasma exhibited a higher maximum concentration (C_{max}) compared to milk (37.3 ± 1.9 vs. 27.3 ± 2.9 ng/mL, respectively; P=0.02; Table 3.4). There was no difference in time to maximum letrozole concentrations (T_{max}) between plasma and milk (2.3 ± 0.4 vs. 2.7 ± 0.3 days, respectively; P=0.52; Table 3.4). There was no difference in the elimination half-life ($t_{1/2}$) for letrozole between plasma and milk 14.6 ± 1.6 vs. 18.2 ± 1.4 hours, respectively; P=0.12; Table 3.4). There was no effect of treatment (P=0.23) or interactions (P=0.32) for the ovulatory follicle diameter during treatment between LRD and control cows (Fig. 3.2A). A treatment-by-day interaction in CL diameter during treatment was attributed to a

larger average CL diameter in the control group at the time of device insertion (P<0.01; Fig. 3.2B). The mean interval from device insertion to ovulation was not different between LRD and Control groups (mean 176 ± 11 hours; P=0.16), but the interval was less variable in the LRD group (residuals: 0 ± 0 vs. 42.7 ± 9.2 hours; P<0.001; Table 3.5). No effect of treatment or interactions were detected in post-ovulation CL diameter or plasma progesterone (Fig. 3.3)

Table 3. 1**.** Precision and accuracy data from LC-MS/MS derived standard curve for letrozole in bovine milk.

Concentration added (ng/mL)	Concentration determined (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
5 (LLOQ)	5.16 ± 0.20	4.0%	103.2%
7.5	7.99 ± 0.06	0.7%	106.6%
10	10.50 ± 0.2	1.9%	105.0%
20	19.40 ± 0.17	0.9%	97.0%
35	33.83 ± 0.42	1.2%	96.7%
50	49.77 ± 0.75	1.5%	99.5%
75	76.20 ± 1.32	1.7%	101.6%
100	93.90 ± 5.05	5.4%	93.9%

n=3 bovine milk samples per concentrations were used, except for the lower limit of quantification (LLOQ, n=5)

Table 3. 2**.** Precision and accuracy data of the LC-MS/MS method for quality control samples of letrozole in bovine milk (n=5 per sample).

Concentration added (ng/mL)	Concentration determined (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
15	13.82 ± 0.47	3.4%	92.1%
25	24.10 ± 1.09	4.5%	96.4%
80	77.98 ± 2.96	3.8%	97.5%

Table 3. 3**.** Total milk production, component percentage and concentration of letrozole determined in the milk by tandem mass spectrometry (mean ± SEM). Cows were given a letrozole releasing device immediately following milking on Day 0 for 4 days (n=6 cows).

Day	Milk produced (kg)	Fat %	Protein %	Letrozole in milk (ng/mL)
0	6.12 ± 0.91	8.10 ±0.83	4.27 ± 0.24	-
0.5	5.52 ± 0.76	7.76 ± 0.94	4.40 ± 0.30	15.98 ± 1.20
1	4.84 ± 0.61	7.42 ± 1.04	4.34 ± 0.31	19.90 ± 0.99
1.5	4.84 ± 0.85	8.10 ± 1.28	4.50 ± 0.39	24.18 ± 1.26
2	4.76 ± 0.66	7.62 ± 1.13	4.49 ± 0.47	25.55 ± 1.17
2.5	4.69 ± 0.64	7.87 ± 1.29	4.54 ± 0.44	25.32 ± 1.17
3	4.76 ± 0.70	8.23 ± 1.55	4.61 ± 0.55	25.83 ± 1.27
3.5	5.07 ± 0.62	8.07 ± 1.29	4.53 ± 0.47	22.83 ± 1.00
4	5.22 ± 0.62	7.31 ± 1.18	4.40 ± 0.45	19.43 ± 1.15
4.5	4.76 ± 0.56	6.92 ± 0.98	4.40 ± 0.41	12.39 ± 0.54
5	4.99 ± 0.51	6.36 ± 0.56	4.18 ± 0.31	8.24 ± 0.30
5.5	5.14 ± 0.51	6.25 ± 0.77	4.18 ± 0.33	6.08 ± 0.28
6	5.22 ± 0.53	6.23 ± 0.50	4.15 ± 0.31	Below lower limit of quantification

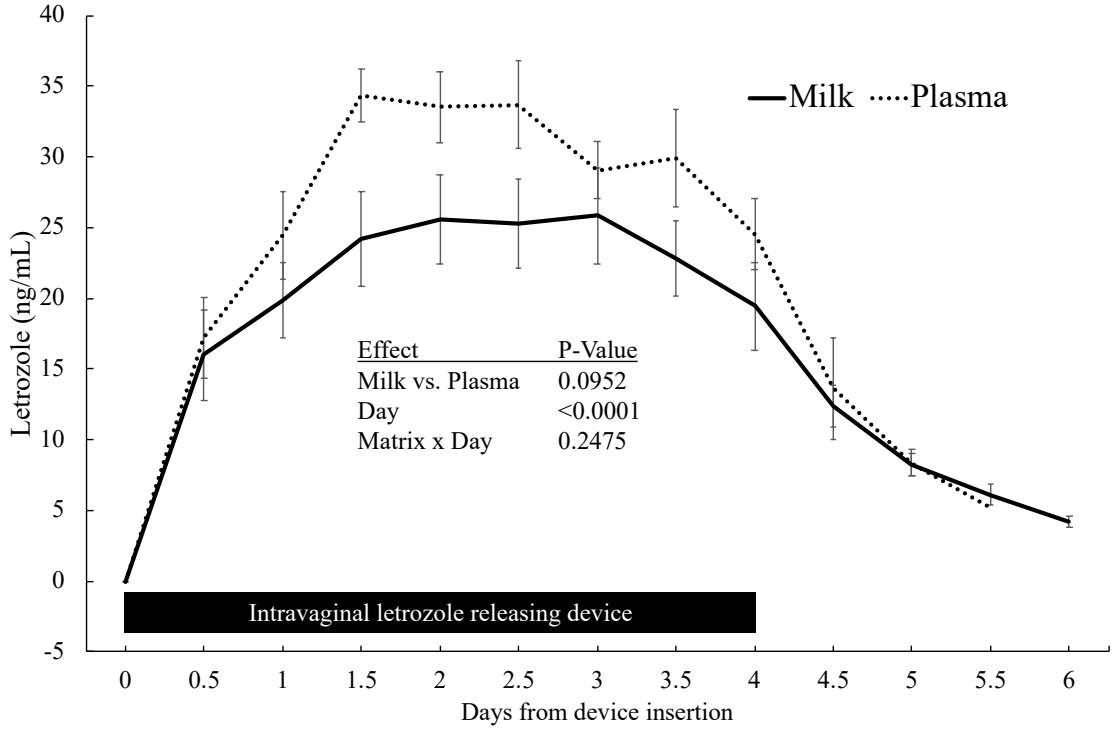

Figure 3. 1**.** Concentrations of letrozole (mean ± SEM) in milk and plasma in lactating Holstein cows treated for 4 days with an intravaginal letrozole releasing device (n=6/sample). Letrozole concentrations were quantified by a tandem mass spectrometry with a lower limit of quantification of 5 ng/mL in milk.

Table 3. 4**.** Pharmacokinetic data (mean ± SEM) from lactating Holstein cows treated for 4 days with an intravaginal letrozole releasing device. Elimination half-life ($t_{1/2}$) was determined from letrozole concentrations after the device was removed on Day 4.

Endpoint	Plasma	Milk	P-Value
T_{max} (days)	2.3 ± 0.4	2.7 ± 0.3	0.52
C_{max} (ng/mL)	37.3 ± 1.9	27.3 ± 2.9	0.02
$T_{1/2}$ (hours)	14.6 ± 1.6	18.2 ± 1.4	0.12
AUC_{last} (hours x ng/mL	3008 ± 234	-	-

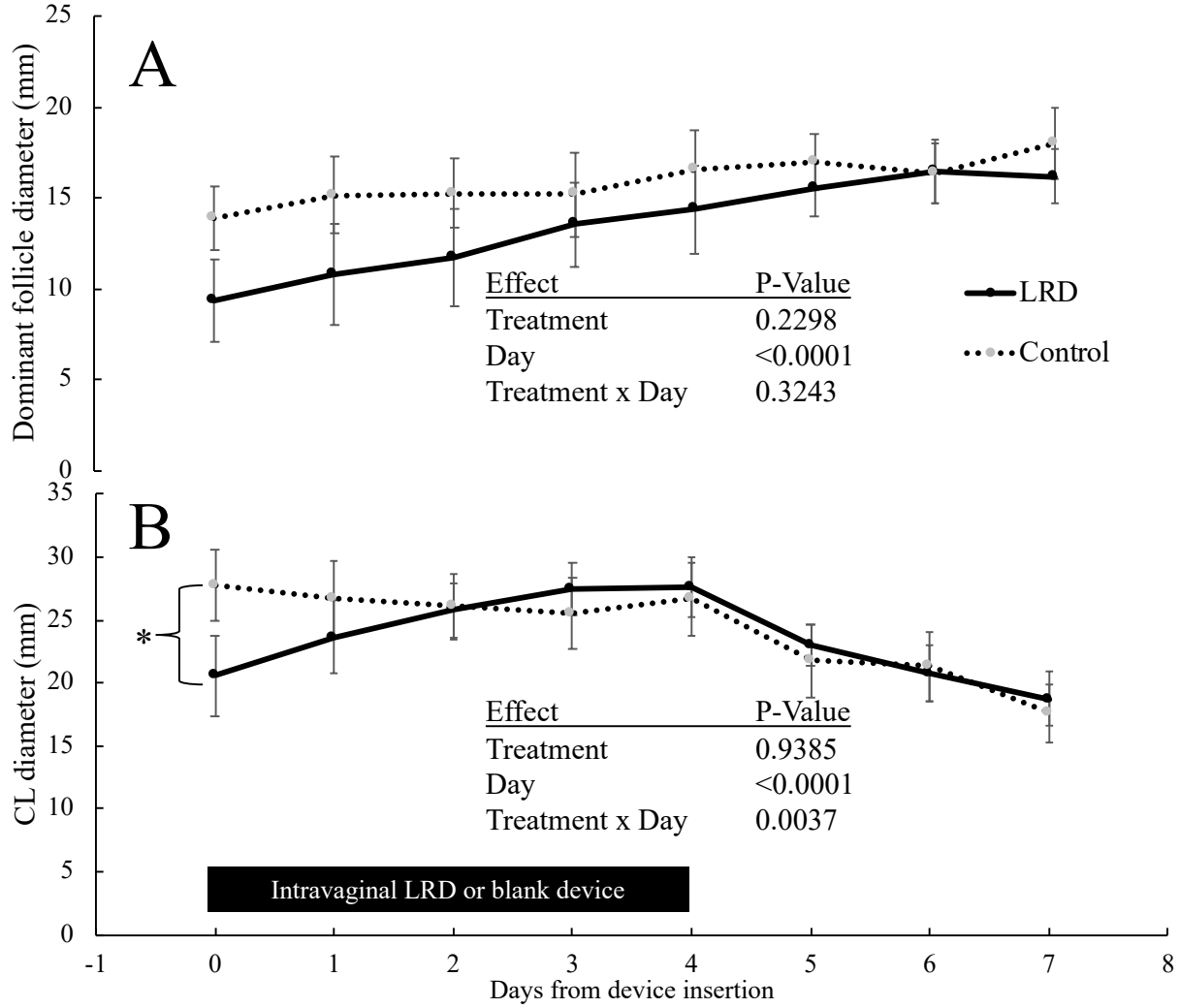

Figure 3. 2**.** Mean (± SEM) ovulatory follicle diameter (A) and CL diameter (B) in lactating Holsteins given either a letrozole releasing device (LRD) or a blank intravaginal device (Control) for 4 days (n=6/group). Cows were given 500 µg cloprostenol im on Day 4 and 4.5 followed by 100 µg gonadorelin im on Day 6.5. *Values tended to be different (P=0.10)

Table 3. 5**.** Ovulation data (mean ± SEM) from cows treated with either an intravaginal letrozole releasing device (LRD) or a blank intravaginal device for 4 days (n=6/group). The device was removed on Day 4 and 500 µg cloprostenol was given on Day 4 and 4.5 followed by 100 µg gonadorelin (GnRH) im on Day 6.5.

Endpoint	LRD	Control	P-value
Time of ovulation (hours after device insertion)	192 ± 0	160 ± 21	0.16
Ovulation synchrony (residuals; hours)	0 ± 0	42.7 ± 9.2	<0.001
Ovulated before GnRH (Day 6.5)	0/6 (0%)	2/6 (33.3%)	0.97
Ovulatory follicle diameter (mm)	16.2 ± 1.5	19.5 ± 1.6	0.16

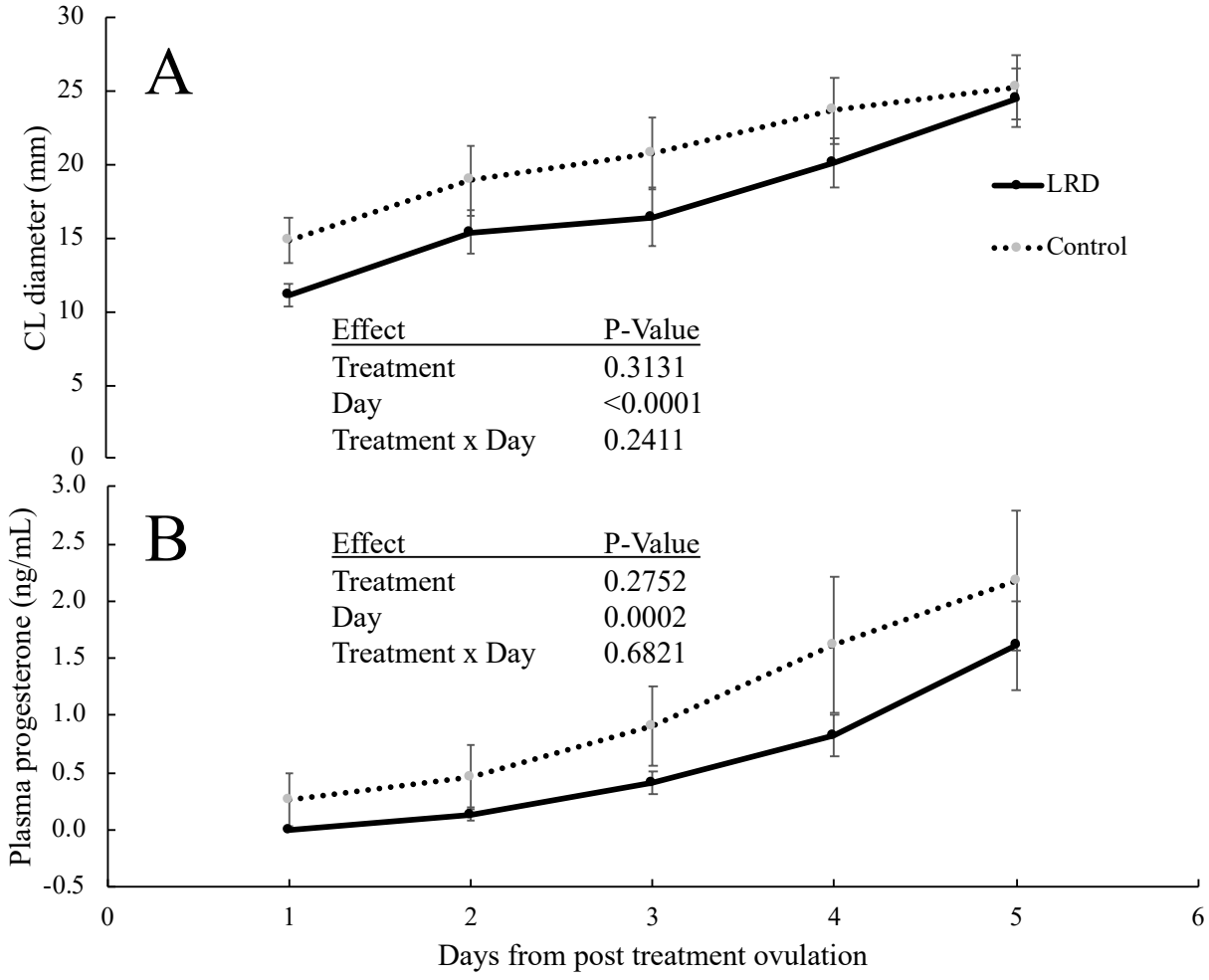

Figure 3. 3**.** Mean (± SEM) CL diameter (A) and plasma progesterone concentration (B) following post-treatment ovulation (mean ± SEM) in cows treated for 4 days with either an intravaginal letrozole releasing device (LRD) or a blank device (control).

3.5. Discussion

Evidence that letrozole is secreted into milk following vaginal delivery is an important consideration for the continued development of its use for synchronization in dairy cattle. Our quantitative analysis showed that milk letrozole fell below the LLOQ (5 ng/mL) within two days following LRD removal, and this rapid elimination provides optimism for its future use in lactating dairy cattle. Limited data on aromatase inhibitors for use in food-producing species makes it difficult to speculate on the tolerance (maximum residue level) for letrozole. However, an acceptable daily intake could be established from toxicity studies done in rodents. If a maximum residue limit of 5 ppb (ng/mL) for letrozole was established, results of the present study suggest that a 4-day LRD regimen would result in a total withdrawal period of 6 days (12 milking sessions). At current Canadian milk prices (August 2019; Dairy Farmers of Ontario, 2019), a cow producing 40 kg of milk per day at 3.5%, 3.0%, and 5.65% fat, protein and other solids, respectively would result in a loss of $28.95 CAD per day or $173.71 CAD for a 6-day withdrawal period. Caution should be exercised when evaluating elimination time of letrozole as low producing, cull cows were used in the present study (average 9-11 kg/day), and there was a strong negative correlation between milk production and milk letrozole concentration. For the endogenous steroid progesterone, metabolic clearance rate is highly correlated to liver blood flow (R=0.92) and liver blood flow was positively correlated to dry matter feed intake (Sangsritavong et al., 2002). Similarly, in pasture-based cows treated with a intravaginal progesterone device, progesterone concentrations were lower in cows fed *ad libitum* compared to feed-restricted cows (Rabiee et al., 2001a), although no effect of circulating progesterone was observed between differing milk yields (Rabiee et al., 2001b). From these studies, it was hypothesized that elimination of steroids (predominantly progesterone) in dairy cows was

attributed to liver metabolism (from increased feed intake) rather than excretion into milk (Sangsritavong et al., 2002). As progesterone exhibits similar size and properties to letrozole (i.e., 314 vs. 283 g/mol; lipophilic), this method of elimination may also exist for letrozole. Future studies on the pharmacokinetics of letrozole in high producing dairy cows are warranted to test this hypothesis.

Feeding non-saleable milk following letrozole treatment to calves may be a viable option; however, an understanding of the potential effects of lowering circulating estrogen concentrations in calves will be important. If calves were fed 6-8 L of milk daily from the period of maximum letrozole concentration found in the present study (~25 ng/mL), they would consume approximately 150 to 200 μg of letrozole per day. In post-menopausal women, the daily oral dose of letrozole is 2.5 mg, but 100 μg /day resulted in a 95% suppression of plasma estradiol and estrone from baseline within two weeks of treatment (Demers, 1994). Secretion of LH is regulated in part by the negative feedback of estradiol in calves. In one study, plasma LH concentrations were measured in bull calves that were either castrated, castrated plus given an estradiol implant, or left intact at 6 weeks of age. The castrated plus estradiol group exhibited the lowest mean LH concentration from 1 to 7 weeks following castration (Wise et al., 1987). In a study examining the effect of GnRH treatment in 4- to 6-week-old bull calves, it was concluded that LH (and subsequent androgen production) was necessary for initiating testicular development and sperm production following puberty (Chandolia et al., 1997). Similarly, heifer calves treated twice daily from 4- to 6-weeks of age with GnRH had a reduced age at puberty of six weeks compared to control heifers (Madgwick et al., 2005). Therefore, suppression of estradiol by giving letrozole may lead to an increase in circulating LH concentrations in prepubertal calves which may shorten the interval to puberty.

In comparison to our previous studies (Yapura et al., 2012, 2013, 2015), letrozole failed to produce a larger preovulatory follicle in the present study. This may have been due to the difference in milk production between the LRD and control groups, or due to lower plasma letrozole concentrations compared to previous studies (Yapura et al., 2015). Previous studies indicated a positive correlation between the size of the preovulatory follicle and milk production (r=0.55; Lopez et al., 2005). This may result from the reduced plasma concentrations of progesterone seen in high producing cows (Lopez et al., 2005), which has been associated with increased LH pulsatility (Adams et al., 1992a). This may have confounded the comparison in the present study since LRD-treated cows produced approximately 25% of total milk compared to controls (i.e., 11 kg vs. 43 kg/day).

The plasma elimination half-life of lactating Holsteins in the present study was approximately half of that reported for nulliparous beef heifers (i.e., 15 vs. 33 hours; Yapura et al., 2014). A similar increase in clearance rate for lactating dairy cows has been observed with the use of the antibacterial agent, enrofloxacin (fluoroquinolones; Idowu et al., 2010). When treated at 5 mg/kg body weight iv, dairy cows have a shorter elimination half-life than beef steers (i.e., 3.7 vs. 5.2 hours). In humans, approximately 60% of letrozole is protein bound to albumin (Requena et al., 2008), thus, protein binding may explain the shorter elimination half-life in lactating cows. The 40% of letrozole unbound to albumin may be transferable in milk in lactating cows. The degree of drug transfer into breast milk in women is indirectly influenced by the drug's protein binding ability (i.e., ibuprofen is 99% protein-bound in plasma, with limited diffusion into breast milk; Hotham and Hotham, 2015; Hale and Rowe, 2017). Conversely, lipid-soluble drugs may dissolute into the fat droplets of milk as seen with citalopram in women (Rampono et al., 2000). In humans, metabolism of letrozole has been shown to occur in the liver

by the CYP-450 isoenzymes, CYP3A4 and CYP2A6, into inactive carbinol metabolites (Bhatnagar, 2007). Letrozole and its metabolites are excreted mainly via urine (~90%; U.S. Food and Drug Administration, 2007). Additional studies are needed to better understand elimination and metabolism mechanisms of letrozole and its metabolites in milk, plasma, and urine in high-producing lactating dairy cows.

In summary, results of the present study document the letrozole milk residue profile after treatment of lactating dairy cows with an intravaginal letrozole-releasing device using LC-MS/MS. Maximum letrozole concentrations were greater in plasma than in milk, but no difference in elimination half-life was observed. This study confirmed that letrozole is excreted into milk and can be used for future studies in the veterinary approval process of letrozole as a novel estrous synchronization strategy in cattle.

3.6. Acknowledgements

The authors thank the animal care staff at the Western College of Veterinary Medicine and the Rayner Research Dairy Farm for their care of the cows in the study. We thank Susan Cook, Kim Tran, and Danielle Carriere at the University of Saskatchewan for assistance with radioimmunoassays. Special thanks to Carlos Leonardi and Rodrigo Carrasco for their assistance with cow handling and data collection. The research was supported by grants from Alberta Agriculture and Forestry, Venco Animal Health, a subsidiary of Dechra Pharmaceuticals PLC, and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 4

4. THE BOVINE MODEL TO TEST THE OVARIAN SUPEROVULATORY EFFECTS OF LETRZOLE

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Relationship of this study to the dissertation

Results of previous studies suggest that letrozole increased the superstimulatory response in humans. The hypothesis for the apparent increased response was that letrozole increased the expression of follicle-stimulating hormone-receptors in the ovary. Using the bovine model, we used an intravaginal LRD developed in Chapter 2 to test the hypothesis that treatment with letrozole and FSH will increase the superstimulatory response and embryo production in cows.

Authors' Contributions

As first author, EMZ participated in designing the experiment, manufacturing of intravaginal devices, collecting and analyzing data, performing mass spectrometry, and in writing and revising the manuscript. RJM assisted in experimental design, interpretation of data, and in revising the manuscript. As senior author, GPA provided the overall intellectual knowledge, experimental design, analysis and interpretation of data, as well as revising the manuscript.

4.1. Abstract

The aromatase inhibitor, letrozole, has been used in the treatment of infertility in women by inducing mild ovarian superstimulation or augmenting the ovarian response to FSH treatment. The effect has been attributed to an apparent upregulation of FSH-receptors on granulosa cells as a result of increased androgen concentrations. The objective of the study was to determine if treatment with the aromatase inhibitor, letrozole, will increase the number of antral follicles present at wave emergence, and if letrozole will potentiate the superovulatory response during gonadotropin treatment using a bovine model. In Experiment 1, sexually mature beef heifers (n=8) at random stages of the estrous cycle underwent ultrasound-guided transvaginal ablation of all follicles ≥5 mm to synchronize emergence of a new follicular wave. On Day 0 (wave emergence), the number of antral follicles ≥2 mm were counted, and an intravaginal letrozole-releasing device (LRD) was placed intravaginally. On Day 5, the LRD was removed, and transvaginal follicle ablation was done to permit antral follicle count at the subsequent wave emergence (Day 6.5). In Experiment 2, non-lactating Holstein cows (n=30) were given two luteolytic doses of prostaglandin 12 hours apart and scanned daily by transrectal ultrasonography to detect ovulation. Transvaginal follicle ablation was done 5 to 8 days after ovulation to synchronize wave emergence, and either an LRD or a blank device was placed for 5 days (n=15/group). On the expected day of wave emergence (Day 0), cows in both groups were given 8 doses of 50 mg pFSH im at 12-hour intervals, and prostaglandin on Days 3 and 3.5. The vaginal devices were removed at the time of the second prostaglandin treatment, and paint was applied to the tailhead to facilitate the detection of estrus. On Day 5, cows were given GnRH (100 μg gonadorelin) im and artificially inseminated 12 and 24 hours later. The ovaries were examined by ultrasonography to record the follicular and luteal response, and ova/embryos were

collected transcervically on Day 12. In Experiment 1, there was no difference in the number of follicles at wave emergence before vs after letrozole treatment (23.2 ± 3.2 vs. 23.5 ± 3.8 follicles; P=0.67). In Experiment 2, there was no treatment effect or treatment-by-day-interaction on the number of follicles >8 mm on Days 3.5, 5 and 6.5. Plasma estradiol concentrations on Day 3.5 were lower and estrus was exhibited later (50.3 ± 1.1 vs 40.7 ± 2.0 hours after first prostaglandin; P<0.001) and with less variance (residuals, 3.1 ± 0.5 vs 6.7 ± 0.9 hours; P<0.01) in the LRD than the sham group, respectively. The number of corpora lutea (CL) on Day 6.5 was lower in the letrozole vs sham group (9.1 ± 1.1 vs 12.3 ± 1.1; P=0.05). The total number of ova/embryos collected per cow did not differ (5.0 ± 1.9 vs 5.4 ± 1.8; P=0.75), but the proportion of transferable embryos tended to be lower (18/30 [60.0%] vs 43/54 [79.6%]; P=0.06) in the letrozole versus sham group, respectively. In summary, LRD treatment did not increase the number of antral follicles at wave emergence and did not improve the superovulatory response or numbers of ova/embryos recovered in the bovine model. Letrozole treatment resulted in more synchronous estrus which may be used in the design of a fixed-time artificial insemination protocol following superstimulatory treatment in cattle.

4.2. Introduction

Letrozole is a third-generation non-steroidal aromatase inhibitor that specifically and reversibly inhibits the enzyme $P450_{aromatase}$ in a dose-dependent manner (Hong and Chen, 2006). The aromatase enzyme is essential for the conversion of androstenedione and testosterone into estrone and estradiol, respectively. Commercially available letrozole (FEMARA®) is labeled for use in hormone-dependent breast cancer in post-menopausal women (Health Canada, 2005);

however, several reports have been published on the use of letrozole in women undergoing ovarian superstimulation for the treatment of subfertility (Mitwally and Casper, 2002; Garcia-Velasco et al., 2005).

Although not critically examined, the putative effect of letrozole in the treatment of subfertility in women is based on the concept that estradiol suppresses gonadotropin release through negative feedback effects on the hypothalamo-pituitary axis. The reduction of circulating estradiol by means of aromatase inhibition is thought to relieve the suppressive effects on follicle-stimulating hormone (FSH) release, thus stimulating the development of more than one ovarian follicle to a pre-ovulatory size (Requena et al., 2008). While this hypothesis remains to be tested in women, a second mechanism has been proposed for the apparent superovulatory effect of aromatase inhibitor treatment based on an increase in intrafollicular concentrations of testosterone and androstenedione as a result of reduced conversion of androgens to estrogens (Garcia-Velasco et al., 2005). In addition, 3-10 days of androgen exposure resulted in increased FSH-receptor expression in granulosa cells (Weil et al., 1998, 1999) of non-human primates, and in knock-out mice, androgens attenuated follicular atresia and enhanced FSH-receptor expression, which then augmented FSH-mediated follicle growth (Sen et al., 2014). Long-term androgen priming has also been found to improve *in vitro* fertilization (IVF) outcome in poor-responder human patients (Moawad and Shaeer, 2012), and daily co-administration of letrozole and FSH during ovarian stimulation improved the IVF outcome compared to gonadotropin treatment alone (Haas et al., 2017).

The bovine model has been used to characterize ovarian follicular dynamics in women and to test mechanistic hypotheses related to the control of follicular growth and ovulation (Adams et al., 2012; Baerwald et al., 2012). In a series of studies using the bovine model to

examine the effects of aromatase inhibitors, there was no support for the hypothesis that treatment results in an increase in circulating FSH; however, through an elevation in circulating LH, treatment altered follicular wave dynamics in a fashion that may be useful for ovulation synchronization in programmed breeding programs (Yapura et al., 2018). To-date, the effects of aromatase inhibitor treatment as an adjunct to gonadotropin treatment for ovarian superstimulation have not been reported.

Using the bovine model, the objectives of the present study were to determine if treatment with the aromatase inhibitor, letrozole, will increase the number of follicles present at wave emergence (Experiment 1) and if letrozole will potentiate the superovulatory response following FSH treatment (Experiment 2).

4.3. Materials and Methods

Experimental protocols were approved by the University of Saskatchewan's Animal Research Ethics Board and were conducted in accordance with the guidelines of the Canadian Council on Animal Care (Animal Use Protocol: #20150076).

4.3.1. Experiment 1

Sexually mature, Hereford-cross heifers (n=8) maintained at the University of Saskatchewan's Livestock & Forage Centre of Excellence were used during April. Heifers weighed 398 ± 24 kg (mean ± SEM) and were allowed free access to water and fed a combination of barley silage and dry alfalfa hay to maintain a body condition score of 3.0 (Scale 1-5, Richards et al., 1986). Prior to enrolment in the study, the ovaries were examined by transrectal ultrasonography to confirm the presence of a corpus luteum (CL; i.e., post-pubertal).

At random stages of the estrous cycle, transvaginal ultrasound-guided follicle ablation was done by aspiration of follicles ≥5 mm in diameter in both ovaries (Bergfelt et al., 1994) using a 5-8 MHz convex-array transvaginal probe (MyLab Alpha, Esaote North America, Fishers, IN, USA) equipped with a disposable 18-ga needle (BD, Mississauga, Ontario, Canada) attached to a 6 mL syringe by silicone tubing (60 cm long x 1.14 mm internal diameter; Cole-Palmer, Montreal, Quebec, Canada). Based on previous studies (Bergfelt et al., 1994), 36 hours after ablation was expected to be the day of new follicular wave emergence (Day 0) and was the day on which a silicone intravaginal letrozole-releasing device (LRD) was inserted (Chapter 2). On Day 5, the LRD was removed, and heifers underwent follicular ablation in the same manner as previously described. The ovaries were examined by transrectal ultrasonography on Day 0 (pre-treatment wave emergence) and Day 6.5 (post-treatment wave emergence) using a 7.5 MHz linear-array probe (MyLab Alpha, Esaote North America, Fishers, IN, USA). The size and relative position of follicles ≥2 mm in diameter were recorded on an ovary sketch sheet. Blood samples were collected daily from Day 0 to 5 and on Day 6.5 by jugular venipuncture into heparinized vacutainer tubes (BD, Mississauga, ON, Canada), and centrifuged at 1500 x *g* for 15 minutes; the plasma was aliquoted and stored frozen at -20ºC. The experimental timeline is displayed in Figure 4.1.

Plasma was analyzed for concentrations of letrozole using LC-MS/MS, as previously described (Chapter 2). Briefly, plasma samples were thawed at ambient temperature and, 100 µL were transferred to a 1.5 mL microcentrifuge tube; the samples were spiked with 50 µL internal standard (200 ng/mL letrozole-d4), and 250 µL of acetonitrile was added, and samples were centrifuged at 15000 x g at 4°C for 15 mins. A total of 200 µL of the supernatant layer was then transferred to a 2 mL autosampler vial and loaded into the LC-MS/MS apparatus for analysis.

4.3.2. Experiment 2

Mature non-lactating, Holstein cows (n=30; age 3 to 10 years) were maintained in outdoor pens at the Western College of Veterinary Medicine, University of Saskatchewan in May. Cows were fed alfalfa hay with free access to water and weighed 763.3 ± 0.7 kg with a body condition score of 2.6 ± 0.1 (Scale 1-5; (Ferguson et al., 1994)). At random stages of the estrous cycle, cows were given two luteolytic doses of prostaglandin (PGF; 500 µg cloprostenol; Vetoquinol N.A., Lavaltrie, QC, Canada) im 12 hours apart and examined daily by transrectal ultrasonography to detect ovulation. On the day of ovulation, antral follicles ≥3 mm were counted and recorded on an ovarian sketch sheet, and cows were ranked numerically by the number of antral follicles ≥3 mm counted on the day of ovulation (Singh et al., 2004). The ranking was used to distribute cows of similar rank evenly (by lottery) between two treatment groups (n=15 per group). At 5 to 8 days post-ovulation, cows underwent ultrasound-guided transvaginal ablation of follicles ≥5 mm and given either an intravaginal LRD or a blank sham device (progesterone-free Cue-Mate device, Bayer Australia, Pymble, NSW, Australia).

Beginning on Day 0 (wave emergence), cows in both groups were given 8 doses of 50 mg pFSH (Folltropin; Vetoquinol N.A.; Lavaltrie, QC, Canada) im at 12-hour intervals. A luteolytic dose of PGF (500 µg cloprostenol im; Bioestrovet; Vetoquinol N.A.; Lavaltrie, QC, Canada) was given on Days 3 and 3.5. The vaginal device was removed, and paint was applied to the tail-head to facilitate estrus detection (Reveal; Cooperative Resources International, Shawano, WI, USA) on Day 3. On Day 5, gonadotropin-releasing hormone (GnRH; 100 µg gonadorelin im; Fertiline, Vetoquinol N.A., Lavaltrie, QC, Canada) was given and artificial insemination was done using proven fertile semen (Semex; Guelph, ON, Canada) 12 and 24

hours later. Estrous behavior was monitored at 8-hour intervals from Day 3.5 to Day 6. Cows were considered in estrus if observed standing to be mounted or if the tail paint was missing at subsequent observation. The ovaries were examined by transrectal ultrasonography on Days 0, 3.5, 5, 6.5, and 12 to record the follicular and luteal response. Ova/embryos were recovered by a nonsurgical transcervical technique by inexperienced embryo transfer course participants by transcervical uterine flush on Day 12 and evaluated for quality (1 to 3: transferable; 4: degenerate; infertile) by IETS standards (Robertson and Nelson, 2010). The experimental timeline is depicted in Figure 4.2.

Blood samples were collected by jugular venipuncture into heparinized 10 mL vacutainer tubes (BD, Mississauga, ON, Canada) on Day -1.5, 0, 3, 5, and 6.5, centrifuged at 1500 x g for 15 minutes and the plasma was aliquoted and frozen at -20°C. Plasma estradiol concentrations were measured by radioimmunoassay at Texas A&M University, as previously described (Kirby et al., 1997). The minimum detection limit was 0.5 pg/mL and the intra- and inter-assay coefficients of variation were 1.6 and 1.5%, respectively.

4.3.3. Statistical analyses

Data were analyzed using SAS Enterprise Guide (Version 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Nominal data were compared between groups by paired t-test and by analysis of variance for repeated measures. Binomial data were compared by chi-square test or GLIMMIX. Data are presented as mean±SEM unless stated otherwise, with significance being defined as $P \leq 0.05$.

4.4. Results

4.4.1. Experiment 1

There was no difference in the number of follicles ≥2 mm at the time of wave emergence before vs after letrozole treatment (23.2 ± 3.2 vs. 23.5 ± 3.8 follicles; P=0.67). Mean plasma letrozole concentrations are shown in Figure 4.1.

4.4.2. Experiment 2

The number of follicles detected at post-ablation wave emergence did not differ between letrozole- and sham-treated groups (P=0.68, Table 4.1). The proportion of letrozole-treated cows that exhibited estrus tended to be lower than sham-treated cows (P=0.07; Table 4.1). However, letrozole-treated cows exhibited estrus later following PGF treatment than sham-treated cows (P<0.001; Table 4.1) and the interval to estrus was less variable (residuals; P<0.01; Table 4.1). The proportion of ovulations (number of CL on Day 12 over the number of follicles ≥3 mm per cow on Day 0) was not different between groups (P=0.15; Table 4.1). The number of ovulations by Day 6.5 was lower in the letrozole than the sham group (P=0.05; Table 4.1), although the number of CL on the day of embryo recovery did not differ (P=0.32; Table 4.1). The total number of ova/embryos collected (P=0.75) and the number of transferable embryos (P=0.56) per cow did not differ between letrozole and sham groups (Table 4.1). There was a tendency for a treatment-by-day interaction for the number of follicles >8 mm due to an increase on Day 5 in the sham group (P=0.08; Figure 4.3A). There was no treatment effect or treatment-by-day-interaction for the number of follicles ≤8 mm on Day 3.5, 5, and 6.5 (Figure 4.3B). A treatment-

by-day-interaction was observed for plasma estradiol during treatment (P=0.04), which was attributed to lower estradiol on Day 3 in the LRD group (Figure 4.4).

No ova/embryos were recovered from 9 cows in the LRD group and 5 cows in the sham group, so comparisons were made based on n=6 and n=10 in the respective groups (Table 4.2). The number of quality 1 embryos was proportionally lower in the letrozole than the sham group (P=0.02). The proportion of transferable embryos tended to be lower (P=0.06), and the proportion of degenerate embryos tended to be higher (P=0.08) in the letrozole vs. sham group. No differences were detected between groups in the proportion of Quality 2 or 3 embryos or unfertilized ova.

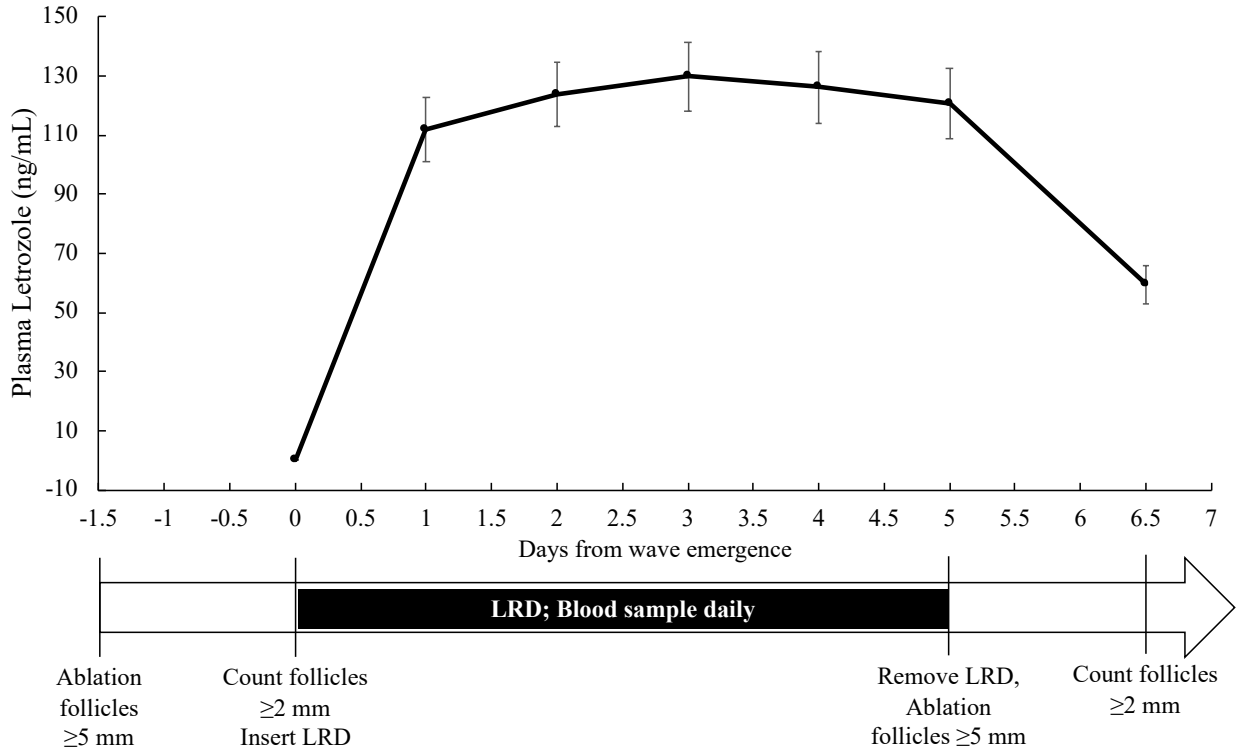

Figure 4. 1. Mean (± SEM) plasma letrozole concentrations in beef heifers (n=8) treated with a letrozole releasing device (LRD) for 5 days (Experiment 1).

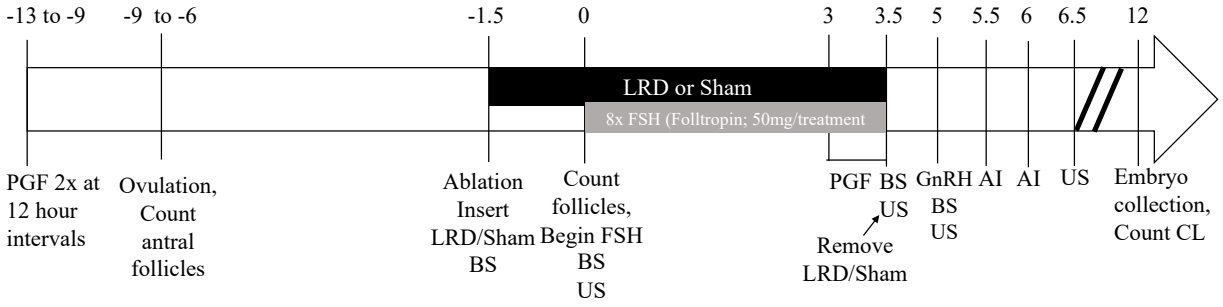

Figure 4. 2**.** Experimental timeline for Experiment 2 in which Holstein cows were given either a letrozole releasing device (LRD) or a sham device and were superstimulated with follicle stimulating hormone (FSH). PGF: 500 µg cloprostenol; BS: blood sample; US: transrectal ultrasonography; GnRH: 100 µg gonadorelin; AI: artificial insemination; CL: corpora lutea

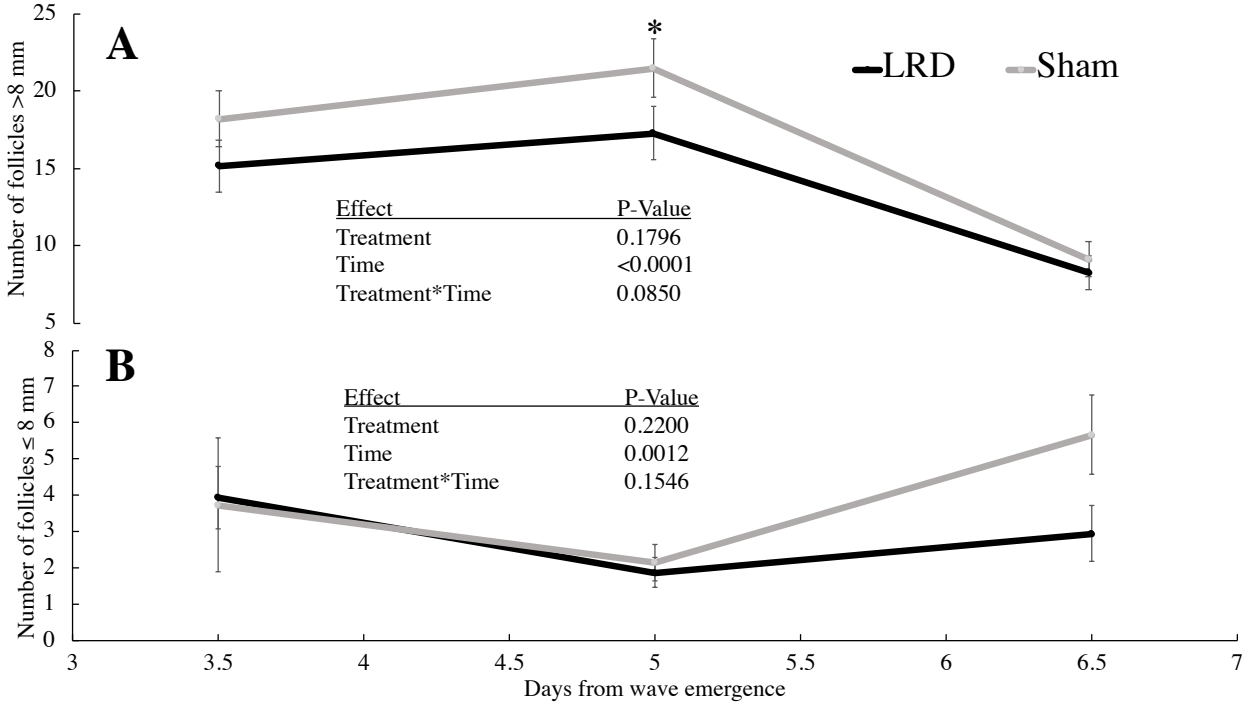

Figure 4. 3**.** Mean (± SEM) number of follicles >8 mm (A) and ≤8 mm (B) in which Holstein cows were given either a letrozole releasing device (LRD) or a sham device and were superstimulated with follicle stimulating hormone (FSH; Experiment 2). *P=0.10

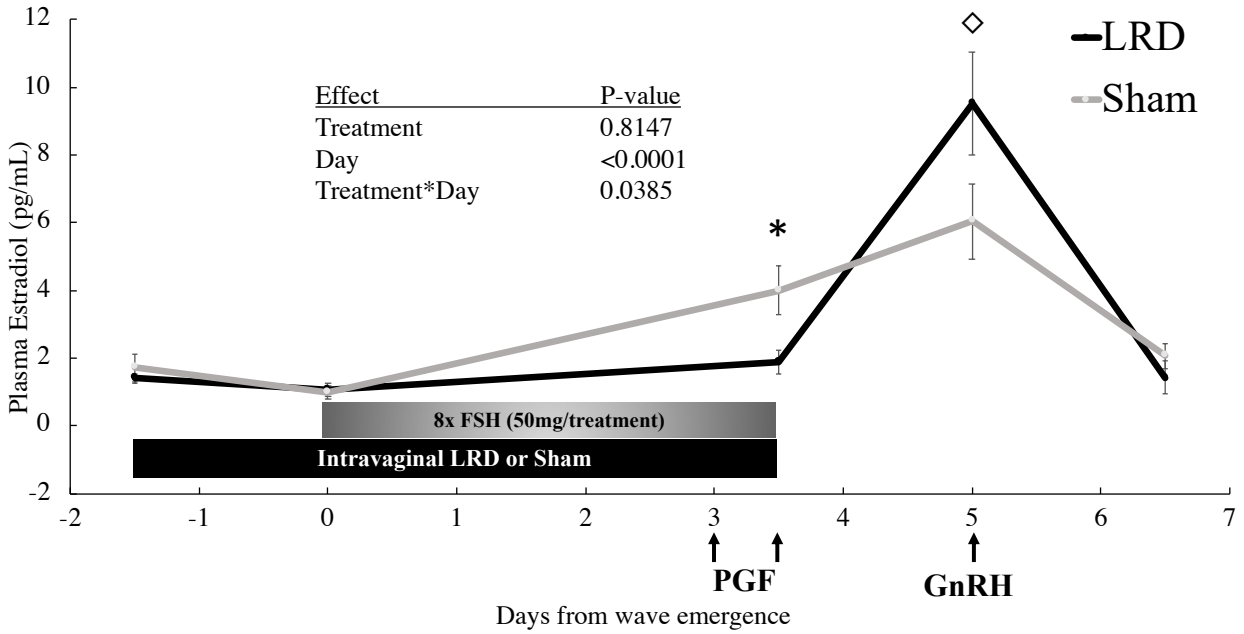

Figure 4. 4**.** Mean (± SEM) plasma estradiol concentrations in which Holstein cows were given either a letrozole releasing device (LRD) or a sham device and were superstimulated with follicle stimulating hormone (FSH; Experiment 2). PGF: 500 ug cloprostenol; GnRH: 100 ug gonadorelin; *P=0.01 ◇P=0.10

Table 4. 1**.** Follicular, luteal, estrus, and embryo responses (mean ± SEM) in which Holstein cows were given either a letrozole releasing device (LRD) or a sham device and were superstimulated with follicle stimulating hormone (FSH; Experiment 2).

	LRD	Sham	P-value
Cows (n)	15	15	-
Follicles ≥3 mm at wave emergence (Day 0)	24.6 ± 3.1	26.5 ± 3.3	0.68
Proportion of cows exhibited estrus	12/15 (80%)	15/15 (100%)	0.07
Interval to estrus from 1st PGF (hrs)	50.3 ± 1.1	40.7 ± 2.0	<0.001
Variance in interval to estrus (hrs)	3.1 ± 0.5	6.7 ± 0.9	<0.01
Proportions of ovulations [x]	0.65 ± 0.02	0.70 ± 0.02	0.15
Ovulations by Day 6.5	9.1 ± 1.1	12.3 ± 1.1	0.05
Number CL day of collection	15.9 ± 2.5	19.0 ± 2.0	0.32
Transferable embryos recovered [y]	3.0 ± 1.2	4.3 ± 1.5	0.56
Infertile ova/degenerate embryos [y]	2.0 ± 0.8	1.1 ± 0.4	0.29
Total structures recovered [y]	5.0 ± 1.9	5.4 ± 1.8	0.75

[x]Total number of corpora lutea (CL) on day of embryo collection over the total number of follicles ≥3 mm at wave emergence (Day 0)

[y]Data derived from cows in which structures were successfully recovered following transcervical uterine flush (n=6 LRD and n=10 Sham)

Table 4. 2**.** Ova/embryo recovery results following superovulation and transcervical recovery in cows treated with an intravaginal letrozole-releasing device (LRD) or a sham device (Experiment 2).

	LRD	Sham	P-value
Number of cows	6	10	-
Total structures recovered	30	54	-
Quality 1	13/30 (43.3%)	38/54 (70.3%)	0.02
Quality 2	3/30 (10.0%)	3/54 (5.6%)	0.46
Quality 3	2/30 (6.7%)	2/54 (3.7%)	0.55
Total transferable embryos	18/30 (60.0%)	43/54 (79.6%)	0.06
Unfertilized ova	4/30 (13.3%)	5/54 (9.3%)	0.57
Degenerate	8/30 (26.7%)	6/54 (11.1%)	0.08

4.5. Discussion

The bovine model has been used extensively for the study of follicular dynamics in women (Adams and Pierson, 1995). It is difficult to compare the ova/embryo production rates

following stimulation between cattle and women, due to the fact that an increased number of follicles leads to a greater chance of ovarian hyperstimulation syndrome (OHSS) which can be life-threatening in women (Rova et al., 2012). Incidences of OHSS have been reported to increase significantly when more than 18 oocytes were recovered (Magnusson et al., 2018). Therefore, care is taken during superstimulation in women and maximizing the number of oocytes and embryos is not the only goal for the use of advanced reproductive technologies in women. In cattle, the main goal for the use of advanced reproductive programs is to maximize the quantity of transferable or freezable embryos from high-valued genetic donors. Gonadotropins (i.e., FSH) given for ovarian superstimulation to maximize embryo production results in substantial expenses for cattle producers. Most recent data show that 94,000 *in vivo* embryo collections and 112,000 oocyte aspirations for *in vitro* fertilization (IVF) were done worldwide in 2016 in cattle (Perry, 2017). A conservative estimate of $100 per superstimulatory treatment (Hasler, 2014) corresponds to over $20.6 million spent annually on superstimulation treatments in cattle. Therefore, cost-effective alternatives for gonadotropins are sought while still maintaining the production of high-quality embryos.

The use of letrozole in the present study did not increase the number of ova/embryos following superstimulation in cattle. In women with low antral follicle counts, letrozole treatment was associated with an increased in the number of oocytes retrieved, perhaps as a result of increased FSH receptor gene expression induced by an increase in intrafollicular androgens (Garcia-Velasco et al., 2005). In women, treatment with 1.25 mg/day of testosterone for 21 days decreased the dosage of FSH needed while increasing the number of oocytes retrieved following aspiration (Kim et al., 2011). Follicular fluid was not sampled in the present study, but the transient drop in plasma estradiol concentrations in letrozole-treated cows may not

have been sufficient to alter intraovarian androgen concentrations sufficiently to affect FSH receptor expression. The role of androgens in ovarian follicular dynamics is not well understood. Early research with monkeys revealed that short-term treatment with testosterone increased the number of preantral follicles (Vendola et al., 1998). Recent studies in mice indicated that testosterone decreased the expression of anti-Müllerian hormone (AMH) as well as increased the size of preantral follicles (Laird et al., 2017). Due to the inhibitory effect of on preantral follicle recruitment, as well as their responsiveness to FSH (Visser et al., 2006), it may be hypothesized that androgens control follicular development through a paracrine manner.

A shortcoming of the present study is the lack of frequent blood sampling (every 15 minutes for 8 hours) during superstimulation to quantify luteinizing hormone (LH) pulse-frequency. To the best of our knowledge, the low estradiol environment model during superstimulation on LH pulse frequency and its subsequent role in embryo quality has not been tested. As shown in a previous study (Yapura et al., 2011), letrozole treatment increased plasma LH concentrations due to the release of hypothalamic estradiol feedback. Therefore, in the present study, it may be hypothesized that LH profiles differed between groups. When pulses of LH were induced (with a small dosage of exogenous GnRH: i.e., 0.25 to 1 pulse per hour) during superstimulation with FSH, no difference was detected in the number of large follicles present at the time of induced LH surge. Following a bolus of GnRH given to induce a preovulatory LH surge, only 1 out of 5 cows ovulated and this was attributed to a decreased LH surge. The inhibition of the LH surge was unexpected and was hypothesized to be due to a desensitization of the pituitary gland by estradiol, GnRH or LH itself (Price et al., 1999). Although not investigated in the present study, the expected increase in LH in the letrozole group may have led to desensitization of the pituitary and thus, decreased numbers of ovulations. Pulse amplitude and

frequency of LH has been reported to be lower in superstimulated vs. unstimulated which was attributed to increased estradiol in superstimulated animals (Roberge et al., 1995). Individual follicles in superstimulated cattle exhibit lower levels of estradiol (although systemic estradiol was higher) present in the follicular fluid compared to unstimulated follicles (Assey et al., 1994). This difference of estradiol may contribute to the reduced developmental competence of superovulated oocytes. Therefore, as letrozole-treated cows in the present study were hypothesized to have lower follicular fluid levels of estradiol, this may explain the lower transferable embryo rate observed in this group. Although this conclusion should be exercised with caution, due to the low recovery rate of embryos by inexperienced practitioners in the present study.

For a superovulatory protocol to be successful in cattle, correct timing of artificial insemination (before ovulation) and subsequent *in vivo* fertilization must be achieved to maximize embryo numbers. In the present study, the onset of estrus following PGF was lengthened by approximately 10 hours but was more synchronous in the letrozole-treated group due to delayed estradiol release following LRD removal. No letrozole-treated cows exhibited estrus before 48 hours following PGF (range 48-56 hours) and three cows failed to exhibit estrus, even though superstimulation was achieved (i.e., ≥ 6 CL present on Day 12). Plasma estradiol was higher on Day 5 (i.e., 12 hours before insemination) in the letrozole group, which may benefit sperm transport following insemination (Hawk and Cooper, 1975). The interval to estrus (i.e., at the time of peak estradiol) has been shown to be variable among superstimulated cattle (D'Occhio et al., 1997), and ovulation has been reported to occur earlier in donors with ≥4 vs. <4 CL at the time of ova/embryo collection (Bo et al., 2006). Therefore, the timing of the endogenous LH surge and subsequent ovulations are also variable among superstimulated cattle.

Unlike for ovarian stimulation protocols in women, protocols for cattle do not generally involve the use of a GnRH antagonist or agonist (Depalo et al., 2012), although these substances have been shown to be efficacious in preventing the endogenous LH surge (D'Occhio et al., 1997). Therefore, without the use of GnRH-antagonists, AI before ovulation is essential for *in vivo* fertilization and subsequent embryo production in cattle.

With the knowledge attained from this study, future studies should investigate the effect of letrozole at a later stage during superstimulation. By increasing LH pulsatility, letrozole treatment immediately after follicular ablation may have affected early follicular growth. A regimen in which letrozole treatment is initiated when follicles are approximately 7 to 8 mm in diameter (i.e., LH-dependent follicles; Ginther et al., 2001) might increase superstimulation and subsequent ovulatory response. Due to the competition of multiple dominant follicles for LH, a higher number of follicles may continue to grow and eventually ovulate (Mapletoft et al., 2002). Due to its short half-life, FSH must be given once or twice per day to women and cattle, respectively (Mapletoft et al., 2002). Therefore, a superstimulation protocol could be developed with FSH given as a single dosage at the onset of wave emergence, followed by letrozole treatment 2 days later. The FSH may allow for simulation until the follicles reach the 7 to 8 mm size, then letrozole treatment would increase endogenous LH concentrations and continue to increase the size and ovulatory potential of the follicles.

In conclusion, the results of the present study involving the bovine model did not support the hypothesis that letrozole treatment potentiates the superovulatory effects of FSH treatment. Letrozole treatment was not associated with either an increase in the number of antral follicles at the time of wave emergence or in the ovarian superstimulatory response and ova/embryo production in cattle. However, letrozole treatment resulted in more synchronous estrus, which

may be useful in the design of a fixed-time artificial insemination protocol following superstimulatory treatment in cattle.

4.6. Acknowledgements

The authors thank Vetoquinol for the donation of hormones used in the experiments. We thank Laurence Boligon de Araujo, Marcos Colazo, Ana Rita Krause, Carlos Leonardi, Alix Nelson, and Will Lillico for assistance with cattle handling and data interpretation. The authors thank Ky Pohler, Sydney Reese, and Gessica Franco from Texas A&M University for estradiol assay assistance. The research was supported by grants from Alberta Agriculture and Forestry, Venco Animal Health, a subsidiary of Dechra Pharmaceuticals PLC, and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 5

5. THE EFFECT OF AN AROMATASE INHIBITOR ON THE OVARIAN - OOCYTE ENVIRONMENT IN BEEF HEIFERS

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Relationship of this study to the dissertation

In this chapter, we used an intravaginal letrozole-releasing device developed in Chapter 2 to test the effect of letrozole on the local ovarian environment and the ability of the cumulus-oocyte complexes to undergo *in vitro* maturation and *in vitro* fertilization. The hypothesis was that letrozole treatment would result in a lower proportion of cumulus-oocyte complexes that would undergo maturation and that letrozole treatment would result in a lower proportion of embryos produced.

Authors' Contributions

As first author, EMZ participated in designing the experiment, collecting and performing statistical analysis on data, performing mass spectrometry, and in writing and revising the manuscript. CJ performed reverse transcriptase polymerase chain reaction (RT-PCR). JS assisted in the experimental design and statistical analysis. MLZ assisted in data collection and revising the manuscript. RD assisted with RT-PCR, interpretation of data, and manuscript revisions. As senior author, GPA provided the overall intellectual knowledge, experimental design, analysis and interpretation of data, as well as revising the manuscript.

5.1. Abstract

The use of the aromatase inhibitor, letrozole has allowed for a new approach for ovarian follicular control in cattle. To date, little has been published on the effects of letrozole on the local ovarian environment and the cumulus-oocyte complex (COC). The objectives of this study were to characterize the local effect of letrozole on the ovary (Experiment 1), *in vitro* maturation of COC (Experiment 2), and *in vitro* embryo production (Experiment 3) in beef heifers. Three to four days after ovulation, ultrasound guided transvaginal follicle ablation was done to induce emergence of a new follicular wave 1 day later (wave emergence = Day 0) and heifers were given an intravaginal letrozole-releasing device (LRD) or a sham device for 4 days immediately after ablation. In Experiment 1, heifers were given a 4-day LRD or sham device and underwent follicle sampling either before the LH surge (i.e., immediately after device removal on Day 3) or after the LH surge on Day 5.5 (i.e., PGF on Day 3 followed by GnRH on Day 5) in a 2x2 factorial design. Follicular fluid was analyzed for letrozole concentrations by tandem mass spectrometry (LC-MS/MS), which was partially validated as a secondary objective of the study. Granulosa cell mRNA from dominant and subordinate follicles was analyzed for expression of HSD3B, LHCGR, STAR, INHBA, FSHR, CYP11A1, CCND2, NPR2, and CYP19A1 by polymerase chain reaction (PCR). In Experiment 2, a 2x2 crossover experimental design was used to compare a 4-day LRD or sham device followed by COC aspiration done on Day 4 and *in vitro* maturation of COC for 0 or 24 hours. The COC were then fixed and oocytes were examined for nuclear maturation. A subset of heifers underwent frequent blood collections to determine plasma LH secretion on Day 3 (i.e., device removal). In Experiment 3, heifers were given either a 4-day LRD or sham device followed by COC aspiration 1 day after device removal. The COC underwent *in vitro* maturation, fertilization, and culture and were evaluated for embryo

development. Continuous data were analyzed by t-test or ANOVA and proportional data were analyzed by chi-square. In Experiment 1, no effect of treatment was observed for gene expression from granulosa cells derived on Day 3. Follicular fluid concentrations of letrozole did not differ (P=0.37) between dominant and subordinate follicles, but a day effect was observed (P<0.001). A significant positive correlation was shown between plasma and follicular concentrations of letrozole at the time of sampling (R=0.9889, P<0.0001). In Experiment 2, the mean LH area under the curve (AUC) was greater in LRD vs sham device (148.2 ± 22.4 vs 74.2 ± 11.8 mins x ng/mL, respectively; P<0.01). A higher proportion of oocytes undergoing maturation for 24 hours in the LRD group continued to the MII stage compared to the other groups (P<0.05). In Experiment 3, the proportion of blastocysts over the total COC was greater in the LRD vs sham group (17/160 [10.6%] vs 9/209 [4.3%], respectively, P=0.02) but no difference was detected in embryo quality 7 days after fertilization. When follicular aspiration data were combined from Experiments 2 and 3, a lower number of total follicles (12.7 ± 0.7 vs 16.1 ± 1.0; P<0.01) and COC (6.3 ± 0.6 vs 8.8 ± 0.9; P=0.02) were collected in the LRD vs sham group, respectively. In summary, letrozole was detected in follicular fluid but did not affect granulosa cell function. Letrozole treatment increased the LH AUC and resulted in a greater proportion of oocytes in the MII stage following 24 hours of maturation. Finally, letrozole did not affect *in vitro* fertilization nor embryo quality, and thus, should not adversely affect fertility *in vivo*.

5.2. Introduction

Aromatase inhibitors have been used off-label for infertility treatment in women during the past 20 years (Mitwally and Casper, 2002). The non-steroidal aromatase inhibitor, letrozole,

acts by competitively binding to the heme group of the aromatase enzyme inhibiting the conversion of androgens to estrogens throughout the body (Chumsri et al., 2011). By lowering circulating estrogen concentrations, the hypothalamus is released from its negative feedback permitting a subsequent rise in gonadotropin release. Due to the pulsatile secretory pattern, gonadotropins are challenging to assess in women due to the requirement of frequent sampling.

Utilizing non-human models to test the effects of letrozole has allowed for a better understanding of the physiological role of letrozole in women experiencing infertility. In a mouse model, letrozole induced an ovarian response similar to polycystic ovarian syndrome (PCOS) in women (Kauffman et al., 2015). Treatment with the aromatase inhibitor, letrozole, reduced estrogen in follicular fluid (Haas et al., 2018) and serum (Pereira et al., 2016) and reduced the negative feedback of estrogens on the hypothalamus. As in women experiencing PCOS, treatment with letrozole in monkeys led to hypersecretion of luteinizing hormone (LH) followed by an overproduction of androgens from the theca cells (Kraynak et al., 2017). We have used the bovine model to study the effect of letrozole on ovarian function (reviewed in Yapura et al., 2018). To date, experiments in cattle have examined the effects of letrozole on follicular and luteal function (Yapura et al., 2013, 2014), synchronization of ovulation (Yapura et al., 2016), co-superstimulation with exogenous FSH ((Zwiefelhofer et al., 2019); Chapter 4), and development of a novel letrozole-impregnated silicone intravaginal device (Chapter 2). Reduced plasma estradiol concentrations following letrozole treatment in cattle have resulted in increased plasma LH concentrations (Yapura et al., 2011, 2012) which have resulted in an increase in dominant follicle diameter, lengthened interwave intervals, and increased progesterone concentrations following ovulation. To date, no studies have assessed the pulsatile secretory pattern of LH in response to letrozole treatment.

If hypersecretion of LH does occur following letrozole treatment, as has been shown in women with PCOS, early intrafollicular maturation or atresia of the cumulus-oocyte complex (COC) may also occur as seen persistent cystic follicles in dairy cows and buffalo (Hamilton et al., 1995; Raghu et al., 2002). In cattle, superstimulation with porcine FSH containing low concentrations of LH increased the ovulation rate and the number of recovered and transferable embryos compared to products with high LH content (Chupin et al., 1984). Chronic exposure of preantral follicles to LH has been shown to decrease expression of FSH-receptors which has led to reduced follicular growth in the rat (Orisaka et al., 2013). Whether LH is beneficial or detrimental to early follicular development is still not understood.

Studies on whether hypothalamic release from estradiol negative feedback (from letrozole) elicits follicle-stimulating hormone (FSH) release from the pituitary have varied. Sources claim an increase in FSH (Requena et al., 2008), a decrease in FSH (Kauffman et al., 2015) or increased mRNA expression in FSH-receptors in the ovaries (from increased intraovarian androgens; (Weil et al., 1999; Kauffman et al., 2015) following letrozole treatment. It has been hypothesized that LH secretion from the pituitary is favored over FSH secretion following letrozole treatment (Burt Solorzano et al., 2012).

Studies on local ovarian effect of letrozole has focused on steroid release into follicular fluid (Haas et al., 2018) and blood circulation (Schoolcraft et al., 2008). Intravaginal treatment with our novel silicone intravaginal letrozole releasing device (LRD; Chapter 2), may have a more profound local effect on the ovary due to arterio-venous anastomosis networks (i.e., bypassing systemic circulation). Therefore, letrozole concentrations may be increased in follicular fluid compared to circulation, because of passage (similar to prostaglandin F_{2a} (PGF)) through the utero-ovarian venoarterial pathway in ruminants (Lee et al., 2010). To our

knowledge, no studies have quantified letrozole in follicular fluid following treatment in any species.

The objectives of this study were to use the bovine model to investigate the effect of letrozole treatment in cattle on 1) the local ovarian environment using follicular fluid analyses and granulosa cell gene expression, 2) LH release and nuclear maturation of oocytes following follicular aspiration, and 3) *in vitro* embryo production following follicular aspiration and *in vitro* maturation, fertilization, and culture. We hypothesized that intravaginal treatment with letrozole would result in increased letrozole concentrations in follicular fluid compared to plasma, an increase in FSH-receptor mRNA in granulosa cells, increased LH release and premature maturation of the COC leading to a reduced capacity for embryo developmental.

5.3. Materials and Methods

Experiments were approved by the University of Saskatchewan's Animal Research Ethics Board following the guidelines of the Canadian Council on Animal Care. Sexually mature, Hereford-cross heifers used for the study were maintained at the University of Saskatchewan's Livestock and Forage Centre of Excellence Goodale Farm. Heifers were kept in outdoor pens and fed barley silage with *ad libitum* access to alfalfa hay and water during the experimental period of April to July.

5.3.1. Experiment 1-Effects of letrozole on the local ovarian environment in cattle

5.3.1.1. Animals and treatments

Hereford-cross heifers (n=31), 11 to 16 months of age were chosen from a herd of 70 based on the detection of a corpus luteum (CL) ≥20 mm diameter by transrectal ultrasonography (7.5 MHz linear array probe, MyLab Alpha, Esaote North America, Fishers, IN, USA). The heifers were given two luteolytic doses of PGF (500 μg cloprostenol im, Bioestrovet, Vetoquinol N.A., Lavaltrie, QC, Canada) 12 hours apart and gonadotrophin-releasing hormone (GnRH; 100 μg gonadorelin im, Fertiline, Vetoquinol N.A., Lavaltrie, QC) 3 days later. The ovaries were examined by transrectal ultrasonography once per day for the following 2 days after administration of GnRH, and the heifers that ovulated were included in the experiment (n=31). Transvaginal ultrasound-guided ablation of follicles ≥5 mm was done 3 to 4 days after ovulation to synchronize follicular wave emergence; the day after ablation was considered the day of wave emergence (Day 0). Heifers were assigned randomly to one of four groups (n=7-8 heifers per group) in a 2x2 factorial design to compare the effects of treatment (LRD vs. sham device) and follicle stage (pre- vs. post-LH surge) on the follicular environment. An intravaginal device (LRD or sham) was inserted immediately after follicle ablation (Day -1), and was removed on Day 3, followed immediately by ultrasound-guided follicle sampling in pre-LH surge groups. Heifers in the post-LH surge groups were given PGF at the time of vaginal device removal (Day 3), GnRH on Day 5, and follicular sampling was done on Day 5.5. (Supplementary Fig. 5.1). Ovarian follicular and luteal dynamics were monitored by transrectal ultrasonography on Days -1, 3, 4, 5, and 5.5.

5.3.1.2. Follicle sampling

Follicle sampling was done following caudal epidural anesthesia was induced (5 mL of 2% lidocaine; Lurocaine, Vetoquinol N.A., Lavaltrie, QC) and the perineum was disinfected

using an iodine-based detergent and solution. The vagina was lavaged by 5 alternating infusions of 60 mL saline and disinfectant solution (1% wt/vol; Virkon; Vetoquinol N.A., Lavaltrie, QC) to remove mucopurulent vaginal discharge due to the presence of the intravaginal device. Aspiration of the follicular contents of the dominant and subordinate follicles was done by transvaginal ultrasound-guided puncture (5-8MHz convex-array probe; MyLab Alpha, Esaote North America Inc, Fishers, IN, USA) with an 18-ga short-beveled inverted needle (Watanabe Tecnologia Aplcada Ltda, Cravinhos, SP, Brazil) connected to a 6 mL syringe via silastic tubing. The dominant follicle was aspirated first and individually; care was taken to relieve the vacuum in the syringe after follicle collapse and before the needle was withdrawn back into the vaginal cavity to minimize the possibility of aspirating any vaginal residue. The follicular aspirate was expelled into a 35 mm Petri dish, and a new needle and tubing were installed before aspiration of pooled subordinate follicles (n=2-3) in a similar manner to minimize contamination. The aspirate was searched by stereomicroscope (10x magnification) to locate and discard the cumulus-oocyte complexes (COC). The granulosa cells and follicular fluid were placed in RNase-free 2 mL micro-centrifuge tubes, and centrifuged (1500 x g for 15 minutes) to form a pellet. The follicular fluid portion was removed and aliquoted into 1.5 mL RNase-free micro-centrifuge tubes and frozen at -20°C. The pellet was resuspended in 0.5-1 mL of RNA Later (Life Technologies, Carlsbad, California, USA) at 4°C overnight, and then stored frozen at -80°C.

5.3.1.3. RNA extraction and cDNA Synthesis

Total RNA extraction from the granulosa cell isolate was done using the Rneasy micro kit (Qiagen, Venlo, Netherlands) in accordance with the manufacturer's instructions. Extracted RNA was analyzed using spectrophotometry (NanoDrop; Thermo Scientific, Waltham,

Massachusetts, USA) for concentration and quality. Samples with a 260/280 ratio below 1.55 were excluded (n=1), and samples of a concentration below 15 ng/μL (n=8) were concentrated with an RNA Clean and Concentrator 5 Kit (Zymo Research, Irvine, California, USA) in accordance with manufacturer's instructions to reach a concentration suitable for the reverse transcription procedure.

From each sample, an aliquot containing 200 ng RNA was utilized for reverse transcription to complement DNA with the iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, California, USA) to produce a cDNA sample for qPCR. This synthesis was done in accordance with the manufacturer's protocol.

5.3.1.4. Reference Genes and Normalization

Eight reference genes (GAPDH, RPL19, RPLPO, EIF2B2, H2AFZ, B2M, SF3A1, and RFN20) were considered for normalization based on previous studies for the cell type being examined (Khan et al., 2016). Primers were verified using NCBI BLAST as well as qPCR melt curves from previously prepared bovine granulosa cell cDNA. Following primer verification, qPCR was done for each of the potential reference genes using SYBR Green Supermix (Wisent Bio Products, St-Bruno, Quebec, Canada) for the 27 samples (LRD dominant follicle n=6, LRD subordinate follicle n=6, Sham dominant follicle n=7, Sham subordinate follicle n=8). A 2 μL aliquot of cDNA from each sample was combined with 5 μL of SYBR Green Supermix, 2 μL Rnase free H_2O, and 1 μL primer in a well of a 384 well white PCR plate (Diamed Lab Supplies Inc., Mississauga, Ontario, Canada) kept on ice. Once complete, the plate was sealed with an adhesive cover and centrifuged at 2000 x *g* for 2 minutes, then analyzed with a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). Final reference gene selection was

determined using geNorm analytic software (Qbase+, Biogazelle, Gent, Belgium), with the geometric mean of *RPL19, RPLPO, EIF2B2,* & *H2AFZ* producing the lowest level of variance (geNormV=0.22). Additional confirmation of reference genes was done through independent geNorm analysis of results from dominant and subordinate follicles, each of which included these reference genes in their normalization recommendation.

5.3.1.5. qPCR Analysis of Genes of Interest

Details of the primers for the four reference and nine genes of interest are provided in Supplementary Tables 3 and 4. Primers for nine genes of interest (HSD3B, LHCGR, STAR, INHBA, FSHR, CYP11A1, CCND2, NPR2, and CYP19A1) were verified in the manner described above. A 2 μL aliquot of cDNA from each sample was combined with 5 μL of SYBR Green, 2 μL Rnase free H_2O, and 1 μL primer in a well of a 384 well PCR plate kept on ice. Once complete, the plate was centrifuged at 2000 x *g* for 2 minutes, then analyzed with a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Industries).

5.3.1.6. Follicular fluid LC-MS/MS and radioimmunoassay analysis

Follicular fluid was analyzed for concentrations of letrozole by tandem mass spectrometry (LC-MS/MS) with a method that was partially validated as part of this study. Follicular fluid from abattoir-derived ovaries was aspirated, centrifuged (3200 x *g* for 15 minutes at 4°C), aliquoted, and stored frozen at -20°C until use. A stock solution of 1 mg/mL of letrozole was prepared by dissolving 50 mg of letrozole in 50 mL of 100% methanol. A standard curve consisting of concentrations of 1, 2, 4, 8, 16, 32, 64, 128, and 256 ng/mL was prepared by serial

dilution in pooled follicular fluid (i.e., removing half of higher concentration and diluting by half using blank follicular fluid). A total of 100 µL of each standard was pipetted into a 1500 µL plastic microcentrifuge tube. Standards were then spiked with 50 µL letrozole-D4 (200 ng/mL in 100% acetonitrile, letrozole-D4, Toronto Research Company, Toronto, ON) to serve as an internal standard for quantifying peak height and area against letrozole, vortexed for 3 seconds, and 250 µL of high-pressure liquid chromatography (HPLC) grade acetonitrile was added. Standards were vortexed for 5 seconds and centrifuged (15,000 x *g* for 15 min. at 4°C). Following centrifugation, 200 µL of supernatant was transferred to an HPLC autosampler vial and loaded into the HPLC/LC-MS/MS machine (QTRAP 4000; SCIEX; Framingham, Massachusetts, USA) autosampler for analysis. Partial validation parameters included linearity, accuracy, and precision.

Separation by HPLC (Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) was done with an analytical column (50 x 2.1 mm, 3 um particle size, Thermo Scientific Betasil C18; Thermo Scientific, Waltham, MA, USA) at 35°C. HPLC flow rate, solvent conditions, and LC-MS/MS conditions were done as previously described (Joshi et al., 2011).

Linearity was tested by running three standard curves independently of each other. Ratio counts vs. actual concentration were plotted and the r^2 values were calculated. The mean (± SEM) r^2 value was 0.998 ± 0.002. The calibration curve required a correlation coefficient of $r^2 \geq 0.99$. The acceptance criteria for each calculated standard was 15% difference from the nominal value of the non-zero calibrators (2, 4, 8, 16, 32, 64, 128, and 256 ng/mL) except the lower limit of quantification (LLOQ) which was set at 20%. Accuracy and precision were calculated by running three independently spiked quality control (QC) samples (3, 10, and 100 ng/mL) 18 times (6 samples per day on 3 separate days). Accuracy was calculated by the percentage of true

concentration of letrozole recovered by the assay. Precision was expressed as the relative standard deviation using the following formula: %RSD=(SD x 100)/mean. The acceptance criteria for precision were <20% LLOQ and <15% for the remaining concentrations. The acceptance criteria for accuracy was 100 ± <20% for LLOQ and 100 ± <15% or higher for the remaining concentrations.

Follicular fluid concentrations of estradiol were analyzed by radioimmunoassay as previously described (Dias et al., 2013). Intra-assay coefficients of variation (CV) for low (15 ng/mL) and high (40 ng/mL) samples were 10 and 12%, respectively.

5.3.2. Experiment 2 –Frequent blood sampling for gonadotropin release and maturation status of COC following letrozole treatment

5.3.2.1. Animals and treatments

Hereford-cross heifers in which a CL was detected by transrectal ultrasonography (n=24) were given PGF 12 hours apart followed by GnRH 3 days later as described in Experiment 1. The ovaries were examined by transrectal ultrasonography once daily for 3 days following GnRH to confirm ovulation. Three days after ovulation, heifers underwent transvaginal ultrasound-guided ablation of all follicles ≥5 mm to synchronize follicular wave emergence (Day 0). Heifers were randomly distributed by the lottery system and given a 4-day intravaginal device (LRD or sham device) immediately following ablation. On Day 3. devices were removed and transrectal ultrasonography was done on Days 3 and 4 for analysis of ovarian structures. On Day 4 heifers underwent ultrasound-guided transvaginal follicular aspiration.

5.3.2.2. COC Collection

Collection of COC was done by transvaginal ultrasound-guided aspiration of all follicles ≥ 3 mm in diameter using an 18-ga short, and inverted beveled needle (Watanabe Tecnologia Aplcada Ltda, Cravinhos, SP, Brazil) connected to a 50 mL conical Falcon tube by silastic tubing. Before aspiration, caudal epidural anesthesia was induced by 5 mL of 2% lidocaine (Lurocaine, Vetoquinol N.A., Lavaltrie, QC) and the perineum was washed by betadine detergent and 70% alcohol. Follicular contents were aspirated by a vacuum pump (BV 003i Digital Vaccum Pump, Watanabe Tecnologia Aplcada Ltda) at a flow rate of 16 mL/min and the collection medium consisted of Dulbecco's phosphate-buffered saline (DPBS), 0.15% ET surfactant (Vetoquinol N.A., Lavaltrie, QC, Canada), and 200 IU/L heparin. Follicular aspirates were poured from the Falcon tube into an ova/embryo filter (Emcon filter, Agtech, Manhattan, KS, USA) and rinsed with collection medium without surfactant. Once rinsed, all contents were poured into a 90 mm petri dish and searched by stereo microscopy at 10x magnification. Groups were facilitated for a maximum of 12 heifers per COC collection day (6 letrozole, 6 control). Each animal was collected twice in a crossover design, with a washout period of 12 days following letrozole treatment (Supplementary Fig. 5.2).

At the time of recovery, COC were classified according to the appearance of the cumulus cell layers and the ooplasm. The cumulus layer was classified as compact good (>3 layers of granulosa cells tightly surrounding the oocyte), compact regular (1-3 layers of granulosa cells surrounding the oocyte), expanded (cumulus cells expanded or partially dissociated), denuded (oocyte without cumulus cells), or degenerate. Degenerate oocytes were excluded from further processing. Oocytes were pooled into four treatments, coinciding with their treatment and

maturation times (LRD 0-hour, LRD 24-hour, Control 0-hour, Control-24 hour); oocytes in the 0-hour group were denuded immediately and fixed as described below, and oocytes in the 24-hour group were placed in maturation medium for 24 hours before denuding and fixation.

5.3.3. In vitro maturation (IVM) and COC nuclear maturation status

The medium used for the maturation of oocytes in the 24-hour group was BO-HEPES-IVM (IVF Bioscience, Falmouth, Cornwall, UK). The COC were washed 3 times in IVM medium before being placed into a portable incubator (LabMix; Watanabe Tecnologia Aplcada Ltda, Cravinhos, SP, Brazil) for transport to the laboratory and were maintained at 38.8°C for 24 hours. Oocytes were denuded (at 0 or 24 hours after collection) by repeated pipetting in 0.3% hyaluronidase (wt/vol) in Ca^{++} and Mg^{+} -free DPBS, washed in 0.1% (wt/vol) polyvinyl alcohol (PVA) PBS 3x, and fixed in 4% (vol/vol) paraformaldehyde/PBS for 15 minutes. Following fixation, oocytes were washed in 0.1% PVA PBS 3x and stored in 4°C until staining. Staining was done as previously reported (Cervantes et al., 2016) and examined under an epifluorescence laser microscope (Zeiss Axioskop 5 Carl Zeiss Ltd., Toronto, ON, Canada). Nuclear maturation stage was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) as shown in Figure 5.1.

5.3.4. Experiment 3 –In vitro fertilization following letrozole treatment

5.3.4.1. Animals and treatments

Using the same synchronization protocol as Experiment 2 (Supplementary Fig. 5.2), Hereford-cross beef heifers (n=33) selected from a larger group of heifers were used in a

crossover design to facilitate treatment with both an LRD and a sham device. As in Experiment 2, COC were collected by transvaginal follicular aspiration, degenerate COC were discarded, and the remaining COC underwent *in vitro* maturation for 24 hours followed by *in vitro* fertilization.

5.3.4.2. In vitro maturation, fertilization, and culture

The COC were washed twice in 0.5 mL BO-WASH (IVF Bioscience) and once in 0.25 mL BO-HEPES-IVM (IVF Bioscience). The grades of COC were pooled by treatment and placed in 0.5 mL tubes (Sarstedt, Germany) containing BO-HEPES-IVM (IVF Bioscience) and placed in a portable incubator (LabMix; Watanabe Tecnologia Aplcada Ltda, Cravinhos, SP, Brazil) at 38.8°C and transported to the laboratory for a maturation time of 21-24 hours.

After 21-24 hours of *in vitro* maturation in the incubator, COC were removed from the maturation tubes and washed once in a preheated and equilibrated 500 μL well of BO-IVF (IVF Bioscience, United Kingdom). The group of oocytes was transferred into a preheated and equilibrated 90 μL drop of BO-IVF with 0.5 mL overlaying mineral oil in a four well plate. The dish was placed in an incubator (Miri® Benchtop Multi-room Incubator, Esco Medical ApS, Denmark) at 38.8°C 5% CO_2. A volume of 2 mL of BO-SemenPrep (IVF Bioscience) was warmed in two separate 15 mL tubes at 37.0°C. Two 0.25 mL Holstein semen straws (Semex, Guelph, ON, Canada) were thawed in 37.0°C water for 1 minute. The straws were dried, and each emptied into one of the 15 mL tubes containing the BO-SemenPrep. Tubes containing the semen were then centrifuged for 5 minutes at 328 x *g*. The supernatants were removed until 300 μL of SemenPrep/sperm solution remained, in which 2 mL of preheated SemenPrep was added; the sperm pellet was resuspended and centrifuged for 5 minutes at 328 x *g*. The supernatant of SemenPrep was removed until 300 μL remained, and the 2 sperm solutions were combined and

mixed with a pipette. Each 90 μL drop of BO-IVF with COC was fertilized with the prepared sperm to attain a final concentration of 2 x 10^6 sperm/mL. Oocytes and sperm were co-incubated at 38.8°C in 5% CO_2 for 18 hours.

Following 18 hours after fertilization, presumptive zygotes were denuded in BO-WASH (IVF Bioscience) and washed in one 500 μL well of preheated and equilibrated BO-IVC (IVF Bioscience) at 38.8°C in 6% O_2, 6% CO_2 and 88% N_2. Each set of presumptive zygotes were placed in a preheated and equilibrated 90 μL drop of BO-IVC with 0.5 mL overlaying mineral oil in a four well plate and incubated at 38.8°C in 6% O_2, 6% CO_2 and 88% N_2. Presumptive zygotes were evaluated 56 hours after fertilization and classified as 1 cell (unfertilized ova) or cleaved (≥2 cells). On Day 7, (Day 0=IVF) embryos were evaluated and classified by quality and grade under the IETS grading system (Stringfellow and Givens, 2010).

5.3.5. Blood sampling, LC-MS/MS, and radioimmunoassays

Blood samples were taken by jugular venipuncture into heparinized tubes (Vacutainer; Becton Dickinson and Co., Franklin Lakes, NJ), subsequently centrifuged (<2 hours after collection; 1,500 x *g*) and plasma was aliquoted into polypropylene tubes and frozen at -20°C until assay. In Experiment 1, blood samples were taken on Day 3 in all groups and twice daily from Day 4 to Day 5.5 in the post-LH group. In Experiments 2 and 3, blood samples were taken at the time of follicular aspiration. On a subset of heifers (n=6/group) in Experiment 2, frequent blood samples were taken for analysis of serum LH and FSH concentrations. Samples were collected via indwelling jugular catheters (Bergfelt et al., 1997) taken every 15 minutes for 8 hours starting immediately following device removal on Day 3. Blood samples were taken by a 12-mL syringe and placed into 12 x 75 mm Borosilicate glass tubes (Fisher Scientific; Hampton,

New Hampshire, USA), allowed to clot, centrifuged (1500 x *g*, 15 minutes) within 45 minutes of collection and serum was placed in polypropylene tubes and frozen at -20°C until analysis. Collections were done in two replicates to facilitate use of time.

Plasma concentrations of letrozole were analyzed by LC-MS/MS using a method that was partially validated in Chapter 2. The lower limit of quantification was 1 ng/mL.

Plasma concentrations of estradiol were determined by radioimmunoassay as previously described (Kirby et al., 1997) at Texas A&M University. The intra-assay coefficient of variation (CV) was 1.5%, and the minimum detection limit was 0.5 pg/mL.

Plasma concentrations of progesterone were determined by a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA, USA) with a minimum detection limit of 0.1 ng/mL. Intra-assay CV for low, medium, and high samples were 11.9, 6.0, and 5.8%, respectively. Inter-assay CV for low, medium, and high samples were 8.4, 6.8, and 1.7%, respectively.

Serum concentrations of LH were analyzed by radioimmunoassay as previously described (Honaramooz et al., 2000) with a minimum detection limit of 0.1 ng/mL, and intra-assay CV for low and high samples were 7.4 and 4.9%, respectively. Serum concentrations of FSH were analyzed by radioimmunoassay as previously described (Rawlings et al., 1984) with a minimum detection limit of 0.13 ng/mL, and intra-assay CV of 5.6%.

5.3.6. Analysis of LH and FSH

The area under the concentration-time curve until the final serum sample (AUC_{last}) was determined using the linear trapezoidal rule for both LH and FSH. Luteinizing hormone pulses were identified using the previously described criteria (Goodman and Karsch, 1980): 1) the peak

occurred within two samples of the previous nadir, 2) pulse amplitude exceeded assay sensitivity (i.e., ≥0.1 ng/mL), and 3) the peak was 2 standard deviations (assay variability) above the preceding and following nadirs. Pulse amplitude was defined as the peak concentration subtracted from the previous nadir concentration occurring within 2 sample time points.

5.3.7. Statistical analyses

Data were analyzed using SAS Enterprise Guide (Version 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Continuous data were compared by t-test or 2x2 analysis of variance using the PROC MIXED procedure. Analysis of variance for repeated measures was used for profile data, and correlations between letrozole in follicular fluid and plasma was done with PROC CORR. Proportional data were compared by Chi-square. Data are presented as mean±SEM, and significance was defined as P≤0.05.

5.4. Results

5.4.1. Experiment 1 – Effects of letrozole on the local ovarian environment in cattle

Partial validation for determining the concentration of letrozole in follicular fluid, the standard curve and quality control samples all passed and the accuracy and precision for the standard curve and quality control samples are shown in Supplementary Tables 5.1 and 5.2. No interactions were found between the daily dominant follicle and CL diameter. A tendency (P=0.09) for CL treatment was due to slower luteolysis following PGF in the LRD group (Fig. 5.2). An increase in plasma progesterone concentrations on Day 3, 4, and 4.5 in the LRD group led to a treatment (P=0.04) and treatment by day interaction (P=0.003; Fig. 5.3). A treatment effect for plasma estradiol concentrations following PGF was attributed to lower (P=0.001)

concentrations in the LRD group and no effect of treatment (P=0.22) or interactions (P=0.15) were detected between groups for plasma LH concentrations following PGF between groups. On Day 3, the LRD group tended (P=0.07) to have higher progesterone than the sham group (Table 5.1). Plasma estradiol concentrations were increased (P<0.05) on Day 5.5 (post-LH) vs. Day 3 (pre-LH), and the LRD group had lower (P<0.05) estradiol concentrations on Day 5.5 (Table 5.1). Similarly, plasma estradiol concentrations in the dominant follicle was greater on Day 5.5 than 3 and tended (P=0.07) to be lower in the LRD group on Day 3. Follicular fluid concentrations of letrozole did not differ (P=0.37) between dominant and subordinate follicles, but a pre- vs. post-LH treatment difference was observed (P<0.001; Table 1, Fig. 5.4). There was a highly significant positive correlation between plasma and follicular concentrations of letrozole at the time of sampling (R=0.9889, P<0.0001).

Granulosa cells from the post-LH group had low-quality RNA, and we were unable to do PCR; therefore, only gene expression data from Day 3 was only assayed (i.e., pre-LH dominant and subordinate follicles). No treatment or treatment by follicle interactions was detected for any gene expression data. No follicle effects for gene expression from granulosa cells were shown for HSD3B, LHCGR, INHBA, FSH, or STAR (Table 2). A follicle effect for gene expression was shown for Cyp11A1 (P<0.001), CCND2 (P<0.01), NPR2 (P=0.05), and Cyp19A1 (P<0.0001; Table 5.2).

5.4.2. Experiment 2 - Frequent blood sampling for gonadotropin release and maturation status of COC following letrozole treatment

The serum LH concentration profiles from frequent blood samples from four individual heifers given both LRD and sham treatments are shown in Figure 5.5. The LH AUC_{last} was greater in LRD vs sham (P<0.01; Table 5.3). Number of LH pulses per 8-hour interval did not

differ (P=0.56) between groups, but overall pulse amplitude (P<0.001), LH pulse amplitude >0.5 ng/mL (P=0.03), and LH pulse amplitude >1 ng/mL (P=0.02) were all greater in the LRD group (Table 5.3). Plasma estradiol concentrations were lower on Day 3 in the LRD vs. sham group (P=0.03; Table 5.3).

The total number of follicles aspirated tended (P=0.07) to be fewer in the LRD group, and the LRD group tended (P=0.10) to have a greater number of degenerate COC (Table 5.4).

A total of 35 oocytes (3-12 per group) out of 238 oocytes that were collected were lost during the fixing and staining procedure, and data from the remaining oocytes were used for statistical comparison of nuclear maturation among groups (Table 5.5). There were more oocytes in the MI stage in the sham 24-hour group compared to the other groups (P<0.05). The LRD 24 hour group yielded a higher proportion of oocytes that were in MII compared to the other groups (P<0.05; Table 5.5) and the sham 24-hour group had a higher proportion of oocytes in the MII stage compared to both 0-hour groups (P<0.05; Table 5.5).

5.4.3. Experiment 3 - In vitro fertilization following letrozole treatment

One heifer was excluded from the LRD group because the vaginal device was lost during treatment. There was a greater number of follicles aspirated (P=0.02), compact regular- (P=0.01) and total-COC per heifer (P=0.03) in the sham group (Table 5.6). The COC collection rate was higher in the sham vs. LRD group (61.6% vs. 51.7%, respectively; P<0.01; Table 5.6).

The proportion of cleaved zygotes from total COC submitted to IVF tended to be higher in the sham vs. LRD group (P=0.07; Table 5.7). The proportion of blastocysts from total COC submitted to IVF was higher in the LRD vs. sham group (P=0.02; Table 5.7). The proportion of

embryos from compact COC tended to be higher in LRD vs. sham (P=0.06; Table 5.7). The proportion of embryos from cleaved COC was greater in LRD vs. sham (P=0.02; Table 5.7).

When follicular aspiration data from Experiment 2 and 3 were combined, the mean number of compact regular COC (P=0.02), total compact COC (P=0.01) and total COC collected (P=0.02) were lower in the LRD group than in the sham group. (Table 5.8).

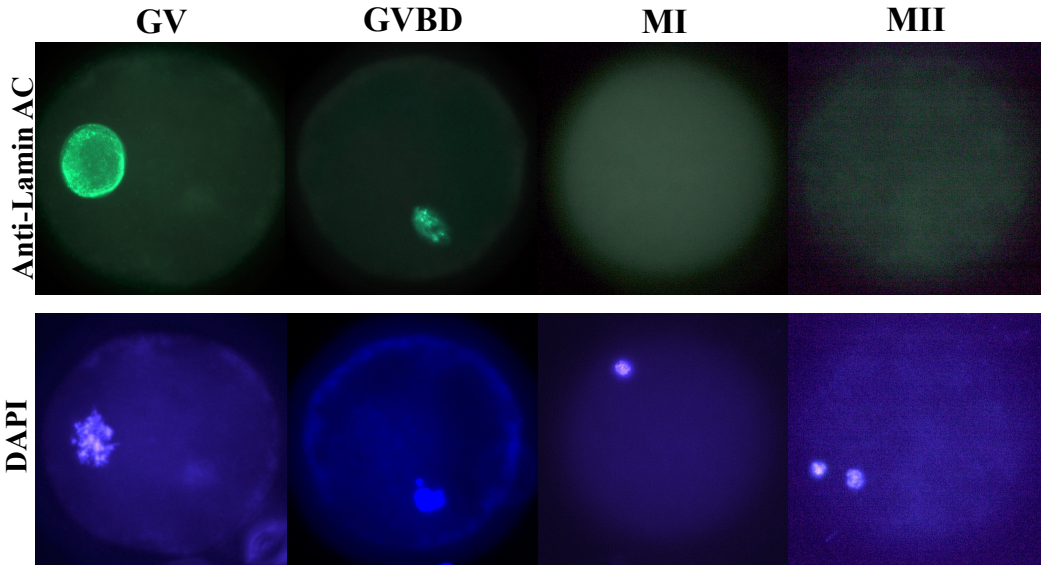


Figure 5. 1**.** Cattle oocytes at different stages of nuclear maturation: germinal vesicle (GV), germinal vesicle break-down (GVBD), metaphase I (MI), metaphase II (MII). Oocytes were stained with anti-Lamin AC/DAPI (Experiment 2). Seen visually by green fluorescence following Anti-Lamin AC staining is the nuclear envelope (only visible in GV and GVBD) while DNA appears with blue fluorescence (DAPI; Diminophenylindole).

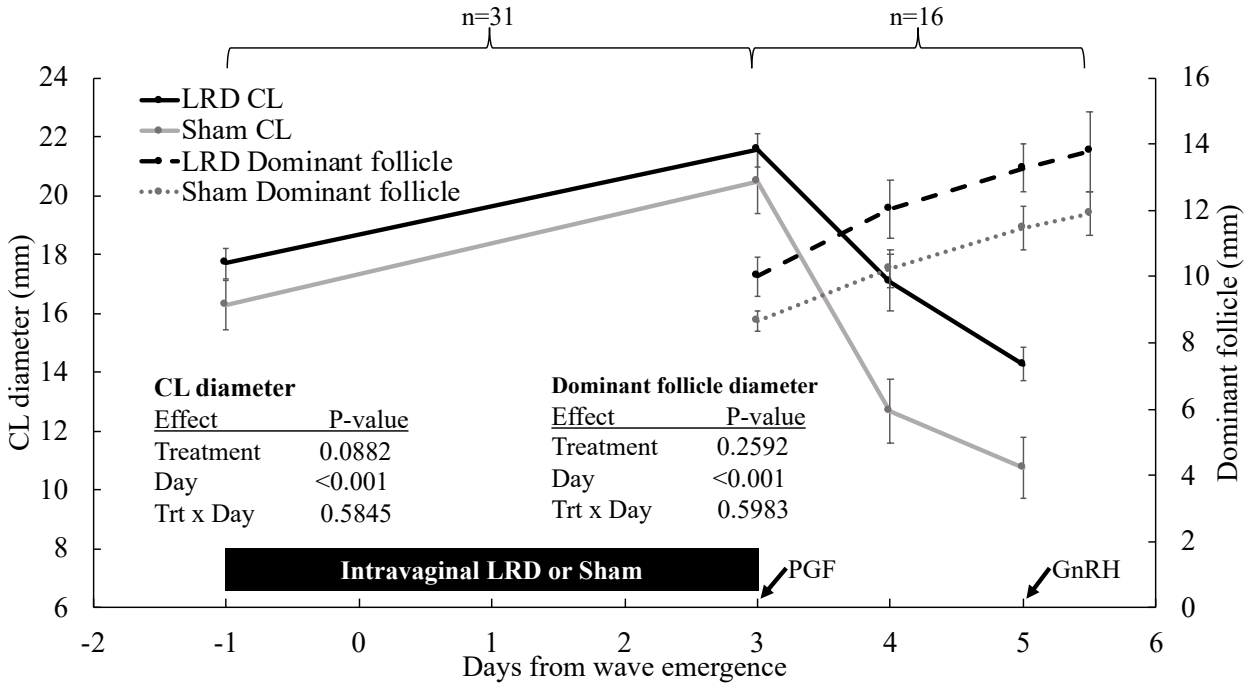


Figure 5. 2. Dominant follicle and corpus luteum (CL) diameter profiles from Experiment 1. Ultrasound guided follicular ablation of all follicles ≥ 5mm was done on Day -1 and heifers were given a letrozole releasing device (LRD) or a sham device (n=15 and 16/group, respectively). Mean ± SEM

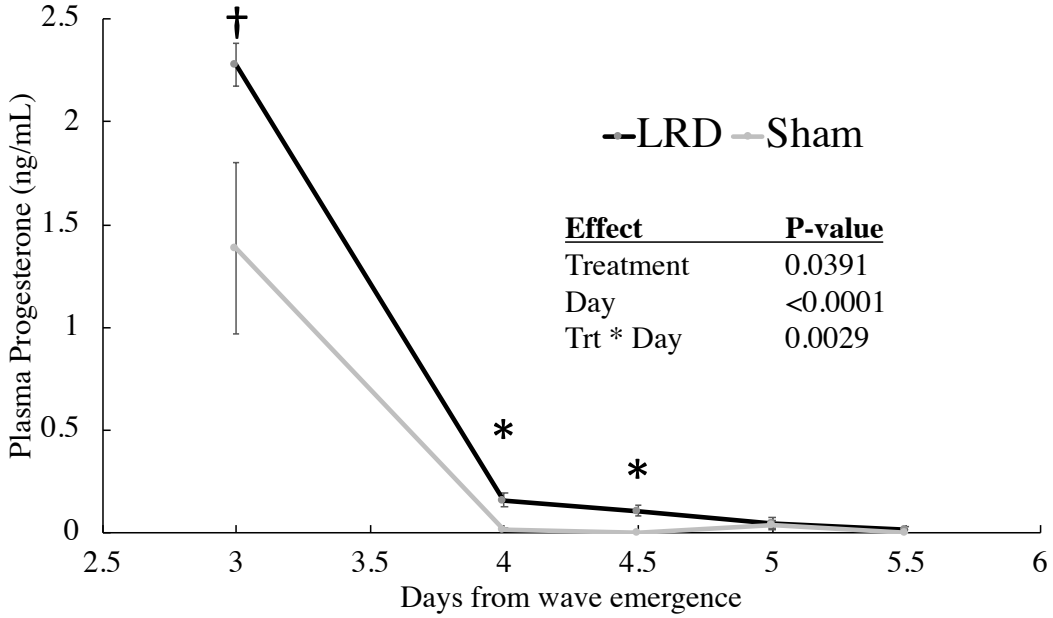


Figure 5. 3**.** Plasma progesterone concentrations in heifers (n=7 or 8/group) following device removal and 500 µg cloprostenol (PGF) on Day 3 and 100 µg gonadorelin (GnRH) on Day 5 (Experiment 1). Heifers were given either a 4-day letrozole releasing device (LRD) or a sham device on Day -1 immediately after ultrasound-guided transvaginal ablation of follicles ≥ 5mm.

†Among days, values tended to be different (P=0.08); *Among days, values were different (P<0.01). Mean ± SEM

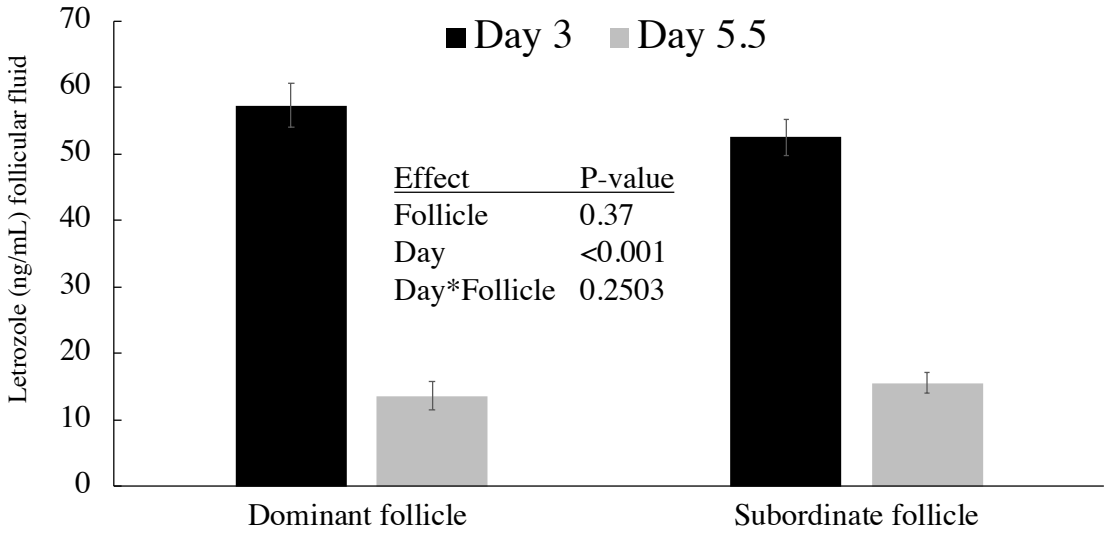


Figure 5. 4**.** Follicular fluid concentrations of letrozole assessed by tandem mass spectrometry (LC-MS/MS). Follicular fluid was collected by ultrasound-guided follicular aspiration from dominant and subordinate follicles on Day 3 or Day 5.5 following ultrasound-guided follicular ablation of all follicles ≥ 5mm on Day -1 (Experiment 1). Mean ± SEM

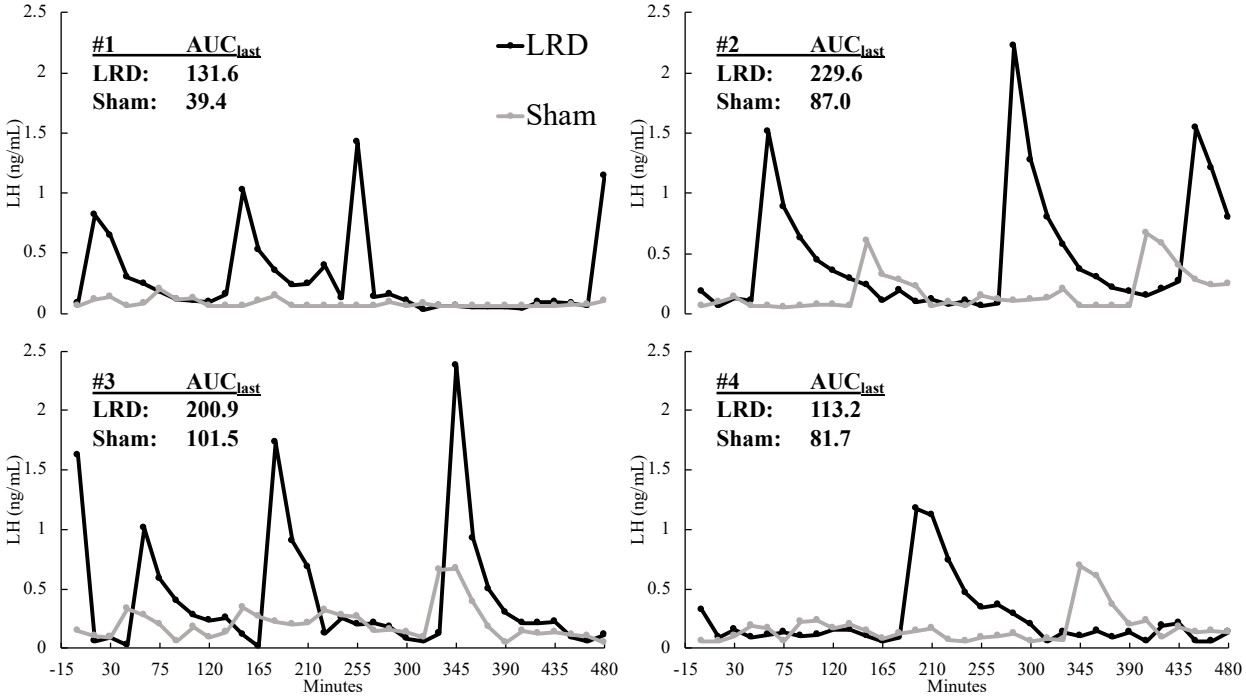


Figure 5. 5**.** Individual serum luteinizing hormone (LH; ng/mL) from four heifers that underwent frequent blood sampling done every 15 mins for 8 hours beginning immediately after a 4-day treatment with either a letrozole-releasing device (LRD) or a sham device. Total area under the curve (AUC_{last}) is indicated for each heifer while under each treatment (Experiment 2).

Table 5. 1**.** Ovarian and endocrine endpoints between heifers given either a 4-day letrozole releasing device (LRD) or a sham device in Experiment 1. Mean ± SEM

	Day of Sampling			
	Day 3		Day 5.5	
	LRD	Sham	LRD	Sham
Heifers	7	8	8	8
Dominant follicle diameter (mm)	9.3 ± 0.6 $^{a, x}$	9.2 ± 0.5 $^{a, x}$	13.8 ± 1.2 $^{a, y}$	11.5 ± 0.7 $^{a, y}$
CL diameter (mm)	21.0 ± 0.6 $^{a, x}$	23.0 ± 1.0 $^{a, x}$	14.7 ± 0.6 $^{a, y}$	11.4 ± 1.2 $^{b, y}$
Plasma progesterone (ng/mL)	4.1 ± 0.9 $^{a, x}$ *	3.0 ± 0.4 $^{a, x}$ *	0.02 ±0.02 $^{a, y}$	0.00 ± 0.00 $^{a, y}$
Plasma estradiol (pg/mL)	1.7 ± 0.3 $^{a, x}$	2.1 ± 0.4 $^{a, x}$	1.9 ± 0.3 $^{a. y}$	3.9 ± 0.6 $^{b, y}$
Dominant follicle estradiol (ng/mL)	36.6 ± 14.8 $^{a, x}$*	107 ± 27.7 $^{a, x}$*	171 ± 30.4 $^{a, y}$	190 ± 39.5 $^{a, y}$
Subordinate follicle estradiol (ng/mL)	4.1 ± 1.8 $^{a, x}$ *	21.2 ± 6.2 $^{a, x}$ *	20.1 ± 12.9 $^{a, x}$	11.1 ± 9.1 $^{a, x}$
Plasma letrozole (ng/mL)	70.4 ± 3.9 ^{x}	-	22.9 ± 2.2 ^{y}	-
Dominant follicle letrozole (ng/mL)	57.3 ± 3.3 ^{x}	-	13.6 ± 2.7 ^{y}	-
Subordinate follicle letrozole (ng/mL)	52.5 ± 2.1 ^{x}	-	15.6 ± 1.5 ^{y}	-

Heifers underwent ultrasound-guided transvaginal ablation on Day -1 followed immediately by insertion of either an LRD or a sham device for 4 days. Heifers either had follicles sampled immediately after device removal on Day 3 (pre-LH surge) or were given PGF at the time of

device removal on Day 3, followed by GnRH on Day 5, and follicle sampling on Day 5.5 (post-LH surge).
Within rows, values with different superscripts between treatments [ab] or between days [xy] indicate significance (P<0.05); *P= 0.05 to 0.10.

Table 5. 2**.** Relative gene expression from granulosa cells derived from heifers treated for 4 days with either a letrozole releasing device (LRD) or a sham (n=8/group) in Experiment 1. Mean ± SEM

	LRD		Sham	
Gene	Dominant	Subordinate	Dominant	Subordinate
HSD3B	1.3 ± 0.5	2.3 ± 1.1	1.8 ± 0.6	2.7 ± 1.1
LHCGR	0.9 ± 0.3	1.0 ± 0.5	2.2 ± 1.2	1.6 ± 0.7
STAR*	1.5 ± 1.1	5.9 ± 3.0	2.2 ± 1.8	7.7 ± 3.5
INHBA	0.8 ± 0.2	0.3 ± 0.1	1.1 ± 0.2	0.7 ± 0.5
FSHR	1.2 ± 0.2	1.8 ± 0.8	1.5 ± 0.7	3.4 ± 1.6
Cyp11A1	1.2 ± 0.2[a]	0.3 ± 0.1[b]	1.4 ± 0.3[a]	0.6 ± 0.2[b]
CCND2	1.3 ± 0.5[a]	0.2 ± 0.1[b]	0.9 ± 0.1[a]	0.3 ± 0.1[b]
NPR2	1.3 ± 0.6[a]	6.0 ± 3.0[b]	2.1 ± 1.5[a]	7.4 ± 3.4[b]
Cyp19A1	1.2 ± 0.2[a]	0.3 ± 0.1[b]	1.4 ± 0.3[a]	0.6 ± 0.2[b]

Genes were normalized to geometric mean of reference genes (Experiment 1).
[ab]Within rows, values with no common superscript are different between follicle groups (P≤0.05)
*Values tended to differ between follicle groups (P=0.06)

Table 5. 3**.** Serum endpoints from frequent blood sampling in heifers (n=6/group) that received an intravaginal letrozole releasing device (LRD) or sham device in Experiment 2. Mean ± SEM

	LRD	Sham	P-value
LH AUC_{last} (mins x ng/mL)	148.2 ± 22.4	74.2 ± 11.8	0.01
LH pulse amplitude (ng/mL)	0.9 ± 0.1	0.3 ± 0.1	<0.001
LH pulses per 8 hours	3.3 ± 0.4	3.0 ± 0.3	0.56
LH pulses >0.5 ng/mL	2.5 ± 1.4	0.8 ± 0.8	0.03
LH pulses >1 ng/mL	1.5 ± 0.6	0 ± 0	0.02
FSH AUC_{last} (mins x ng/mL)	136.7 ± 7.6	143.2 ± 14.7	0.70
CL diameter (mm)	19.7 ± 1.0	20.1 ± 1.1	0.78
Serum progesterone (ng/mL)	4.8 ± 0.7	4.1 ± 0.3	0.34
Serum estradiol (pg/mL)	2.5 ± 0.3	4.1 ± 0.5	0.03
Serum letrozole (ng/mL)	69.3 ± 4.2	-	-

AUC_{last}: cumulative area under the curve; LH: luteinizing hormone; FSH: follicle-stimulating hormone; CL: corpus luteum

Table 5. 4**.** Mean (± SEM) plasma steroids, follicular and cumulus-oocyte complex (COC) endpoints in heifers treated with either a letrozole releasing device (LRD) or a sham vaginal device in Experiment 2.

	LRD	Sham	P-Value
Heifers (n)	24	24	-
Plasma progesterone (ng/mL)	3.7 ± 0.7	3.8 ± 0.3	0.91
Plasma estradiol (pg/mL)	3.4 ± 0.3	3.5 ± 0.3	0.91
Dominant follicle size at aspiration (mm)	10.3 ± 0.3	10.1 ± 0.3	0.50
Total follicles aspirated	10.2 ± 0.9	13.7 ± 1.6	0.07
Compact good COC	0.5 ± 0.2	1.0 ± 0.3	0.18
Compact regular COC	2.4 ± 0.6	3.3 ± 0.6	0.28
Compact COC Total	2.9 ± 0.6	4.3 ± 0.7	0.15
Expanded COC	0.4 ± 0.2	0.5 ± 0.1	0.52
Denuded COC	0.7 ± 0.2	1.0 ± 0.3	0.42
Degenerate COC	0.6 ± 0.3	0.2 ± 0.1	0.10
COC collected per heifer	4.1 ± 0.8	5.9 ± 0.8	0.24
COC collection rate (COC/follicles aspirated)	110/234 (47.0%)	141/329 (42.9%)	0.35

Table 5. 5**.** Nuclear status of oocytes from heifers (n=12 heifers/group) treated with either a letrozole releasing device (LRD) or sham device for 4 days in Experiment 2.

	LRD 0 hours	LRD 24 hours	Sham 0 hours	Sham 24 hours
GV	25/38 (65.8%)[a]	1/49 (2%)[b]	33/52 (63.5%)[a]	0/64 (0%)[b]
GVBD	11/38 (29.9%)[a]	0/49 (0%)[b]	16/52 (30.8%)[a]	2/64 (3.1%)[b]
MI	1/38 (2.6%)[a]	6/49 (12.2%)[a]	2/52 (3.8%)[a]	27/64 (42.2%)[b]
MII	1/38 (2.6%)[a]	42/49 (85.7%)[b]	1/52 (1.9%)[a]	35/64 (54.7%)[c]

Following collection, cumulus-oocyte complexes (COC) in the 0-hour group were immediately denuded and fixed with 4% paraformaldehyde. COC in the 24-hour group were placed into maturation medium for 24 hours prior to fixation.

A total of 35 oocytes were lost during the fixing/staining procedure (n=3-12/group); GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II

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

Table 5. 6**.** Mean (± SEM) plasma steroids, follicular and cumulus oocyte complex (COC) endpoints in heifers treated with either a letrozole releasing device (LRD) or a sham vaginal device in Experiment 3.

	LRD	Sham	P-Value
Heifers (n)	32	33	-
Plasma progesterone (ng/mL ± SEM)	5.0 ± 0.5	4.2 ± 0.3	0.12
Plasma estradiol (pg/mL ± SEM)	6.4 ± 0.8	6.6 ± 0.6	0.83
Dominant follicle size at aspiration (mm)	10.1 ± 0.2	10.1 ± 0.2	0.84
Total follicles aspirated	14.6 ± 0.8	17.9 ± 1.0	0.02
Compact good COC	1.2 ± 0.2	1.6 ± 0.3	0.28
Compact regular COC	3.7 ± 0.6	6.3 ± 1.0	0.03
Compact COC Total	4.9 ± 0.7	7.9 ± 1.0	0.01
Expanded COC	0.3 ± 0.1	0.5 ± 0.2	0.39
Denuded COC	2.1 ± 0.3	2.3 ± 0.4	0.72
Degenerate COC	0.3 ± 0.1	0.3 ± 0.1	0.75
COC collected per heifer	7.5 ± 0.9	11.0 ± 1.3	0.03
COC collection rate (COC/follicles aspirated)	241/466 (51.7%)	363/589 (61.6%)	<0.01

Table 5. 7**.** Cleavage and embryo rates following *in vitro* fertilization on Day 0 in heifers treated with a letrozole releasing device (LRD) or a sham vaginal device in Experiment 3.

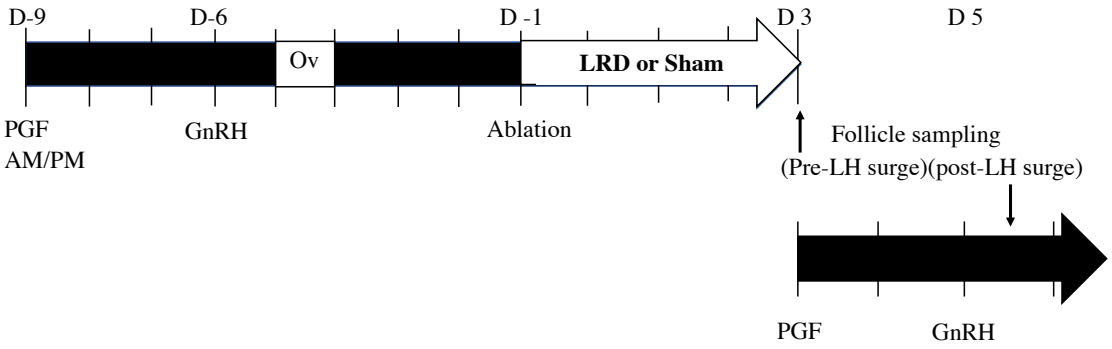
	LRD	Sham	P-value
Cleavage/total COC	109/177 (61.6%)	167/238 (70.2%)	0.07
Cleavage/compact COC	109/128 (85.2%)	167/186 (89.8%)	0.22
Morulae/total COC	28/160 (17.5%)	39/209 (18.7%)	0.77
Blastocysts/total COC	17/160 (10.6%)	9/209 (4.3%)	0.02
Embryos/total COC	45/160 (28.1%)	48/209 (23.0%)	0.26
Embryos/compact COC	45/118 (38.1%)	48/173 (27.7%)	0.06
Embryos/cleaved COC	45/97 (46.4%)	48/150 (30%)	0.02

Cleavage was assessed 56 hours after co-culture with sperm and embryo evaluation was done on Day 7.
COC: cumulus-oocyte complex

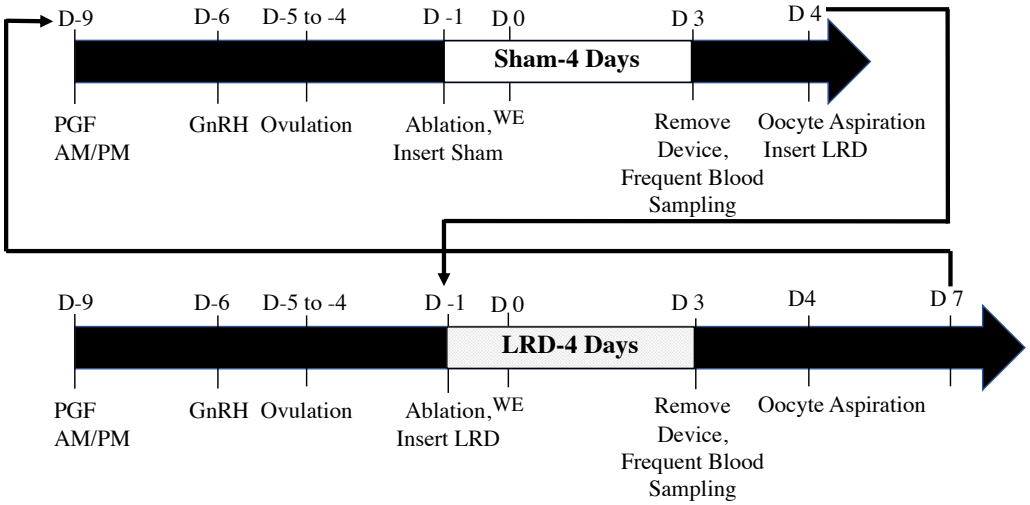
Table 5. 8**.** Mean (± SEM) plasma steroids, follicular and cumulus oocyte complex (COC) endpoints in heifers treated with either a letrozole releasing device (LRD) or a sham vaginal device in Experiment 2 and 3 combined.

	LRD	Sham	P-Value
Heifers (n)	56	57	-
Plasma progesterone (ng/mL)	4.5 ± 0.4	4.0 ± 0.2	0.34
Plasma estradiol (pg/mL)	5.2 ± 0.5	5.3 ± 0.4	0.80
Dominant follicle size at aspiration (mm)	10.2 ± 0.2	10.1 ± 0.2	0.54
Total follicles aspirated	12.7 ± 0.7	16.1 ± 1.0	<0.01
Compact good COC	0.9 ± 0.2	1.4 ± 0.2	0.10
Compact regular COC	3.1 ± 0.4	5.0 ± 0.6	0.02
Compact COC total	4.0 ± 0.5	6.4 ± 0.7	0.01
Expanded COC	0.3 ± 0.1	0.5 ± 0.1	0.28
Denuded COC	1.5 ± 0.2	1.7 ± 0.3	0.51
Degenerate COC	0.4 ± 0.1	0.3 ± 0.1	0.27
COC collected per heifer	6.3 ± 0.6	8.8 ± 0.9	0.02
COC collection rate (COC/follicles aspirated)	351/700 (50.7%)	504/918 (54.9%)	0.06

Supplementary Figures and Tables



Supplementary Figure 5.1. Experimental treatment and sampling timeline (Day 0 = day of ovarian follicular wave emergence). At the time of ultrasound-guided transvaginal ablation, heifers were given either a 4-day letrozole releasing device (LRD) or a sham device. Follicle sampling was done on Day 3 (pre-luteinizing hormone [LH] surge) or Day 5.5 (post-LH surge) in Experiment 1 (4 groups; n=7 to 8/group). PGF: 500 μg cloprostenol; GnRH: 100 μg gonadorelin; Ov: Ovulation



Supplementary Figure 5.2. Experiment timeline for Experiments 2 and 3. On Day -1, heifers underwent ultrasound-guided transvaginal ablation of follicles ≥5 mm to induce wave emergence and were given either a 4-day letrozole releasing device or a sham device. Devices were removed on Day 3 and on Day 4 heifers underwent ultrasound-guided transvaginal oocyte aspiration. Both experiments utilized a crossover design to facilitate individual heifers (n=24 to 33) receiving both treatments at different times.
PGF: 500 μg cloprostenol; GnRH: 100 μg gonadorelin

Supplementary Table 5.1. Precision and accuracy data from LC-MS/MS derived standard curve for letrozole in bovine follicular fluid.

Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
1 (LLQ)	0.96 ± 0.14	14.3%	96.5%
2	2.01 ± 0.26	13.0%	100.5%
4	4.25 ± 0.24	5.6%	106.3%
8	7.78 ± 0.61	7.9%	97.3%
16	16.37 ± 1.71	10.5%	102.3%
32	31.13 ± 1.40	4.5%	97.3%
64	62.27 ± 4.97	8.0%	97.3%
128	126.67 ± 2.08	1.6%	99.0%
256	260.33 ± 7.77	3.0%	101.7%

Each standard curve was independently run on 3 separate days (n=3/sample; lower limit of quantification (LLQ) n=18).

Supplementary Table 5.2. Precision and accuracy data from LC-MS/MS method for quality control samples of letrozole in bovine follicular fluid.

Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
3	2.94 ± 0.55	18.6%	98.3%
12	10.87 ± 1.57	14.3%	90.6%
120	114.47 ± 17.93	15.7%	95.4%

Each standard curve was independently run on 3 separate days (n=18/sample).

Supplementary Table 5.3. Reference genes, primer sequences and polymerase-chain reaction efficiency.

Gene	Accession no.	Oligo	Primer sequence (5' to 3')	Amplicon size (BP)	Efficiency (%)
GAPDH	NM_001034034.2	Forward	ACCCAGAAGACTGTGGATGG	178	73.2
		Reverse	CAACAGACACGTTGGGAGTG		
RPL19	NM_001040516.2	Forward	GCCAACTCCCGTCAGCAGA	154	100.3
		Reverse	TGGCTGTACCCTTCCGCTT		
RPLPO	NM_001012682.1	Forward	GGCGACCTGGAAGTCCAACT	149	97.0
		Reverse	CCATCAGCACCACAGCCTTC		
EIF2B2	NM_001015593	Forward	CATGAGATGGCAGTCAATTTGT	219	95.3
		Reverse	CTTGAACATAGGAGCACAGACG		
H2AFZ	NM_174809.2	Forward	GGTAAGGCTGGGAAGGACTC	181	99.7
		Reverse	CGGTGAGGTACTCCAGGATG		
B2M	XM_002691119.4	Forward	GCGTCCTCCAAAGATTCAAG	154	95.8
		Reverse	CAGGTCTGACTGCTCCGA		
SF3A1	NM_001081510	Forward	TGTGTCCCTCTTGCTGAGTTT	194	116.4
		Reverse	ATTCCTGGTTTCACGTCTCCTA		
RFN20	NM_001081587	Forward	TCCAGGTTCTCCTCGCTAATAC	153	95.8
		Reverse	GAGCCTGAAGAAGAGGTTCAAA		

Supplementary Table 5.4. Genes of interest, primer sequences and polymerase-chain reaction efficiency.

Gene	Accession no.	Oligo	Primer sequence (5' to 3')	Amplicon size (BP)	Efficiency (%)
HSD3B	XM_010803103.3	Forward	GCTAATGGGTGGGCTCTGAA	140	101
		Reverse	TGATTGGTCAGGATGCCGTT		
LHCGR	XM_015473421	Forward	GCTAATGCCTTTGACAACCTCC	91	98.2
		Reverse	TTGTAAACGCTCCAGCCTCA		
STAR	NM_174189.3	Forward	GAGATGGCTGGAAGAAGGTG	101	91.4
		Reverse	GCCAGATAACCCCATCTCAA		
INHBA	NM_174094.4	Forward	CTTCAGCTGCTCCTCTTGCT	157	92.8
		Reverse	TGACTCCAGGATCTCCACCT		
FSHR	XM_024998263.1	Forward	GCCAAAGGATTTGACGTGAT	167	100
		Reverse	GTGATGGCCAGGATGCTAAT		
Cyp11a1	NM_176644.2	Forward	CAGTGTCCCTCTGCTCAACCTCC	99	96.8
		Reverse	TTATTGAAAATTGTGTCCCATGCG		
Cyp19a1	NM_174305.1	Forward	GTGTCCGAAGTTGTGCCTATT	148	91.1
		Reverse	GGAACCTGCAGTGGGAAATGA		
NPR2	XM_024995444.1	Forward	CTGCTCCTAAGCTGGGTGAGT	99	95.2
		Reverse	GGTCATCTGTGCGAGCAT		
CCND2	XM_024992177.1	Forward	TGAGGAGCAGAAGTGCGAAG	199	99.2
		Reverse	AGGCTTGATGGAGTTGTCGG		

5.5. Discussion

In Experiment 1, letrozole treatment affected luteolysis as evinced by the larger CL and higher plasma progesterone concentrations following PGF treatment and device removal. As the terminal half-life of plasma letrozole in heifers has been reported to be 33 hours (Yapura et al., 2013) letrozole would be expected to be present in the circulation inhibiting estradiol production for at least that long after LRD removal. The low concentrations of plasma estradiol may explain the reduced rate of luteolysis in the LRD group. In cattle undergoing natural luteolysis (i.e., approximately 15 to 18 days after ovulation), exogenously administered estradiol has been shown to be associated with decreased plasma progesterone and increased plasma prostaglandin metabolite concentrations (Araujo et al., 2009). The estradiol receptor in the endometrium is

stimulated by endogenous estradiol, which in turn stimulates oxytocin receptor synthesis in the endometrium (Robinson et al., 2001). Oxytocin releases from the posterior pituitary and CL binds to the oxytocin receptor and PGF is released from the endometrium leading to the luteolytic cascade (Silvia et al., 1991; Mann et al., 2001). In the present study, luteolysis was induced with the prostaglandin F_{2a} analog, cloprostenol, which has a half-life of approximately 3 hours in cattle (Reeves, 1978). It may be hypothesized that the exogenously administered cloprostenol induced oxytocin release from the CL, but a lack of oxytocin receptors in the endometrium, because of the reduced estradiol environment, prevented the endogenous release of PGF. As estradiol began to increase slowly after removal of the LRD, oxytocin receptors were likely synthesized in the endometrium leading to the production of endogenous PGF and eventual luteolysis. Frequent blood sampling for plasma progesterone and prostaglandin metabolite following PGF in LRD-treated heifers is needed to test this hypothesis.

Interestingly, in the present study, letrozole concentrations were highly correlated in plasma and follicular fluid, although letrozole concentrations were lower in the follicular fluid portion. To our knowledge, this is the first study reporting letrozole concentrations in follicular fluid. Exogenous drugs or metabolites such as cotinine (a metabolite of nicotine) in women have been shown to be highly correlated between follicular fluid and serum. (Younglai et al., 2002). Serum and follicular fluid concentrations of steroids do not correlate in women, regardless of age (Smitz et al., 2007; von Wolff et al., 2017), although in the present study, plasma and follicular fluid concentrations of estradiol were correlated ($r=0.51$; $P<0.01$).

In the present study, there were no differences between treatments in expression of any of the genes analyzed in granulosa cells. Previous studies in letrozole-treated mice showed an upregulation of Cyp19 and FSHR, but no differences in LHR expression (Kauffman et al., 2015).

The idea that concentrations of androgens positively correlate to FSHR expression in granulosa cells (Weil et al., 1999), has been used to improve ovarian stimulation in women combining letrozole and FSH (Garcia-Velasco et al., 2005). The use of letrozole in combination with FSH did not increase the superovulatory response in cattle (Chapter 4), which is consistent with a lack of difference in mRNA expression in FSHR in the present study. It may be argued that the dose of letrozole given did not affect gene expression and an increased dosage may be expected to elicit an effect. It has been reported that a single 2.5 mg letrozole tablet taken orally resulted in peak circulating concentrations of letrozole of approximately 40 ng/mL in healthy postmenopausal women (Vanol et al., 2016). Circulating concentrations of letrozole in women who took repeated 2.5 mg per day for 6 months ranged from 57 to 290 ng/mL (Precht et al., 2012). Therefore, the plasma letrozole concentrations (mean 70 ng/mL) obtained in heifers during this study fall within range used to reduce estrogen levels for breast cancer treatment and ovarian stimulation in women (Mitwally and Casper, 2004); a prolonged elevation of intraovarian androgens may be needed to elicit a change in FSHR gene expression.

Treatment with letrozole decreased estradiol and increased LH pulsatility during the frequent 8-hour sampling period, although no changes were observed in serum FSH concentrations. This lack of difference in serum FSH concentrations following letrozole treatment confirms that inhibin may be the primary regulator of FSH secretion and FSH secretion does not differ following release of the negative feedback on the hypothalamus, but LH secretion is regulated by both estradiol and progesterone (Bergfeld et al., 1996; Gregory and Kaiser, 2004). In the present study, plasma progesterone concentrations were increased in the LRD group, and the concentrations of progesterone did not result in a decreased LH AUC. Therefore, it may be

hypothesized that estradiol may play a more inhibitive role on the LH secretory pattern than progesterone in cattle (reviewed in Price et al., 1999).

The high amplitude of LH pulsatility did not cause premature maturation in the LRD 0-hour maturation group, which did not support our hypothesis in Experiment 2. On the contrary, following 24 hours of maturation, the LRD group had the highest proportion of oocytes in the MII stage (Table 5.5). Similarly in women, co-stimulation with LH and FSH led to a higher number of metaphase II stage oocytes following *in vivo* induced maturation compared to FSH alone (De Placido et al., 2005). Although the increased LH amplitude may have increased oocyte maturation, it could be detrimental in early follicle development. At the time of oocyte aspiration, the number of follicles aspirated, and total number of oocytes recovered was lower in the LRD group. Our experimental protocol utilized transvaginal ablation of all ovarian follicles ≥5 mm in diameter to induce wave emergence approximately 1 day later (Bergfelt et al., 1997). Immediately following ablation of follicles (removal of inhibin and estradiol), FSH is expected to surge, but in our protocol, an LRD was inserted, and LH pulse amplitude may have increased. The dependency of follicles ≤7 mm on FSH is well characterized (Adams et al., 1992b), but the pulse frequency of LH during early follicular growth in cattle is not well defined. In cattle, maximal concentrations of FSH and LH in the circulation have been reported to occur 8- and 24 to 32-hours, respectively after follicle wave emergence (Ginther et al., 1998). Therefore, this lag in maximal LH concentrations may play a role in early follicular development, and high LH during this period may cause atresia in small follicles. Frequent blood samples would need to be taken at the time of device insertion to better understand whether this FSH to LH relationship exists during early follicle development.

In summary, despite estradiol concentrations that were decreased following treatment with an intravaginal LRD, no differences were detected in granulosa cell gene expression (Experiment 1). Treatment with an LRD increased pulse amplitude of plasma LH, but not FSH, and resulted in the highest proportion of COC reaching the metaphase II stage following 24 hours of *in vitro* maturation compared to all other groups (Experiment 2). Finally, letrozole treatment did not impact *in vitro* fertilization rates in heifers (Experiment 3). Future studies assessing the impact of LH on early follicle development should be done.

5.6. Acknowledgements

We thank Laurence Boligon de Araujo and David Moore for assistance in COC collections. CJ was supported through the Western College of Veterinary Medicine's Interprovincial Undergraduate Student Research program and Boehringer Ingleheim. Special thanks to Ky Pohler, Sydney Reese and Gessica Franco from Texas A&M University for and Susan Cook, Kim Tran, and Danielle Carriere at the University of Saskatchewan for assistance in running radioimmunoassays. The study was supported by grants from Alberta Agriculture and Forestry, Venco Animal Health (a subsidiary of Dechra Pharmaceuticals PLC), and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 6

6. DETERMINING THE INTERVAL TO OVULATION AND THE INITIAL DEVELOPMENT OF A FIXED-TIME ARTIFICIAL INSEMINATION PROTOCOL FOLLOWING AROMATASE INHIBITOR TREATMENT IN BEEF CATTLE

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Relationship of this study to the dissertation

In Chapter 5, we revealed that there was no deleterious effect of letrozole treatment on the ability of the oocyte to undergo *in vitro* maturation, fertilization, and culture. We investigated the interval to ovulation and developed a fixed-time artificial insemination protocol following treatment with a letrozole releasing device developed in Chapter 2. We hypothesized that ovulation and P/AI would be higher following letrozole treatment compared to two other fixed-time artificial insemination protocols.

Authors' Contributions

As first author, EMZ devised the experimental design, collected and interpreted data, performed statistical analysis and drafted the manuscript. WL collected and interpreted data in Experiment 3 as well as assisted in data collection in all experiments. As senior author, GPA provided the original experimental design, helped develop the new letrozole releasing device, and revised the manuscript.

6.1. Abstract

Experiments were done to determine the interval to ovulation following a 4-day letrozole treatment (Experiment 1), pregnancy per AI (P/AI) following letrozole treatment and fixed-time artificial insemination (FTAI) in a field trial (Experiment 2), and the effect of presynchronization with prostaglandin F_{2a} (PGF) prior to letrozole treatment with two different letrozole releasing devices (LRD; Experiment 3) on P/AI. In Experiment 1, nulliparous heifers and lactating beef cows (n=30/parity) at random stages of the estrous cycle were given an intravaginal LRD on Day 0 and PGF on Day 4. Cattle were then assigned randomly and given 100 μg GnRH at 48- or 60-hours after PGF, or no GnRH (n=10/parity/treatment). Transrectal ultrasonography was done every 8 hours from Day 6 until ovulation. In Experiment 2, nulliparous heifers (n=71) and lactating beef cows (n=126) at random stages of the estrous cycle were assigned randomly to one of three FTAI protocols, 1) a 4-day LRD, followed by PGF at LRD removal and GnRH concurrently with FTAI at 48 hours after PGF, 2) 2.5 mg estradiol-17β plus 50 mg progesterone (E2P4) in oil and a progesterone releasing intravaginal device (PRID) for 7 days, PGF at device removal and GnRH concurrently with FTAI at 60 hours after PGF or 3) a modified 5-day Cosynch plus PRID, with PGF given at device removal and GnRH concurrently with FTAI at 72 hours after PGF. All cattle were inseminated with frozen-thawed semen and had their ovaries examined by transrectal ultrasonography from FTAI until ovulation. In Experiment 3, nulliparous heifers (n=26) at random stages of the estrous cycle were assigned randomly to either PGF on Day -8 (Presynchronization) or no PGF (Control). On Day 0, heifers were again assigned randomly and given either a newly designed X-LRD or a T-LRD for three days. Devices were removed and PGF was given on Day 3 followed by GnRH and FTAI on Day 5 (48 hours later). On Days 0 and 3 vaginal irritation was assessed visually and assigned to a 5-point

scale where 1 was no irritation and 5 was widespread hyperemia with mucosal erosions. Blood sampling was done at 0, 2, 4, 6, 8, 10, 12 hours, and daily until Day 5 following device placement on a subset of heifers (n=10/group) to determine plasma letrozole concentrations. Binary data were compared by GLIMMIX and continuous data by ANOVA or t-test. In Experiment 1, there was no difference in the proportion of cattle that ovulated following letrozole treatment (overall 54/60 [90%]). Cattle in the GnRH 48-hour group ovulated earlier ($P=0.03$) than either the GnRH 60-hour or no-GnRH groups. Cows in the GnRH 48-hour group ovulated more synchronously ($P<0.05$) than either the Cow 60-hour or Heifer 48-hour groups. In Experiment 2, the dominant follicle was larger ($P<0.001$) and the P/AI was lower in the LRD group compared to both the E2P4 and PRID groups (14/69 [20.3%] vs. 35/58 [60.3%] and 31/69 [44.9%], respectively; $P<0.001$). In Experiment 3, the X-LRD resulted in higher ($P<0.0001$) plasma letrozole concentrations during treatment and decreased ($P=0.14$) vaginal irritation score numerically on Day 3 compared to the T-LRD. The P/AI did not differ ($P=1.0$) for synchronization treatment protocol or between devices. In summary, although ovulation synchrony was achieved following letrozole treatment, the LRD group had the lowest P/AI compared to other estrus synchronization protocols. The X-LRD improved letrozole release and was considered appropriate for future studies for understanding the interval to estrus and ovulation following letrozole treatment in beef cattle.

6.2. Introduction

The aromatase inhibitor, letrozole, was initially developed for the suppression of tumor growth during breast cancer treatment because it prevented estrogen synthesis (Iveson et al., 1993). Letrozole reversibly binds to the P450 heme group of the aromatase enzyme. In addition, letrozole was found to be more effective in inducing ovulation in women with polycystic ovarian syndrome (PCOS) than the selective estrogen receptor modulator, clomiphene citrate (Mitwally and Casper, 2001). Letrozole is used in combination with human menopausal gonadotropin (hMG) for controlled ovarian stimulation in women with PCOS. An increase in fertility with letrozole and hMG co-treatment compared to hMG alone was attributed to lower estradiol levels and increased receptivity of the endometrium (D'Amato et al., 2018).

Currently, two synchronization strategies are employed worldwide for fixed-time artificial insemination (FTAI) in cattle; gonadotropin releasing hormone(GnRH)- and steroid-based protocols (Bo et al., 1993; Pursley et al., 1995). The efficacy of GnRH-based protocols is dependent on the ability of the first administration of GnRH to induce ovulation of a dominant follicle and synchronize the emergence of a new follicular wave. When GnRH is given at random stages of the estrous cycle, 44 to 54% of dairy cows ovulated, resulting in a failure to synchronize follicle wave emergence in half of the cows (Bello et al., 2006; Colazo et al., 2009). To increase the proportion of ovulation in response to the first GnRH administration, presynchronization with prostaglandin (PGF) has been used before initiating the GnRH-based protocol (Presynch; Moreira et al., 2001) and successive GnRH-based protocols (Double Ovsynch; Souza et al., 2008). Steroid-based protocols involve giving estradiol and progesterone to induce follicle wave emergence approximately 4 days later (Bo et al., 1993), followed by luteolysis induced by PGF and FTAI. Steroid-based protocol are short in duration and effective;

>11 million FTAI treatments were administered in Brazil in 2016 utilizing this technology (Mapletoft et al., 2018). With increasing consumer concern over steroid use in food-producing animals, estradiol is prohibited for use in estrus synchronization protocols in food producing animals in the United States, Europe, and in milk-producing animals in Australia and New Zealand, but can still be obtained by veterinarian prescription in Canada (Lane et al., 2008; AETA, 2019).

To fill this void in commercially approved protocols, letrozole has been investigated in our laboratory for ovarian synchronization in cattle (Yapura et al., 2018). Letrozole is delivered through a novel intravaginal device ((Yapura et al., 2015); Chapter 2) that has been used for ovulation induction (Yapura et al., 2016) and co-superstimulation with follicle-stimulating hormone ((Zwiefelhofer et al., 2019); Chapter 4). We have characterized follicular fluid concentrations of letrozole in cattle treated with an intravaginal letrozole releasing device (LRD) and the subsequent decline of estradiol production in both dominant and subordinate follicles (Chapter 5). Letrozole-treatment in cattle has been shown to have no deleterious effects on granulosa cell function, nuclear maturation of the oocyte, the ability of oocyte to undergo *in vitro* fertilization (Chapter 5). In an early estrus synchronization study (Yapura et al., 2016), 92% of heifers ovulated following a 4-day regimen of letrozole treatment, with PGF at device removal and GnRH concurrently with FTAI 24 hours later. However, only 3 of 43 became pregnant following FTAI (Yapura, 2015). The reason behind the low P/AI may have been due to the short interval from PGF to GnRH (24 hours) resulting in short-lived corpora lutea (CL) following ovulation as previously described (Rantala et al., 2009).

Intravaginal administration of letrozole was found to be effective because of continuous prolonged drug release and ease of delivery ((Yapura et al., 2015); Chapter 2). Giving a drug

over multiple days by im or iv administration is labor intensive and can be avoided by utilization of intravaginal drug delivery. However, mucopurulent discharge is commonly observed at the time of intravaginal device removal in cattle (Walsh et al., 2008; Yapura et al., 2015). No differences in P/AI have been reported in cattle with or without evidence of mucopurulent discharge following removal of an intravaginal progesterone device (Villarroel et al., 2004), but intravaginal devices have been associated with increased vaginal pH and decreased fertility following natural breeding in sheep (Martinez-Ros et al., 2018). As cattle producers may perceive vaginal discharge following device removal as undesirable in estrus synchronization protocols, minimizing vaginitis may make an intravaginal device more attractive for commercial use.

We hypothesized that letrozole-based synchronization protocols for FTAI will result in a higher P/AI compared to commonly used protocols, and a newly developed LRD will increase letrozole release and decrease vaginitis. The objectives of these studies were to 1) determine the interval to ovulation following letrozole treatment, 2) compare P/AI following letrozole treatment compared to other commonly used estrus synchronization protocols and 3) develop a new intravaginal letrozole-releasing device to maximize letrozole release and minimize vaginitis.

6.3. Materials and Methods

Procedures were approved by the University of Saskatchewan's Animal Research Ethics Board in accordance with the guidelines of the Canadian Council on Animal Care. Cattle were housed at the University of Saskatchewan's Livestock and Forage Centre of Excellence Goodale Farm in outdoor pens. Cattle were fed a mixed ration consisting mainly of barley silage and had *ad libitum* access to alfalfa hay and water. Experiments 1 utilized nulliparous Hereford-cross

heifers (n=30; weight: 395 ± 8 kg; BCS: 3.3 ± 0.06 on 1-5 scale (Richards et al., 1986) and lactating beef cows (n=30; weight: 640 ± 15 kg; BCS: 2.9 ±0.08) >42 days postpartum during the month of May. In Experiment 2, sexually mature, nulliparous, Hereford-cross heifers (n=71; weight: 438 ± 6 kg; BSC: 3.2 ± 0.2) and lactating beef cows (>42 days postpartum; n=126; weight: 666 ± 9 kg; BCS: 3.2 ± 0.03) were used during the months of June and July. Experiment 3 was conducted during the months of August and September and utilized non-pregnant, nulliparous heifers (n=26) previously used in Experiment 2.

6.3.1. Experiment 1 – Interval to ovulation following letrozole treatment in beef heifers and cows

Cattle were given an LRD at random stages of the estrous cycle (Day 0) for 4 days followed by a luteolytic dose of PGF (500 μg cloprostenol im; Estrumate, Merck, Kirkland, QC) on Day 4. Following PGF, cattle were assigned randomly to one of three groups and given GnRH (100 μg gonadorelin im; Fertiline, Vetoquinol N.A., Lavaltrie, QC) at 48- (Day 6) or 60-hours after device removal and PGF (Day 6.5) or no GnRH (n=10 cows and 10 heifers per group; Figure 6.1). Beginning on Day 6, transrectal ultrasonography (7.5 MHz linear array transrectal probe; MyLab Alpha; Esaote North America, Fishers, IN) was done at 8-hour intervals for 4 days or until ovulation was detected to measure the diameter of the ovulatory follicle and determine ovulation. Daily ultrasonography was done for 8 days following ovulation to assess CL diameter.

6.3.2. Experiment 2 - Fixed-time artificial insemination concurrently with GnRH at 48 hours after LRD removal

At random stages of the estrous cycle cattle were assigned randomly to one of the following three groups (Figure 6.2): 1) a 4-day LRD, followed by PGF at LRD removal and GnRH concurrently with FTAI at 48 hours after PGF (n=46 cows, 23 heifers), 2) 2.5 mg estradiol-17β plus 50 mg progesterone (E2P4) in oil (Bo et al., 1994) and a progesterone releasing intravaginal device (PRID; Vetoquinol N.A., Lavaltrie, QC, Canada) for 7 days, PGF at device removal and GnRH concurrently with FTAI at 60 hours after PGF (n=40 cows, 18 heifers) or 3) a modified 5-day Cosynch plus PRID with no GnRH given at PRID insertion, PGF given at device removal and GnRH concurrently with FTAI at 72 hours after PGF (n=39 cows, 30 heifers). Cattle were FTAI with frozen-thawed semen as part of another experiment (Yang et al., unpublished). The ovaries were examined for ovulatory follicle diameter by transrectal ultrasonography on the day of PGF and AI, and daily for two days following AI until ovulation was detected (Day 0). A subgroup of cows (n=20/group) underwent transrectal ultrasonography for CL diameter on Day 10 and 20. Pregnancy diagnosis was done 28 days after ovulation by transrectal ultrasonography to confirm the presence of an embryo proper with a heartbeat.

6.3.3. Experiment 3. Development of a new intravaginal LRD

At random stages of the estrous cycle, heifers were assigned randomly and given PGF on Day -8 (Presynchronization; Day 0=device insertion) or no presynchronization (Control) and one of two letrozole releasing devices (a newly designed LRD (X-LRD; Figure 6.3A) or the LRD designed in Chapter 2 (T-LRD; Figure 6.3B) on Day 0. Heifers in the presynchronization group were given a luteolytic dose of PGF im on Day -8. In all groups, the LRD were inserted on Day 0

and removed on Day 3 (Figure 6.4). At that time, heifers were given PGF and fitted with an electronic estrus detection sensor (Heatwatch, Cow Chips, Manalapan, NJ, USA) attached to the tailhead to record the interval to estrus. On Day 5 (i.e., 48 hours after PGF), heifers were given GnRH (100 μg gonadorelin im; Fertiline, Vetoquinol N.A., Lavaltrie, QC, Canada) concurrently with FTAI with frozen-thawed semen (Semex, Guelph, ON, Canada). The ovaries were examined daily by transrectal ultrasonography to monitor follicular and luteal dynamics from Day 0 to 5 and twice daily from Day 5 until ovulation was detected. Vaginoscopy was done on a subgroup of heifers (n=5 per treatment group/4 total groups) on Day 0 and Day 3 to record the appearance of the vaginal wall, the nature of the vaginal secretions, and to collect a vaginal fornix swab sample for cytology. The cytological samples were taken from around the cervical os using a sterile, double-guarded culture swab and a disposable vaginal speculum. The culture swab was then smeared onto a microscope slide that was air-dried, stained with hematoxylin and eosin and examined by wide-field microscopy (20x magnification) for the number of neutrophils per 100 cells. The gross appearance of the vaginal wall was scored on a 5-point scale where 1 was no irritation and 5 was widespread hyperemia with mucosal erosions. The vaginal discharge was also scored on a 5-point scale where 1 was clear mucus, 2-3 mucopurulent, and 5 was frank purulent material. The interval to onset of estrus (hours from PGF to onset of estrus) was derived from HeatWatch software. The return to estrus rate was determined by the number of heifers that returned to estrus within 10 days of FTAI. Pregnancy diagnosis was done 28 days after ovulation by transrectal ultrasonography as described in Experiment 2.

6.3.4. Blood sampling, radioimmunoassays, and LC-MS/MS

In all experiments, blood samples were taken by jugular venipuncture into heparinized tubes (Vacutainer; Becton Dickinson and Co., Franklin Lakes, NJ) which were centrifuged in <2 hours of collection at 1,500 x *g*, and plasma was aliquoted into polypropylene tubes and frozen at -20°C until assay. In Experiment 1, blood samples were taken on Days 0, 3, 4, 5, 6, and 3x per day from Days 6 to 7.3. Blood samples were then taken 2, 4, 6, and 8 days following post-treatment ovulation. In Experiment 2, blood samples were taken from a subset of cows (n=20/group) at the time FTAI, ovulation, and 10 and 20 days post-ovulation. In Experiment 3, blood samples were taken daily from Day 0 to 5. On a subset of heifers (n=5/group) frequent blood samples were taken for plasma letrozole at 0, 2, 4, 6, 8, 10, 12 hours after device placement.

Plasma concentrations of estradiol were determined by radioimmunoassay as previously described (Kirby et al., 1997) at Texas A&M University. The minimum detection limit was 0.5 pg/mL, and the intra- and inter-assay coefficients of variation (CV) were 2.4 and 3.9%, respectively.

Plasma concentrations of luteinizing hormone (LH) were determined by radioimmunoassay as previously described (Rawlings et al., 1984). The minimum detection limit was 0.1 ng/mL and intra-assay CV for low and high samples were 7.3 and 6.0%, respectively.

Plasma concentrations of progesterone (P4) were determined using a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA, USA) with a minimum detection limit of 0.1 ng/mL. Intra- assay CV for low, medium, and high samples were 9.0, 6.8, and 3.2%, respectively. Inter-assay CV for low, medium and high samples were 14.3,

8.4 and 17.4%, respectively. Plasma concentrations of letrozole were analyzed by LC-MS/MS as previously described (Chapter 2).

6.3.5. Statistical Analyses:

Data were analyzed using SAS Enterprise Guide (Version 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical data (interval to ovulation and estrus, residuals, CL or dominant follicle diameter and steroid levels at defined timepoints, neutrophil counts, and vaginal discharge/irritation scores) were analyzed by 2x3 (Experiment 1 and 2) or 2x2 (Experiment 3) ANOVA. Serial data (CL or dominant follicle diameter profiles and steroid, hormone and letrozole profiles) were analyzed by analysis of variance for repeated measures using the MIXXED procedure. Binary data (ovulation and return to estrus rate, and P/AI) were analyzed by the GLIMMIX procedure using binary distribution and link logit function. If an interaction was detected, post hoc analyses were done using least significant difference. Significance was defined as $P \leq 0.05$, and data are presented as mean ± SEM unless stated otherwise.

6.4. Results

6.4.1. Experiment 1 – Interval to ovulation following letrozole treatment in beef heifers and cows

There was no difference between parity or GnRH treatment for ovulation rate within 120 hours after PGF (total 54/60 [90%]; Table 6.1). An effect of GnRH on the interval from PGF to ovulation was attributed to a shorter interval in the 48-hour group compared to the 60-hour and no GnRH groups (P=0.03; Table 6.1). A tendency (P=0.08) for variation in the interval to ovulation was due to decreased variation in the cow GnRH 48-hour GnRH group compared to

both the cow 60-hour GnRH and heifer 48-hour GnRH groups (P<0.05; Table 6.1). Cattle in the no GnRH group ovulated a larger (P<0.01) follicle than either the 48- and 60-hour GnRH groups (Table 6.1). An effect of GnRH treatment (P<0.001) on plasma estradiol concentrations was due to lower plasma estradiol during treatment in the heifer 60-hour GnRH group (Fig. 6.5). An interaction (P<0.001) for GnRH treatment by day for plasma estradiol in heifers was detected, and individual day comparisons are shown in Fig. 6.5. No differences were detected for plasma estradiol concentrations in cows (Fig. 6.6), plasma LH in heifers or cows, plasma progesterone following post-treatment ovulation in heifers (Fig. 6.7a), or CL diameter following post-treatment ovulation in cows (Fig. 6.8b). A GnRH treatment-by-day interaction (P=0.001) was detected for the CL diameter following post-treatment ovulation in heifers attributed to a decreased diameter in the 60-hour GnRH group 8 days after ovulation (Fig. 6.7b). A GnRH treatment-by-day interaction (P<0.01) was detected for plasma progesterone following post-treatment ovulation in cows. The interaction was due to increased plasma progesterone 4-days following ovulation in the 60-hour GnRH group vs. the 48-hour GnRH group (P<0.05; Fig. 6.8a).

6.4.2. Experiment 2 - Fixed-time artificial insemination concurrently with GnRH at 48 hours after LRD removal

The dominant follicle at device removal was larger (P<0.001) in the LRD vs. E2P4 and PRID groups (Table 6.2) as well as larger (P<0.001) in the cow vs. heifer groups (Table 6.2). The ovulatory follicle on the day of FTAI was larger (P<0.001) in the LRD and E2P4 groups compared to PRID groups (Table 6.2) and the heifer LRD group had the largest ovulatory follicle which led to a significant interaction of parity by FTAI protocol (P=0.05). No differences were detected between FTAI protocols for plasma progesterone (P=0.91) or estradiol (P=0.97) on the

day of AI (Table 6.2). Similarly, no differences for parity (P=0.99) or FTAI protocol (P=0.93) were detected for the proportion of cattle that ovulated following FTAI (Table 6.2). A tendency (P=0.08) for a greater proportion of animals ovulated before FTAI in the LRD and PRID group than the E2P4 group (Table 6.2). A greater (P=0.02) proportion of animals ovulated by 24 hours after FTAI in the E2P4 group than the LRD and PRID group (Table 6.2). The E2P4 group resulted in a longer (P=0.04) interval to ovulation compared to the LRD and PRID groups (Table 6.2). The interval from FTAI to ovulation was also more synchronous (P<0.01) in the E2P4 group compared to both the LRD and PRID groups (Table 6.2) and in heifers compared to cows (P=0.04; Table 6.2). Cattle in the LRD group had a lower (P<0.001) P/AI compared to the E2P4 and PRID groups (Table 6.2). No difference between FTAI protocols or interactions for plasma progesterone or CL diameter following post-treatment ovulation (Fig. 6.9) were observed in cows.

6.4.3. Experiment 3 - Development of a new intravaginal LRD

No differences or interactions were detected between FTAI protocols or devices for the number of neutrophils per 100 cells on Day 0 and 3, vaginal irritation score on Day 3, proportion of heifers that displayed estrus, or the interval from PGF to estrus (Table 6.3). There were no differences between FTAI protocols or devices for ovulation within 48 hours after GnRH, plasma progesterone 10 days following post-treatment ovulation, proportion of heifers that returned to estrus within 10 days of ovulation, or P/AI (Table 6.3). The presynchronization group resulted in a reduced (P=0.001) vaginal discharge score on Day 3 than the control group (Table 6.3). There were no differences in plasma letrozole concentrations among FTAI protocol groups; therefore, their data were combined. The X-LRD resulted in increased (P<0.001) plasma

letrozole compared to the T-LRD throughout treatment (Fig. 6.10a). A device by day interaction ($P<0.0001$) for plasma letrozole was detected and post-hoc analysis revealed that the X-LRD resulted in increased ($P<0.05$) plasma letrozole from 12 hours- to 5 days-following device insertion (Fig. 6.10a). There was no effect of FTAI protocol ($P=0.83$) or device ($P=0.13$) for plasma estradiol concentrations during treatment. The CL diameter during treatment was greater ($P=0.05$) in the presynchronization group and a FTAI protocol-by-device-by-day-interaction ($P=0.03$) was detected for CL diameter and comparisons are shown in Figure 6.11a. A FTAI protocol-by-device-by-day interaction ($P<0.01$) for plasma progesterone was due to a higher concentration of progesterone on Day 0 in the X-LRD Random group (Fig. 6.11b). A FTAI protocol-by-day interaction ($P<0.01$) for dominant follicle diameter was due to an increased diameter on Day 2 and 4 in the presynchronization group (Fig. 6.12).

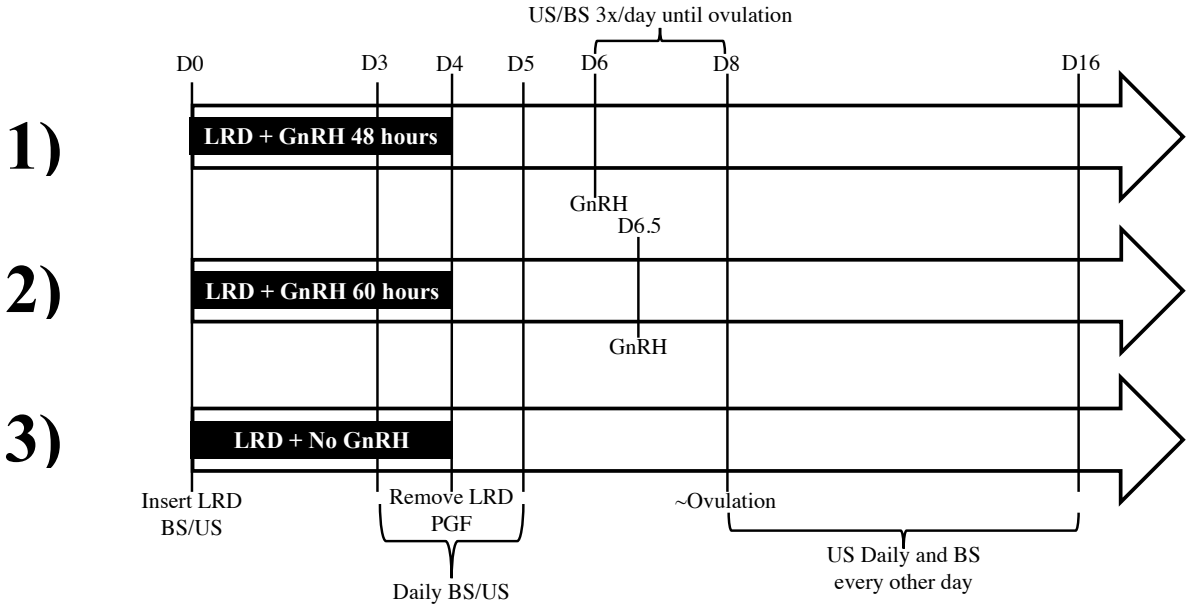

Figure 6. 1**.** Experimental timeline for heifers and cows (n=10/parity/group) treated with a letrozole releasing device (LRD) for 4 days at random stages of the estrous cycle (Experiment 1). On Day 4, cattle were given 500 μg cloprostenol (PGF) and assigned randomly to one of three groups and given 100 μg gonadorelin (GnRH) at 48 or 60 hours after PGF, or no GnRH. Transrectal ultrasonography (US) was done 3x per day until ovulation was detected. BS: blood sampling

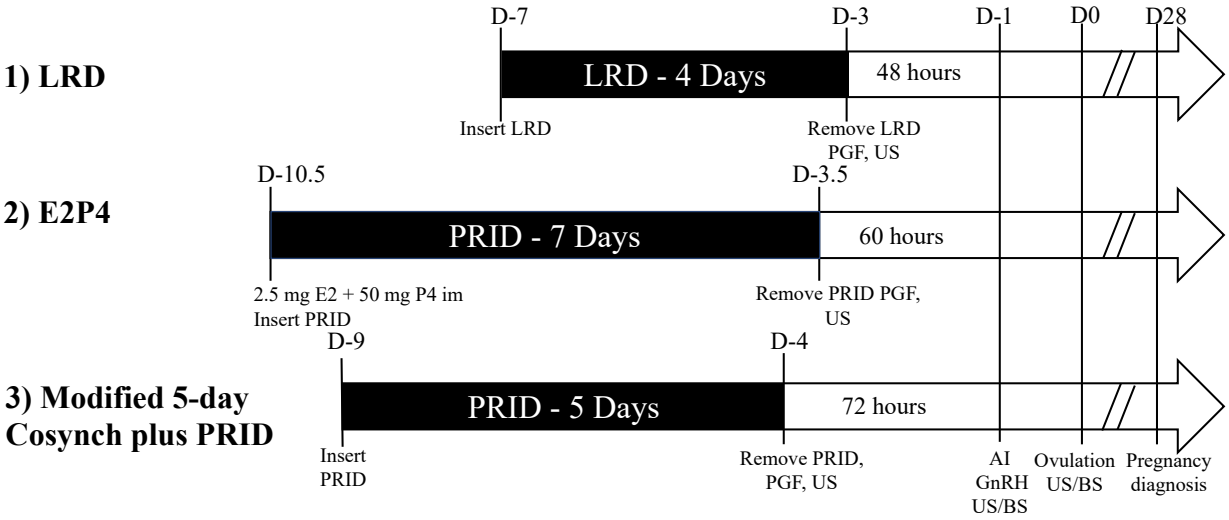

Figure 6. 2**.** Experimental timeline for cattle synchronized for fixed-time artificial insemination. At random stages of the estrous cycle, cattle were assigned randomly to one of three treatment: 1) a 4-day LRD, followed by 500 μg cloprostenol (PGF) at LRD removal and 500 μg gonadorelin (GnRH) concurrently with FTAI at 48 hours after PGF, 2) 2.5 mg estradiol-17β plus 50 mg progesterone (E2P4) in oil and a progesterone releasing intravaginal device (PRID) for 7 days, PGF at device removal and GnRH concurrently with FTAI at 60 hours after PGF or 3) a modified 5-day Cosynch plus PRID with no GnRH given at PRID insertion, PGF given at device removal and GnRH concurrently with FTAI at 72 hours after PGF (Experiment 2) E2: estradiol; P4: progesterone; LRD: letrozole releasing device; US: transrectal ultrasonography; AI: artificial insemination

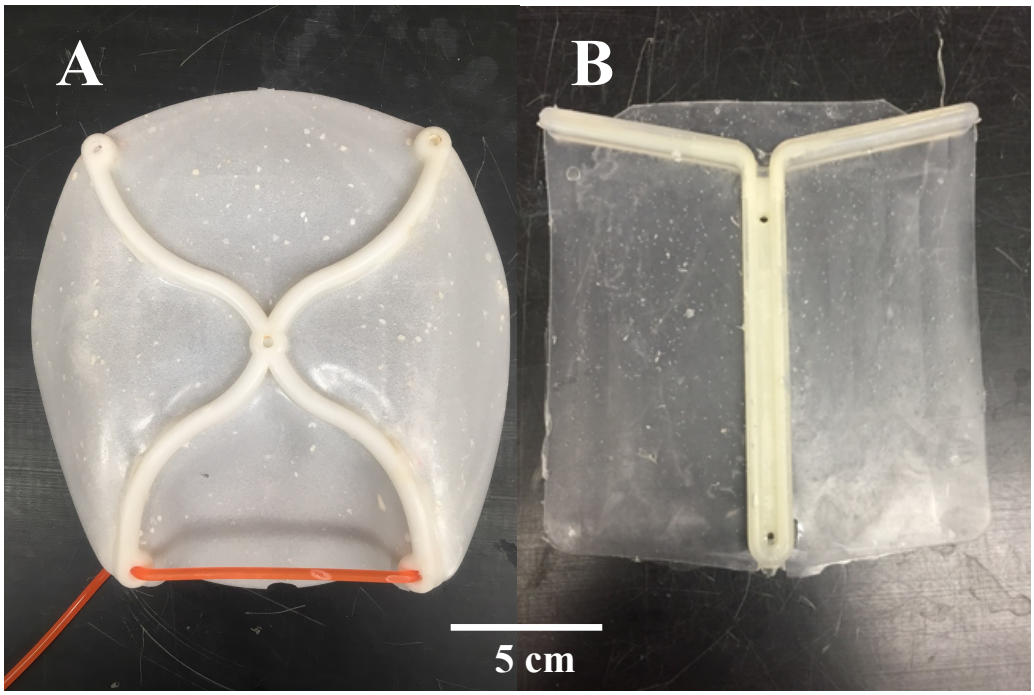

Figure 6. 3**.** A newly designed X-letrozole releasing device (X-LRD; A) compared to the T-LRD (B). The T-LRD was used in Experiment 1, 2 and 3. The X-LRD was developed in Experiment 3 to minimize strain on the vaginal wall while maintaining identical surface areas. The X-LRD exhibits less rigidity on the cranial vagina.

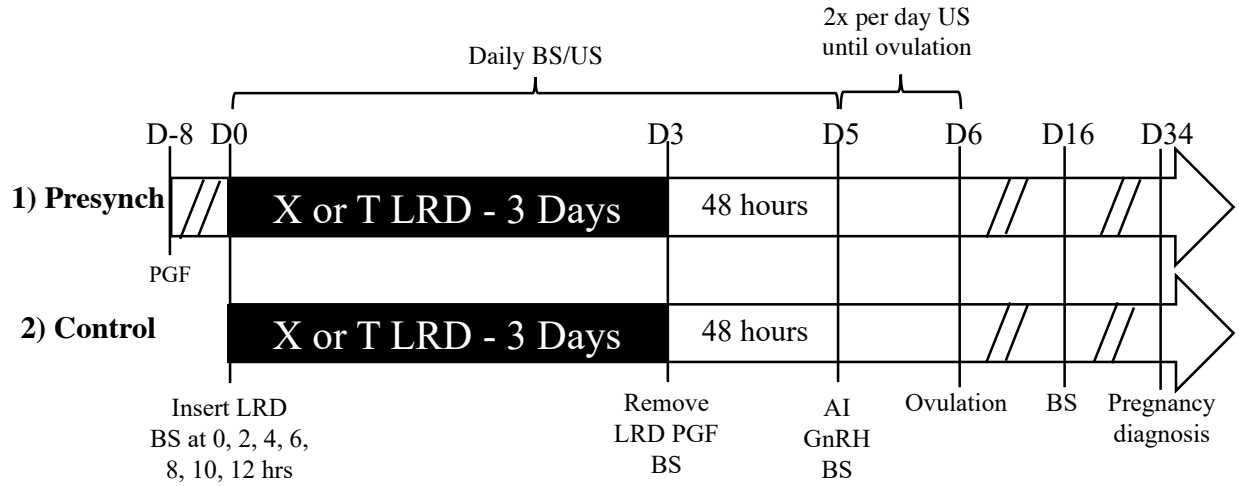

Figure 6. 4**.** Experimental timeline in Experiment 3 for heifers either 1) presynchronized on Day -8 (Presynch) with 500 µg cloprostenol (PGF) or not (Control) prior to insertion of a letrozole releasing device (LRD). On Day 0, heifers were assigned randomly and given either a X-LRD or T-LRD (4 total groups; n=5 to 8 heifers/group) for 3 days. On Day 3, the device was removed and PGF was given followed by 100 µg gonadorelin (GnRH) concurrently with artificial insemination (AI) 48 hours after PGF. Twice daily transrectal ultrasonography (US) was done until ovulation. BS: blood sampling

Table 6. 1**.** Endpoints (Mean ± SEM) for Experiment 1 from cows and heifers given a 4-day treatment of letrozole prior to FTAI.

	Cows			Heifers			P-value		
Endpoint	48 hrs	60 hrs	No GnRH	48 hrs	60 hrs	No GnRH	Parity	GnRH	Parity x GnRH
Animals (n)	10	10	10	10	10	10	-	-	-
Ovulation rate[1]	10/10	10/10	9/10	8/10	9/10	8/10	0.97	0.99	0.99
Interval to ovulation (hrs)[2]	77.6±2.4	83.8±7.5	87.8±5.3	70.0±4.4	82.9±4.7	86.0±4.3	0.41	0.03	0.77
Residuals of ovulation (hrs)[3]	5.6±2.5[a]	19.0±6.3[b]	12.0±4.5[ab]	17.9±2.1[b]	13.0±3.7[ab]	12.2±3.0[ab]	0.39	0.66	0.08
Ovulatory follicle diameter (mm)	16.4±0.6	16.3±0.9	18.4±0.5	14.3±0.7	14.6±1.0	16.3±0.9	<0.01	0.03	0.69

Cattle were given PGF at the timing of device removal and then given GnRH at 48-, 60-hours after PGF, or no GnRH.
[ab]Within rows, values with no common superscript are different (P<0.05)
[1]Proportion of animals that ovulated within 120 hours after PGF
[2]Hours from PGF to ovulation
[3]Variation in ovulation timing

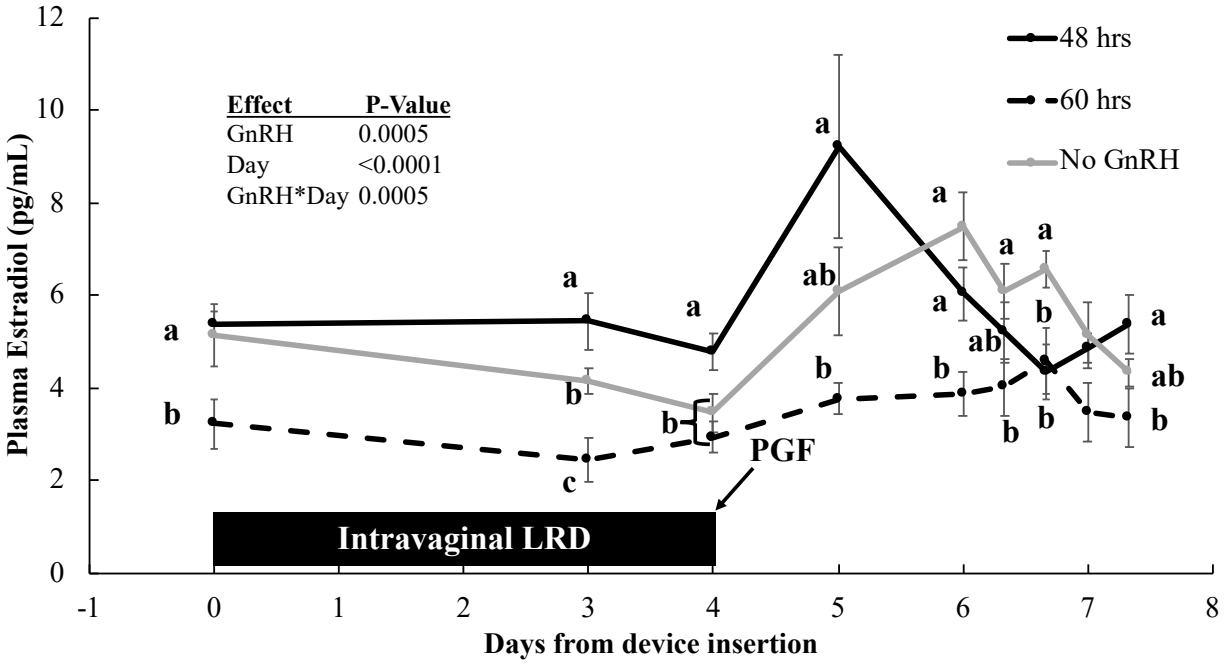

Figure 6. 5**.** Mean (± SEM) plasma concentrations of estradiol in heifers given a letrozole releasing device (LRD) for 4 days. On Day 4, heifers were given 500 µg cloprostenol (PGF) and assigned randomly to one of three groups and given 100 µg gonadorelin (GnRH) at 48 or 60 hours after PGF, or no GnRH in Experiment 1. [abc]Within days, values with no common superscripts are different (P<0.05).

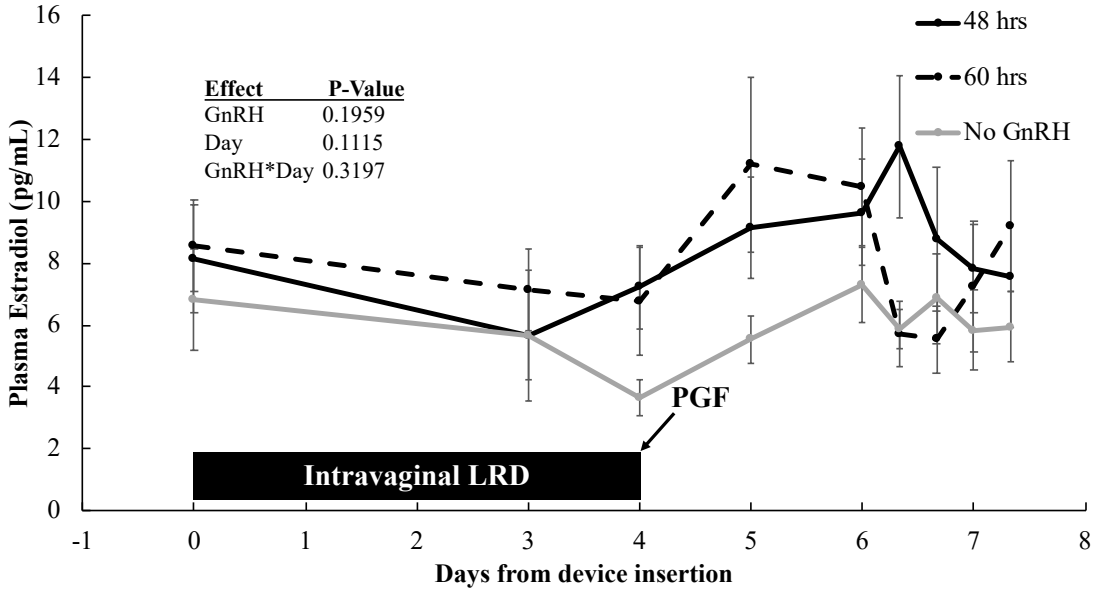

Figure 6. 6**.** Mean (± SEM) plasma concentrations of estradiol in cows given a letrozole releasing device (LRD) for 4 days. On Day 4, cows were given 500 μg cloprostenol (PGF) and assigned randomly to one of three groups and given 100 μg gonadorelin (GnRH) at 48 or 60 hours after PGF, or no GnRH in Experiment 1.

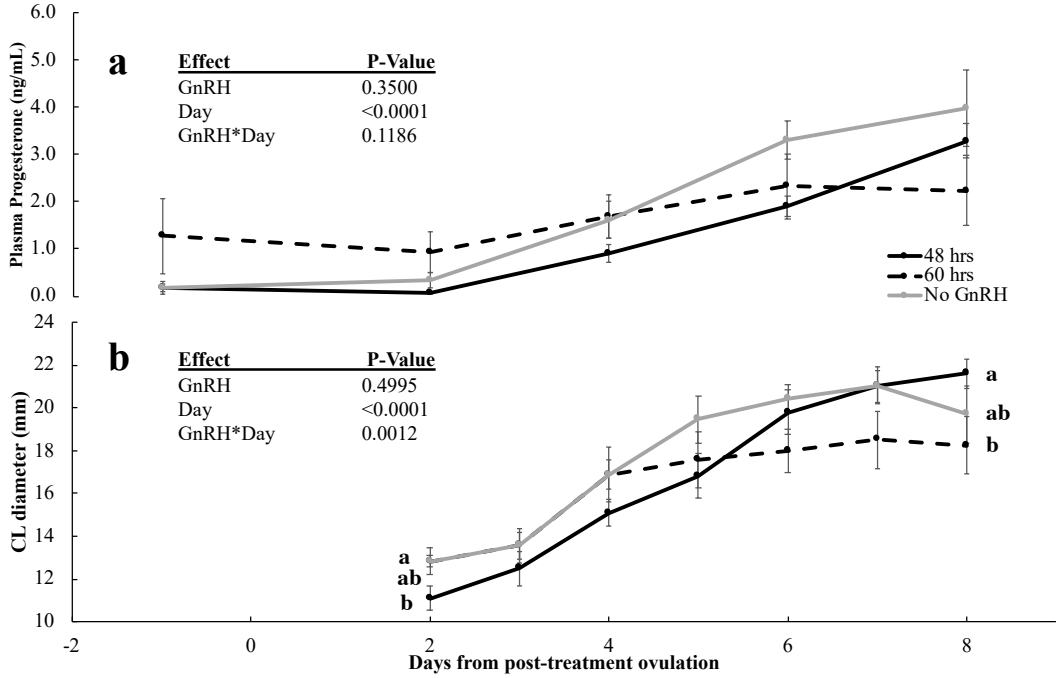

Figure 6. 7**.** Mean (± SEM) plasma progesterone concentration (a) and CL diameter (b) in heifers given a letrozole releasing device (LRD) for 4 days in Experiment 1. Heifers were assigned randomly and given GnRH at 48- or 60-hours after PGF, or no GnRH. Transrectal ultrasonography was done 3x per day to determine ovulation. [ab]Within days, values with no common superscripts are different (P<0.05).

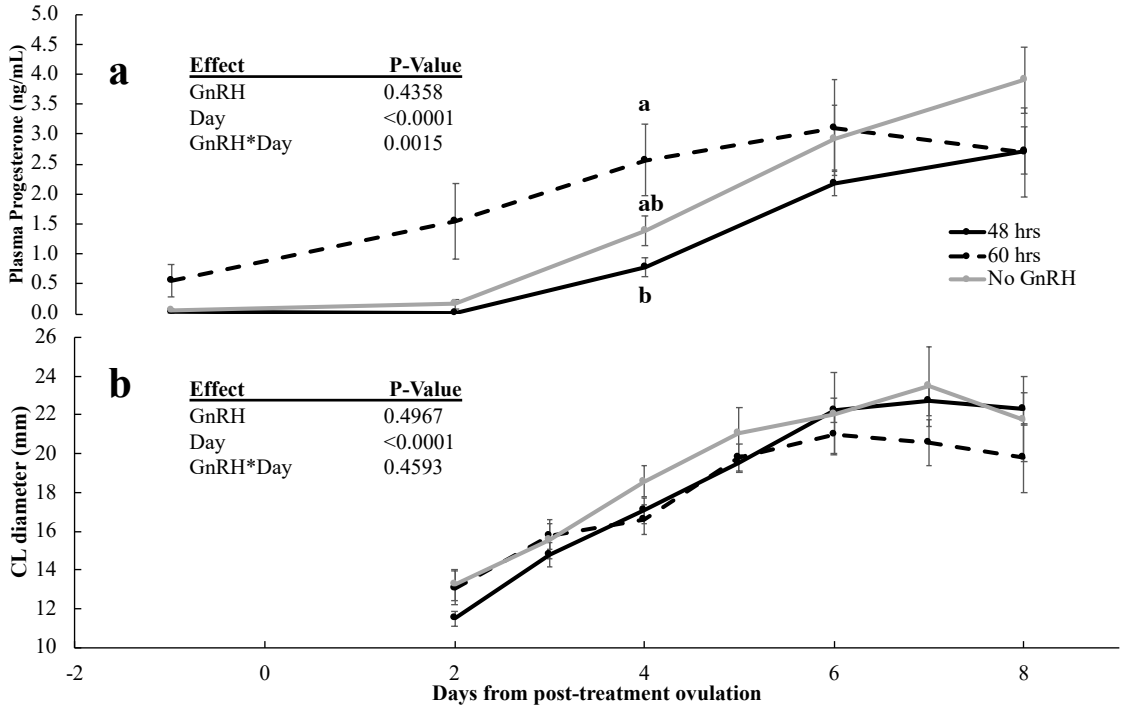

Figure 6. 8**.** Mean (± SEM) plasma progesterone (a) and CL diameter (b) in cows given a letrozole releasing device (LRD) for 4 days in Experiment 1 Cows were assigned randomly and given GnRH at 48- or 60-hours after PGF, or no GnRH. Transrectal ultrasonography was done

3x per day to determine ovulation. [ab]Within days, values with no common superscripts are different (P<0.05).

Table 6. 2**.** Endpoints from heifers and cows given either a 4-day letrozole releasing device (LRD), 2.5 mg estradiol plus 50 mg progesterone and a progesterone releasing intravaginal device (E2P4) for 7 days, or a modified Cosynch plus a progesterone releasing intravaginal device (PRID) for 5 days in Experiment 2. Mean ± SEM

	Cows			Heifers			P-value		
Endpoint	LRD	E2P4	5-day Cosynch	LRD	E2P4	5-day Cosynch	Parity	Protocol	Parity x Protocol
Animals (n)	46	40	39	23	18	30	-	-	-
OF at device removal (mm)	13.9±0.5	12.6±0.4	11.9±0.6	12.9±0.5	10.2±0.4	11.0±0.4	<0.001	<0.001	0.34
OF at FTAI (mm)	14.7±0.4[ab]	14.7±0.3[ab]	14.0±0.3[b]	15.4±0.5[a]	13.9±0.4[bc]	12.9±0.4[c]	0.19	<0.001	0.05
Plasma P4 at FTAI (ng/mL)*	0.11±0.05	0.08±0.04	0.08±0.05	-	-	-	-	0.91	-
Plasma E2 at FTAI (pg/mL)*	7.6±1.2	7.3±0.9	7.6±0.9	-	-	-	-	0.97	-
Ovulated by FTAI	8/46 (17%)	1/40 (3%)	6/39 (15%)	3/23 (13%)	0/18 (0%)	3/30 (10%)	0.36	0.08	0.99
Ovulated by 24 hrs after FTAI	28/46 (61%)	33/40 (83%)	28/39 (72%)	28/39 (78%)	18/18 (100%)	20/30 (67%)	0.17	0.02	0.39
Ovulated by 48 hrs after FTAI	5/46 (11%)	6/40 (15%)	4/39 (10%)	0/23 (0%)	0/18 (0%)	4/30 (13%)	0.14	0.61	0.99
Interval to ovulation (hrs)[2]	26.2±2.3	32.2±1.4	27.1±2.2	26.0±2.4	30.4±0.4	25.3±2.2	0.48	0.04	0.92
Ovulation synchrony (hrs)[3]	39.0±6.1	17.1±3.6	31.8±6.4	28.6±6.7	4.4±0.7	23.4±7.2	0.04	<0.01	0.94
Pregnancy per AI	7/46 (15%)	25/40 (63%)	15/39 (39%)	7/23 (30%)	10/18 (56%)	16/30 (53%)	0.22	<0.001	0.33

LRD: letrozole releasing device; E2P4: 2.5 mg estradiol + 50 mg progesterone in oil; PRID: progesterone releasing intravaginal device; OF: ovulatory follicle; P4: progesterone; E2: estradiol; AI: Artificial insemination;*n=20/synch [1]within 48 hours after FTAI; [2]Timing to ovulation from FTAI; [3]Residual variation of interval to ovulation
[ab]Within rows, values with no common superscript are different (P<0.05)

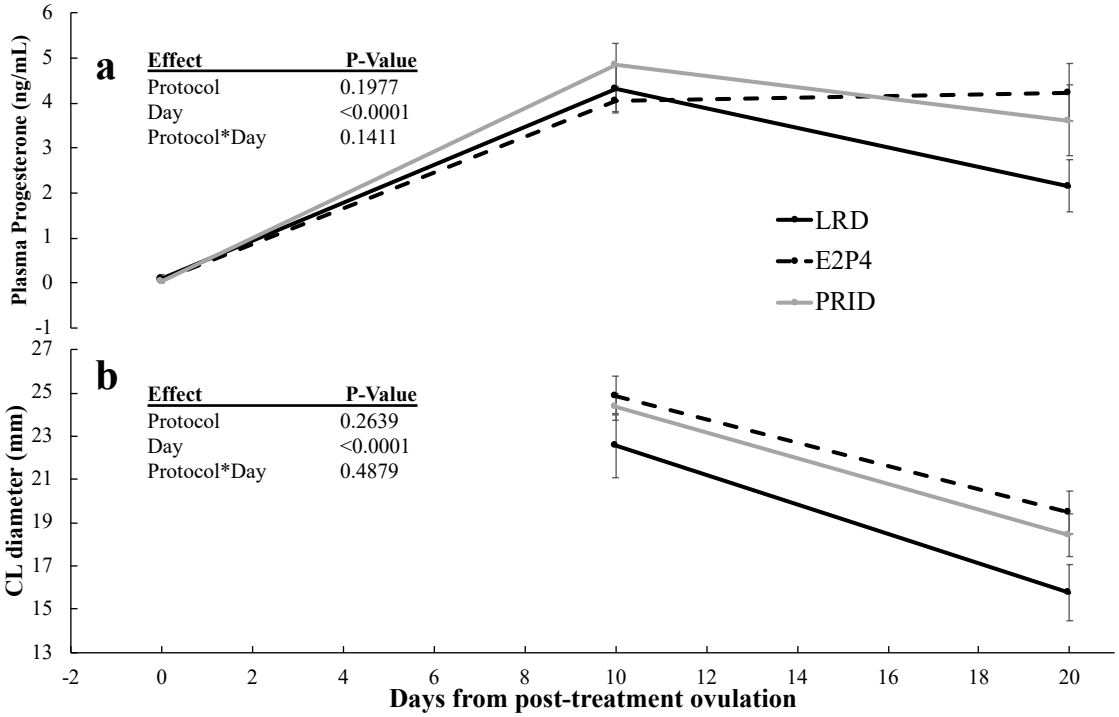

Figure 6. 9**.** Mean (± SEM) plasma progesterone (a) and CL diameter (b) following post treatment ovulation in cows given either: a 4-day letrozole-releasing device (LRD), estradiol plus progesterone (E2P4), or a modified Cosynch plus progesterone releasing intravaginal device (PRID) for 5 days protocol (n=20/protocol). Following FTAI, ultrasonography was done daily for ovulation in Experiment 2.

Table 6. 3**.** Endpoints from Experiment 3 in which heifers were either presynchronized or not with PGF and given either a 3-day X- or T-LRD. Mean ± SEM

	Presynchronization		Control	
Endpoint	X-LRD	T-LRD	X-LRD	T-LRD
Heifers (n)	8	5	5	8
Neutrophils per 100 cells Day 0*	24.6±12.8	38.2±17.8	23.0±10.2	18.4±17.4
Neutrophils per 100 cells Day 3*	43.4±14.8	35.2±11.1	19.0±9.5	17.8±15.4
Vaginal discharge score Day 3*	2.2±0.2[a]	2.2±0.2[a]	2.8±0.2[b]	3.2±0.2[b]
Vaginal irritation score Day 3*	0.8±0.2	1.6±0.4	1.4±0.2	1.8±0.6
Displayed estrus	3/8 (38%)	3/5 (60%)	2/5 (40%)	2/8 (25%)
Onset of estrus (hrs)[1]	48.0±2.9	50.7±2.9	56.0±3.6	54.0±3.6
Interval to ovulation (hrs)[1]	82.5±1.5	84.0±0	72.0±12.0	46.5±18.5
Ovulated within 48 hrs of GnRH	8/8 (100%	4/5 (80%)	4/5 (80%)	6/8 (75%)
P4 10 days after ovulation[2]	2.4±0.8	2.7±1.1	2.5±1.1	4.2±1.4
Early return to estrus[3]	3/8 (38%)	1/5 (20%)	3/5 (60%)	2/8 (25%)
Pregnancy per AI	2/8 (25%)	2/5 (40%)	2/5 (40%)	2/8 (25%)

Heifers were either Presynchronized with 500 µg cloprostenol (PGF) on Day -8 or not (control). Heifers in both groups were either given a 3-day X- or a T-letrozole releasing device (LRD) on

Day 0, followed by device removal and PGF on Day 3. Following PGF, GnRH was given concurrently with fixed-time artificial insemination on Day 5.
*n=5/group; [1]Hours from PGF to estrus or ovulation; [2]Plasma progesterone (P4); [3]Estrus detected by Heatwatch within 10 days from post-treatment ovulation
[ab]Within rows, values with no common superscript among synchronization are different (P<0.001)

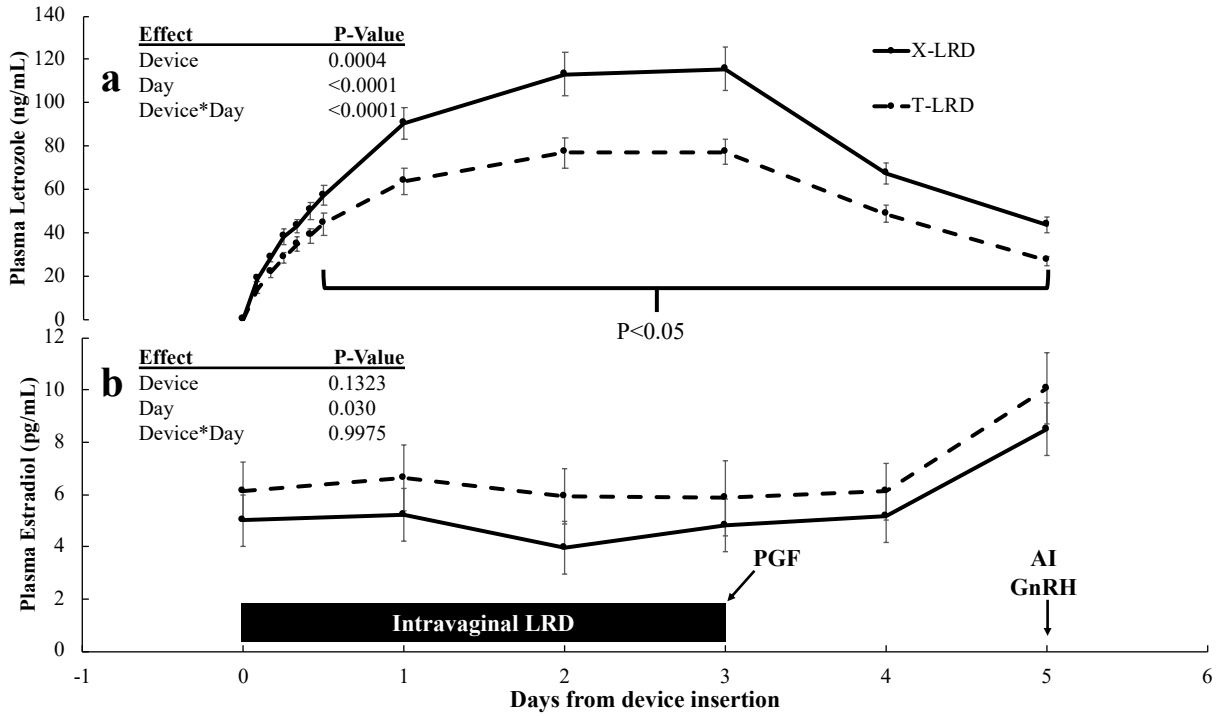

Figure 6. 10**.** Mean ± SEM plasma concentrations of letrozole (a; n=10/device and estradiol (b; n=13/device) between heifers given a newly developed X-letrozole releasing device (LRD) or a T-LRD for 3 days in Experiment 3. Frequent blood samples were taken every two hours from device insertion until 12 hours and then every day until Day 5 for plasma letrozole concentrations. Blood samples were taken daily for plasma estradiol concentrations. On Day 3, 500 µg cloprostenol (PGF) was given and 100 µg gonadorelin (GnRH) was given concurrently with artificial insemination (AI) on Day 5.

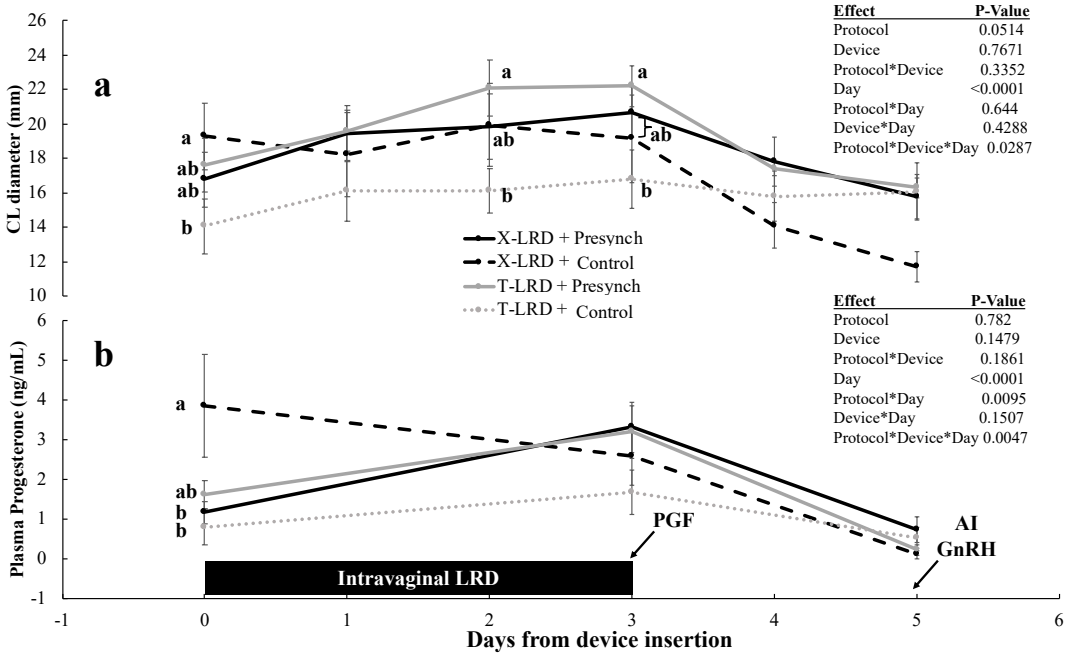

Figure 6. 11**.** Mean (± SEM) CL diameter (a) and plasma progesterone concentrations (b) in heifers either presynchronized with 500 µg cloprostenol (PGF) on Day -8 (Presynch) or not (Control). Heifers were either given a 3-day X- or T-letrozole releasing device (LRD) on Day 0. On Day 3, 500 µg cloprostenol (PGF) was given and 100 µg gonadorelin (GnRH) was given concurrently with artificial insemination (AI) on Day 5 in Experiment 3. [ab]Within days, values with no common superscripts are different (P<0.05).

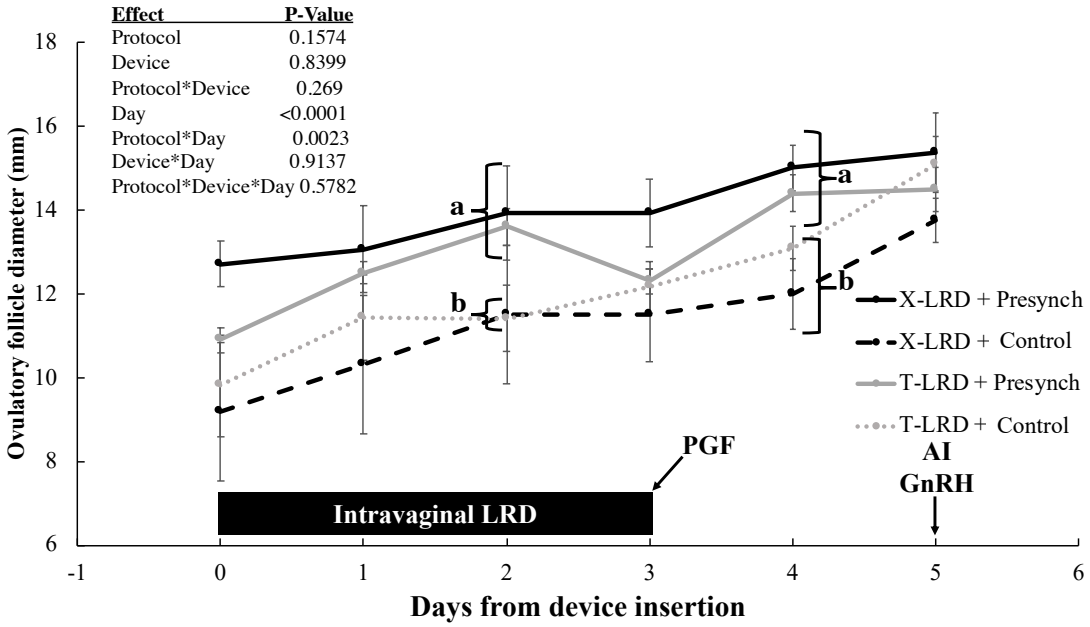

Figure 6. 12**.** Mean (± SEM) ovulatory follicle diameter in heifers either presynchronized with 500 µg cloprostenol (PGF) on Day -8 (Presynch) or not (Control). Heifers in both groups were either given a 3-day X- or T-letrozole releasing device (LRD) on Day 0. On Day 3, 500 µg

cloprostenol (PGF) was given and 100 μg gonadorelin (GnRH) was given concurrently with artificial insemination (AI) on Day 5 in Experiment 3. [ab]Within days, values with no common superscripts are different (P<0.05).

6.5. Discussion

Following results of Experiment 1 showing a high ovulation rate (>90%) following letrozole treatment, the 48-hour interval from PGF to GnRH was chosen for Experiment 2 due to the synchrony, as well as the increased CL diameter following post-treatment ovulation. However, the lower P/AI observed in LRD-treated cattle in Experiment 2 was unexpected. A low P/AI was shown in a previous study (Yapura et al., 2016), where GnRH was given concurrently with FTAI 24 hours after PGF. We expected that by extending the PGF to FTAI interval by an additional 24 hours, it would allow for a rebound in estradiol and increase P/AI. In Chapter 5 it was shown that letrozole treatment did not adversely affect granulosa cell function, oocyte maturation, or the ability of the oocyte to fertilize *in vitro*. Thus, it was expected results should be similar or even improved compared to currently used FTAI protocols. In Experiment 2, the interval from PGF treatment to ovulation was longer in the E2P4 group than in either the LRD or 5-day Cosynch groups. Similarly, later ovulations were also consistent which resulted in more synchronous ovulations in the E2P4 group.

Heifers in the LRD group had the largest ovulatory follicle at the time of FTAI among the other heifer treatments, and numerically a larger dominant follicle than all cow groups. In dairy (Wolfenson et al., 2004) and beef (Tarso et al., 2016) cattle, cows have been shown to ovulate a larger follicle than heifers, but circulating progesterone concentrations following ovulation is generally lower in lactating dairy cows than heifers (Wolfenson et al., 2004). The difference in follicle size and subsequent progesterone production has been explained, at least in part, by the

apparent increase in steroid metabolism in lactating cows (Sangsritavong et al., 2002). High progesterone concentrations during the growth of the ovulatory follicle decreases LH pulse amplitude and frequency (Kinder et al., 1996) which decreases the growth of LH-dependent follicles (i.e., ≥8 mm; Adams et al., 1992a). Similarly, using the ovariectomized cow model, the administration of a combination of estradiol and progesterone at physiological concentrations seen during the luteal phase causes LH-pulse frequency to decrease (Stumpf et al., 1993). With the decreased aromatase production of estradiol, increased LH-pulse amplitude in LRD-treated heifers (Chapter 5) would be expected to cause the development of larger dominant follicles. To our knowledge, this is the first study in which a larger dominant follicle was observed at FTAI in heifers than cows, and suggests a dose-effect of letrozole, whereby decreasing estradiol production and subsequently increasing LH pulse amplitude in cattle with a smaller mass.

Although ovulation rate was high (85%) in Experiment 3, a short-lived CL occurred in 9/26 (35%) heifers with estrus occurring within 10 days of ovulation. Similarly, plasma progesterone 10 days after ovulation averaged 2.9 ng/mL, but 9/26 (35%) were below 1 ng/mL which is below expected concentrations of 4 to 6 ng/mL observed in a previous study (Dadarwal et al., 2013). Low circulating progesterone and short-lived CL were not observed in Experiment 1, potentially due to blood sampling and ultrasonography only being done for 8 days following post-treatment ovulation. Short lifespan CL have been hypothesized to occur as a result of the low concentrations of circulating estradiol at the time of ovulation induction with GnRH. When estradiol does not reach optimal concentrations, synthesis of oxytocin receptors in the endometrium fail to occur. Therefore, following induced ovulation, as estradiol concentrations increase from the dominant follicle of the first follicular wave, oxytocin receptors are synthesized and produce PGF resulting in a short-lived CL (Araujo et al., 2009). In any case, the

development of short lifespan CL following letrozole treatment may explain the low P/AI in the LRD group in Experiment 2. In a previous study, high fertilization rates following estrus detection and AI (≥90%) has been demonstrated by embryo development on Day 8 (Diskin and Sreenan, 1980). However, the highest rate of embryo mortality has been reported to occur 8 to 16 days after AI (Diskin and Sreenan, 1980). Therefore, the functionality and lifespan of the CL and subsequent maintenance of pregnancy may contribute to early embryo mortality. Reduced preovulatory estradiol concentrations following letrozole treatment in Experiment 2 (from the shortened proestrus period) may have led to a short lifespan CL and thus, a low P/AI. Both heifers and cows in the no GnRH group (i.e., endogenous LH-induced ovulation) in Experiment 1 had numerically higher plasma progesterone concentrations on the last day of sampling (8 days after ovulation) suggesting that the administration of GnRH at 48- and 60-hours after PGF did not allow for the normal rise in in estradiol production during the proestrus period. Unfortunately, the HeatWatch system was not used in Experiment 1; it may have provided an indication of the time of onset of estrus and time of the endogenous LH peak.

The newly developed X-LRD outdone the T-LRD in Experiment 3 as shown by higher plasma letrozole concentrations (Fig. 6.10a) and a lower vaginal irritation score (Table 6.3). Although there was no difference in the surface area of both devices, the increase in drug delivery may be because of the design of the X-LRD, specifically the caudal portion (lower half of Figure 6.3a and b). In the T-LRD the “wings” of the caudal portion has been observed to fold up upon each othe while the caudal portion of the X-LRD, once expelled from the applicator, opens up maximizing contact with the vaginal mucosa. Previous studies with the original silicone LRD (Chapter 2) have shown that plasma levels of letrozole are positively correlated to the surface area, which has also been observed with intravaginal progesterone-releasing devices (van

Werven et al., 2013). Similarly, vaginal irritation was greater in the T-LRD because of the rigidity of the two points extending laterally in the cranial vaginal. The X-LRD was designed to distribute its force over 4 points extending laterally in both the cranial and caudal vagina.

In summary, giving GnRH 48 hours after PGF decreased variation in the interval to ovulation in cows following a 4-day letrozole treatment in Experiment 1. However, a 4-day letrozole treatment followed by GnRH given concurrently with FTAI 48 hours after PGF resulted in a lower P/AI compared to an E2P4 and a modified 5-day Cosynch plus PRID protocol in Experiment 2. Lastly, there was no benefit of presynchronization before initiating letrozole treatment for P/AI in Experiment 3. In addition, a newly designed X-LRD increased letrozole drug release and n decreased vaginal irritation numerically. Future studies should address the interval to estrus following a 4-day letrozole synchronization protocol to optimize the time of GnRH administration and pregnancy success.

6.6. Acknowledgements

The authors thank Merck for their donation of Estrumate used in the study. We thank Ky Pohler, Sydney Reese and Gessica Franco at Texas A&M University and Susan Cook, Kim Tran, and Danielle Carriere at the University of Saskatchewan for assistance in running radioimmunoassays. We also thank Steve Yang, Alix Nelson, and the staff at the University of Saskatchewan's Livestock and Forage Centre of Excellence for assistance in data collection and animal handling. The study was supported by Alberta Agriculture and Forestry, Venco Animal Health, a subsidiary of Dechra Pharmaceuticals PLC, and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 7

7. ESTRUS, CORPUS LUTEUM FUNCTION, AND PREGNANCY PER ARTIFICIAL INSEMINATION FOLLOWING AROMATASE INHIBITOR TREATMENT IN BEEF CATTLE

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Relationship of this study to the dissertation

In Chapter 6, we demonstrated that following a letrozole-based synchronization in cattle, the percent of ovulation was high, but following fixed-time artificial insemination, P/AI were low. This was shown by a return to estrus (i.e., short-lived CL) after ovulation in a proportion of heifers in Chapter 6, potentially as a result of low estradiol before induced ovulation. We hypothesized that by extending the period from LRD removal and prostaglandin followed by GnRH-induced ovulation, we would reduce the incidence of short-lived CL and subsequently increase P/AI.

Authors' Contributions

As first author, EMZ devised the experimental design, collected and interpreted data, performed statistical analysis and drafted the manuscript. JS and MGC helped with the development of the experimental design, interpretation of data, and manuscript revisions. MLZ assisted in data collection and manuscript revisions. As senior author, GPA provided the overall intellectual

knowledge, experimental design, analysis and interpretation of data, as well as revising the manuscript.

7.1. Abstract

Experiments were conducted to determine if letrozole treatment interferes with luteolysis and affects the timing of estrus and ovulation (Experiment 1), whether the stage of the estrous cycle at initiation of letrozole treatment affects P/AI (Experiment 2), and to compare a 4-day letrozole protocol vs a 5-day Cosynch plus CIDR protocol for fixed time artificial insemination (FTAI) in beef cattle (Experiment 3). In Experiment 1, heifers were fitted with a HeatWatch sensor and given prostaglandin F_{2a} (PGF). Ovulation (Day 0) was determined by daily ultrasonography, and on Day 3, heifers were given an intravaginal letrozole-releasing device (LRD) or sham device for 4 days (n=16 per group). Half of the heifers in each group were given PGF once on Day 7 (PGF 1x) or twice on Days 7 and 7.5 (PGF 2x). The onset of estrus was determined by HeatWatch software. Following PGF, ultrasonography was done twice daily until ovulation and for 14 days following ovulation for assessment of ovarian structures. In Experiment 2, heifers were assigned to 1 of 3 groups (n=10-11/gp) in which a 4-day LRD treatment was initiated during metestrus (Days 0 to 2), diestrus (Days 7 to 9), or proestrus (Days 15 to 17). PGF was given at device removal and 12 hours later. In Experiment 3, nulliparous (n=69) heifers and multiparous (n=151) cows were given either an LRD for 4 days or 100 µg GnRH plus a CIDR for 5 days (CIDR/Cosynch). At the time of device removal an estrus patch was placed on the tailhead; PGF was given at device removal and 12 hours later. In Experiment 2 and 3, cattle were given GnRH and inseminated with frozen thawed semen 66 hrs after device removal. Pregnancy diagnosis was done 28 to 34 days after ovulation by transrectal ultrasonography. In Experiment 1, no main effects or interactions were detected between device

or PGF treatment for the onset of estrus following PGF (overall 60.0 ± 2.5 h) or CL diameter profiles during luteolysis. The CL diameter following ovulation was greater following ovulation in the PGF 2x than in the PGF 1x groups (P=0.03). In Experiment 2, a greater proportion of heifers in the proestrus group ovulated ≤24 h after device withdrawal than the metestrus and diestrus groups combined (6/11 vs 0/22, respectively; P<0.05). The P/AI tended to be greater in the metestrus than the diestrus group (10/11 vs 6/10; P=0.08), with the proestrus group lower than either (0/11; P<0.05). In Experiment 3, there was no difference in the overall P/AI (total 117/218 [53.7%]) between FTAI protocol (P=0.15) or parity (P=0.24). In summary, letrozole treatment did not interfere with luteolysis, and PGF given twice at a 12-hour interval on Day 7 after ovulation decreased variance in the interval to ovulation. No differences were detected in P/AI following FTAI between a 4-day LRD vs a 5-day Cosynch plus CIDR protocol and letrozole treatment initiated during metestrus and diestrus yielded greater P/AI than starting in proestrus.

7.2. Introduction

Elevated plasma estrogen concentrations near the time of insemination are associated with increased rate of sperm transport in the female reproductive tract (Hawk, 1983). High concentrations of circulating estradiol coincide with peak estrus activity (Lyimo et al., 2000) and cattle that display estrus within 24 hours before fixed time artificial insemination (FTAI) have higher P/AI than cattle that do not exhibit estrus (Perry et al., 2005, 2007). Due to the effect of synchronizing follicular wave emergence, estradiol-based synchronization protocols enabled FTAI in cycling and anestrus cattle (Nunez-Olivera et al., 2014). Although these protocols are in common use in South America, estradiol-based protocols for FTAI are limited in North America by regulations requiring a veterinary prescription (Canada) or complete prohibition (United States; AETA, 2019). Similarly, estrogens were banned for ovarian synchronization in food producing species in Europe, and in milk-producing animals in Australia and New Zealand in the late 2000's (Lane et al., 2008). Therefore, alternatives to estradiol-based protocols are needed for FTAI in cattle in many countries worldwide.

In the United States and Canada, gonadotropin-releasing hormone (GnRH)-based protocols are used extensively for FTAI in both beef and dairy cattle (Colazo and Mapletoft, 2014). Cosynch, or the administration of GnRH concurrently with FTAI, has been adapted for use in beef cattle due to the requirement of only 3 handlings. However, the efficacy of GnRH-based protocols is dependent on ovulation following the first GnRH which is given at a random stage of the estrous cycle. The ovulation rate is low when GnRH is given during early metestrus due to the dominant follicle not acquiring luteinizing hormone receptors (Atkins et al., 2008). During a high progesterone environment, a GnRH-induced LH surge has been shown to be decreased (Colazo et al., 2008; Giordano et al., 2012). Ovulation rates are highest when GnRH is

given on Days 5 to 9 of the estrous cycle when the dominant follicle of the first wave is growing (Vasconcelos et al., 1999). To manipulate this interval, prostaglandin (PGF) is given prior to enrolment into GnRH-based FTAI protocols (Vasconcelos et al., 1999) to increase the number of animals in this stage. However, this requires an additional handling, which may increase the cost of the protocol.

Extensive investigations on the development of non-steroidal aromatase inhibitor-based methods to control ovarian function in women and cattle (reviewed in Yapura et al., 2018) have been done in our laboratory. Recent investigations have examined dose-response relationships, timing, mechanism of action, and drug delivery methods with letrozole. Research has also involved the development of a unique intravaginal letrozole releasing device (Chapter 2) and effects on ovarian superstimulation in cattle (Chapter 4; Zwiefelhofer et al., 2019). No deleterious effects of letrozole on oocyte maturation or *in vitro* fertilization capacity were detected (Chapter 5), and synchrony of ovulation was similar between letrozole-treated groups given GnRH 48 or 60 hours after PGF treatment (Chapter 6; Zwiefelhofer et al., 2018). Despite the synchrony of ovulation, initial breeding trials with the letrozole-based protocol for FTAI at 48 hours after PGF were disappointing (30% P/AI; Chapter 6). It was speculated that the low P/AI may be related to an inadequate duration of proestrus to permit a sufficient increase in estradiol (i.e., PGF to AI/GnRH interval of 48 hours) resulting in short-lived (i.e., 8 to 10 days; Chapter 6) corpora lutea (CL; Mann and Lamming, 2000).

It was hypothesized that increasing the rate of luteolysis and extending the interval from PGF treatment to GnRH would minimize the incidence of short-lived CL and increase the P/AI after FTAI. The objectives were to determine if letrozole treatment interferes with luteolysis and affects the timing of estrus and ovulation (Experiment 1) whether the stage of the estrous cycle at

initiation of letrozole treatment affects P/AI (Experiment 2), and to compare a 4-day letrozole protocol vs a 5-day Cosynch plus CIDR protocol for FTAI in beef cattle (Experiment 3).

7.3. Materials and Methods

Procedures were approved by the University of Saskatchewan's Animal Research Ethics Board in accordance with the guidelines of the Canadian Council on Animal Care. Experiments 1 and 2 involved the use of sexually mature nulliparous Hereford cross heifers (n=32) aged 16 to 18 months of age (weight: 486 ± 8 kg; body condition score [BCS]: 3.3 ± 0.05; 1 to 5 scale; with 1 being emaciated and 5 being obese (Richards et al., 1986)). Heifers were maintained at the University of Saskatchewan's Livestock and Forage Centre of Excellence Goodale Farm during the months of October to January. In Experiment 3, sexually mature nulliparous Hereford cross and Angus heifers (n=69; weight: 412 ± 6 kg; BCS: 2.7 ± 0.04) and multiparous lactating Angus cows ≥42 days post-partum (n=151; weight: 616 ± 8 kg; BCS: 2.6 ± 0.03) were used. Cattle were located at the University of Saskatchewan's Livestock and Forage Centre of Excellence Termuende Ranch (Lanigan, Saskatchewan). Prior to each experiment, cattle were examined by transrectal ultrasonography to confirm a normal reproductive tract and confirm cyclicity by the presence of a CL.

7.3.1. Experiment 1. Estrus response and corpus luteum function following letrozole treatment

A 2x2 factorial design was used to determine the effects of an LRD vs sham device and single vs double administration of PGF on luteolysis (defined as plasma progesterone

concentrations ≤1 ng/mL within 24 hours of PGF), estrous behavior, and ovulation. Prior to enrollment into the study, heifers were fitted with a HeatWatch electronic sensor (CowChips; Manalapan, NJ, USA) to record the time, intensity, and duration of estrus. At random stages of the estrous cycle, heifers were given 2 doses of PGF (500 μg cloprostenol each; Bioestrovet; Vetoquinol N.A., Lavaltrie, QC, Canada) 12 hours apart to induce luteolysis followed 3 days later by GnRH (100 μg gonadorelin im; Fertiline, Vetoquinol N.A., Lavaltrie, QC, Canada) to induce ovulation. The ovaries were examined daily by transrectal ultrasonography (7.5 MHz linear array probe, MyLab Alpha, Esaote North America, Fishers, IN, USA) to determine the day of ovulation (Day 0). On Day 3, heifers were assigned randomly to one of two groups and given either an LRD or a sham device for 4 days. On Day 7, devices were removed and heifers in each group were again randomly assigned to one of two groups and given 500 μg cloprostenol once at the time of device removal or twice at 12 hour intervals (Day 7 and 7.5; 4 groups; n=8/gp; Fig. 7.1). On Day 7, heifers underwent transrectal ultrasonography at 12-hour intervals for 72 hours to measure luteal and follicular dynamics and then daily thereafter to determine ovulation. Daily ultrasonography was done for 14 days following ovulation to assess CL diameter. Data on intervals to onset of estrus (hours from first PGF to first recorded mount), and peak estrus (hours from first PGF to the hour with the greatest number of mounts), estrus intensity (total number of mounts), and duration of estrus (interval from the first to the last mount) were collected by HeatWatch software. The luteolysis rate was defined as the proportion of heifers with plasma progesterone concentrations ≤1 ng/mL within 24 hours of PGF.

7.3.2. Experiment 2. Effect of the stage of the estrous cycle on the efficacy of letrozole-based synchronization for FTAI

To determine the effect of initiating letrozole treatment during different stages of the estrous cycle, heifers were reused from Experiment 1 and assigned randomly to one of three groups (n=10-11/group) in which a 4-day LRD treatment was initiated during metestrus (Day 0 to 2; Day 0 = ovulation), diestrus (Day 7 to 9), or proestrus (Day 15 to 17; Fig. 7.2). Heifers were given PGF at the time of LRD removal and 12 hours later and were inseminated with frozen thawed semen concurrently with administration of GnRH 66 hours after device removal. Beginning at device removal heifers underwent transrectal ultrasonography at 12-hour intervals until ovulation was detected. Transrectal ultrasonography was also done 7 and 14 days after post-treatment ovulation to determine CL diameter and plasma progesterone concentration. Transrectal ultrasonography was used to diagnose pregnancy 28-35 days after ovulation by confirming an embryo proper with a heartbeat. Estrus endpoints from HeatWatch were analyzed as described in Experiment 1.

7.3.3. Experiment 3. Field trial for FTAI

Using a 2x2 factorial design, heifers and cows were synchronized during the month of June using a 4-day letrozole- or a conventional 5-day Cosynch plus CIDR protocol (n= 35 to 78 per group; n=4 groups; Fig. 7.3). At random stages of the estrous cycle, cattle in the letrozole group were given PGF im to induce luteolysis and were given an estrus detection patch (Estrotect, Rockway Inc., Spring Valley, WI, USA) which was placed on the tailhead. Five days after PGF, the estrus detection patch was scored based on color change (0: unchanged, 1: ≤50% color change, 2: >50% color change, 3: missing; Colazo et al., 2018) and an LRD was inserted

for 4 days. Estrus was defined to have occurred if the estrus patch was scored 2 or 3. Cattle in the 5-day Cosynch plus CIDR group were given GnRH im (100 ug gonadorelin im) followed by an intravaginal progesterone device (Eazi-Breed CIDR, Zoetis, Kirkland, QC, Canada) for 5 days. At the time of device removal, cattle in both treatment groups were given PGF im twice at 12-hours apart and an estrus detection patch placed on the tailhead. At 66 hours after device removal, cattle were assigned randomly and FTAI with frozen thawed semen containing ≥10 million progressive motile sperm from one of four different sires, and treated with GnRH. Cattle were examined by transrectal ultrasonography at device removal, AI, and 30 hours after AI to measure dominant follicle diameter and CL diameter and to confirm ovulation. A subsequent CL measurement was done by transrectal ultrasonography 6 days post-treatment ovulation on a subset of cattle (n=20 per treatment per parity). Pregnancy diagnosis was done 28 to 34 days after ovulation by transrectal ultrasonography, as in Experiment 2.

7.3.4. Blood sampling and radioimmunoassays

Blood samples were taken by jugular venipuncture into heparinized tubes (Vacutainer; Becton Dickinson and Co., Franklin Lakes, NJ), centrifuged <2 hours after collection at 1,500 x *g* and plasma was aliquoted into polypropylene tubes and frozen at -20°C until assay. In Experiment 1, blood samples were taken daily from Day 3 to 7, twice daily from Day 7.5 to Day 10 and daily until Day 12. Blood samples were taken every other day from 2 to 14 days after ovulation. In Experiment 2, blood samples were taken daily from Day 0 to 4, twice daily until Day 6.5, Day 6.75, 7, and 7.5 and on Days 7- and 14 following post-treatment ovulation. In Experiment 3, blood samples were taken from a subset of cattle (n=20/group) at the time of AI and 6 days after ovulation.

Plasma concentrations of estradiol were determined by radioimmunoassay as previously described (Kirby et al., 1997). The minimum detection limit was 0.5 pg/mL. Intra- and inter-assay coefficients of variation were 6.8 and 9.2%, respectively.

Plasma concentrations of progesterone were analyzed by a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA, USA) with a minimum detection limit of 0.1 ng/mL. Intra-assay coefficients of variation in Experiments 1 and 2 for low, medium, and high samples were 11.0, 9.1, and 10.8%, respectively. Inter-assay coefficients of variation for low, medium, and high samples were 11.9, 8.2, 10.7%, respectively. Intra-assay coefficients of variation in Experiment 3 for low, medium, and high samples were 6.7, 6.9, and 9.2%, respectively. Inter-assay coefficients of variation for low, medium, and high samples were 11.9, 8.2, and 10.7%, respectively.

7.3.5. Statistical analyses

Data were analyzed using SAS Enterprise Guide (Version 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Serial data (i.e., CL, dominant follicle, progesterone and estradiol profiles) were analyzed by 2x2 ANOVA using a mixed model for repeated measures to examine the main effects of LRD vs Sham and PGF 1x or 2x (Experiments 1 and 2), and heifer vs cow and LRD vs a 5-day Cosynch plus CIDR protocol (Experiment 3). Numerical scale-data (onset of estrus, residuals of onset of estrus, peak estrus, length of estrus, total number of mounts, ovulation timing, residuals of ovulation timing, and size of ovulating follicle) were analyzed by 2x2 ANOVA using the MIXED procedure. Binary were compared by chi-square in Experiments 1 and 2 (luteolysis and ovulation rate, and P/AI), and by logistic regression with the factorial GLIMMIX procedure comparing parity by FTAI protocol (estrus patch detection score,

ovulation rate, estrus rate, double ovulation rate, P/AI) or bull by FTAI protocol using binary distribution and link logit function in Experiment 3. If an interaction was detected, post hoc analyses were done using least significant difference. Significance was defined as P≤0.05 and data are presented as mean ± SEM unless stated otherwise.

7.4. Results

7.4.1. Experiment 1. Estrus response and corpus luteum function following letrozole treatment

One heifer in the LRD PGF 1x group was removed from the experiment due to aggressive behavior. One heifer in the LRD PGF 1x group did not display estrus or ovulate within 7 days after PGF and was included in the luteolysis rate, but was removed from further analysis. No difference was detected among groups in the luteolysis rate as all heifers had plasma progesterone concentrations ≤1 ng/mL within 24 hours of PGF. No difference was detected among groups in onset of estrus, peak estrus, total number of mounts, and ovulation timing (Table 7.1). An interaction between treatments was detected for length of estrus where LRD PGF1x and Sham PGF 2x were longer (P<0.05) than Sham PGF 1x (Table 7.1), while LRD PGF 2x was intermediate. Residuals of onset of estrus tended (P=0.07) to be more synchronous in the PGF 2x group than the PGF 1x group (Table 7.1). Similarly, residuals of the interval to ovulation were more (P=0.01) synchronous in the PGF 2x than the PGF 1x group (Table 7.1). A device by PGF interaction for CL diameter profiles was detected between LRD PGF 1x vs Sham PGF 1x and LRD PGF 1x vs LRD PGF 2x (Fig. 7.4). A device-by-day interaction (P=0.05) was detected from Days 3 to 7 and a tendency (P=0.08) between PGF treatment was evident from Day 7.5 to

10 for plasma progesterone during luteolysis (Fig. 7.5). A larger dominant follicle during treatment in the LRD group led to a device-by-day interaction (P=0.04) for dominant follicle diameter from Days 7 and 7.5 (Fig. 7.6A). There was an effect of device and PGF treatment on plasma estradiol profiles (P=0.04; Fig. 7.6B) with the PGF 1x and Sham groups having increased plasma estradiol. A larger CL following ovulation in the PGF 2x group resulted in a treatment effect (P=0.03); a tendency (P=0.07) of device-by-PGF interaction for post-ovulation CL diameters was due to increased size in the LRD PGF 2x group (Fig. 7.7A). There was no effect of device, PGF treatment, or interactions for plasma progesterone post-ovulation (Fig. 7.7B).

7.4.2. Experiment 2. Effect of the stage of the estrous cycle on the efficacy of letrozole-based synchronization for FTAI

A total of 6 heifers ovulated during LRD treatment or within 24 hours following LRD removal in the proestrus group, which was a greater proportion than in the other groups (P<0.05; Table 7.2). Their data for dominant follicle and CL diameter were truncated on Day 3 following device insertion. There were no differences between metestrus and diestrus groups in the number of double ovulations, intervals to the onset of estrus, estrus to AI, nor in dominant follicle diameter at AI or the degree of synchrony of ovulation (Table 7.2). The total number of mounts during estrus was greater in diestrus than metestrus (P<0.05) and a tendency (P=0.09) between the groups was detected for timing of ovulation following AI/GnRH (Table 7.2). Letrozole treatment initiated during metestrus tended (P=0.09) to increase P/AI (91%) compared to diestrus (60%), and both were higher (P<0.05) P/AI than proestrus (0%; Table 7.2). A treatment (P<0.001) and treatment-by-day interaction (P<0.0001) was shown for CL diameter and dominant follicle profiles during treatment and comparisons among days are shown (Fig. 7.8 & 7.9). A treatment-by-day interaction was detected for CL diameter (P=0.06) and plasma

progesterone (P=0.005) profiles 7- and 14-days following post-treatment ovulation. The CL diameter and plasma progesterone were greater (P<0.01) in the metestrus vs proestrus group and tended (P=0.07) to be greater in the diestrus vs proestrus group (Fig. 7.11).

7.4.3. Experiment 3. Field trial for FTAI

Data from 2 heifers in the LRD group were excluded because of injury and device loss, respectively. A device-by-day and parity-by-day effect on CL diameter was attributed to a larger CL on the day of device removal and 66 hours after device removal for the LRD and Cow groups, respectively (P<0.05; Fig.7.12A). A device-by-parity interaction for dominant follicle diameter was due to a larger dominant follicle in the Heifer LRD group (P=0.01; Fig. 7.12B). A parity effect for ovulation detected prior to FTAI was due to an increased proportion of heifers that ovulated prematurely vs cows (P<0.001; Table 7.3). At FTAI, a greater (P<0.001) proportion of heifers had an estrus detection score of ≥2 than cows (Table 7.3). A lower proportion of cattle in the LRD group at FTAI had an estrus score ≥2 compared to the CIDR group (P=0.01; Table 7.3). A greater (P=0.05) proportion of double ovulations occurred in cows than heifers and in the LRD than CIDR group (P=0.05; Table 7.3). The CL diameter 6 days after ovulation was greater (P<0.001) in cows than heifers with no effect (P=0.13) of protocol (Table 7.3). Plasma progesterone 7 days after AI was greater (P=0.004) in the LRD than the CIDR group and in heifers than cows (P<0.001; Table 7.3). A device-by-parity interaction (P=0.04) for plasma progesterone 7 days after FTAI was attributed to an increased concentration in the Heifer LRD group. Similarly, a device-by-parity-by-day interaction (P=0.01) for plasma progesterone profiles on the day of FTAI and 7 days later was attributed to an increased concentration in the Heifer LRD group (Fig. 7.13). There were no differences for device (P=0.15) or parity (P=0.24) in overall P/AI. Similarly, there were no differences for device (P=0.89) or parity (P=0.57) in

P/AI from cattle with an estrus score ≥2 (overall 90/122 [73.8%]). No difference was detected for device (P=0.58) or parity (P=0.55) for P/AI from cattle with an estrus score <2 (Table 7.3). Cattle with an estrus score ≥2 had a higher (P<0.0001) P/AI compared to an estrus score <2 (Fig. 7.14). A bull interaction (P<0.05; Table 4) was due to an increase in P/AI in heifers obtained with Bull A than Bull B in the 5-day Cosynch plus CIDR group (Table 7.4). No differences were detected between Bull C and D in the cow groups (Table 7.4).

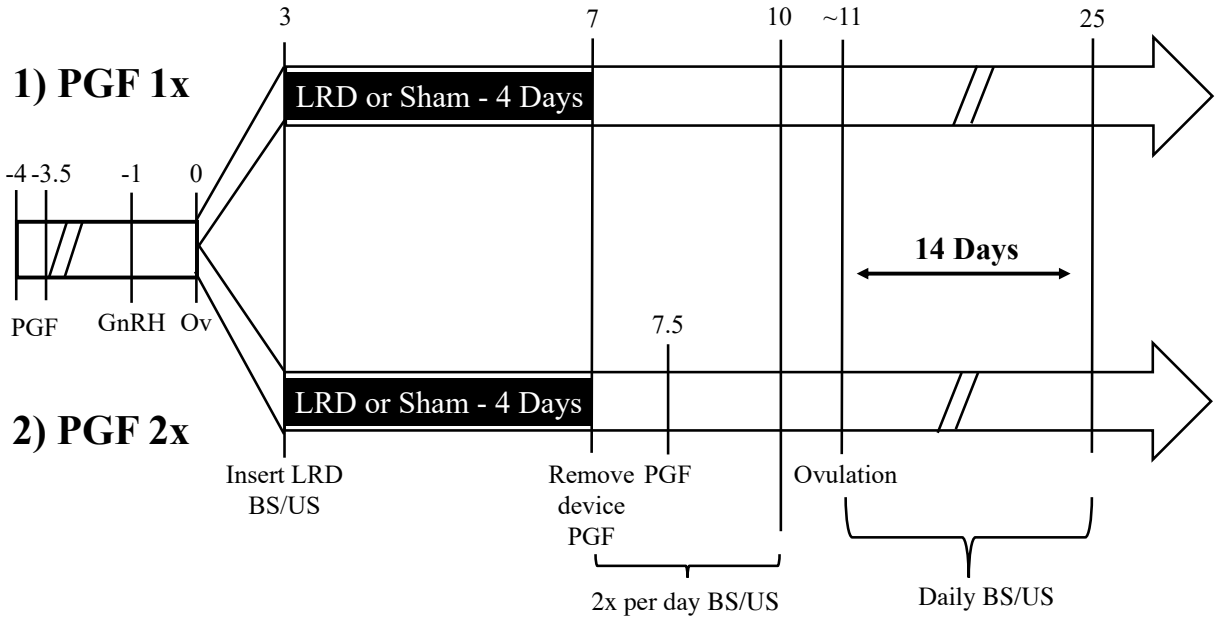

Figure 7. 1**.** Timeline for Experiment 1 in which heifers 3 days after ovulation were either given a 4-day letrozole releasing device (LRD) or sham device. At device removal, heifers were assigned randomly to one of two groups and given either 500 μg cloprostenol once (PGF 1x) or twice at 12-hour intervals (PGF 2x; 4 groups, n=8 heifers/group). Following PGF, blood sampling (BS) and transrectal ultrasonography was done twice daily for 3 days. After ovulation, US and BS was done daily for 14 days.

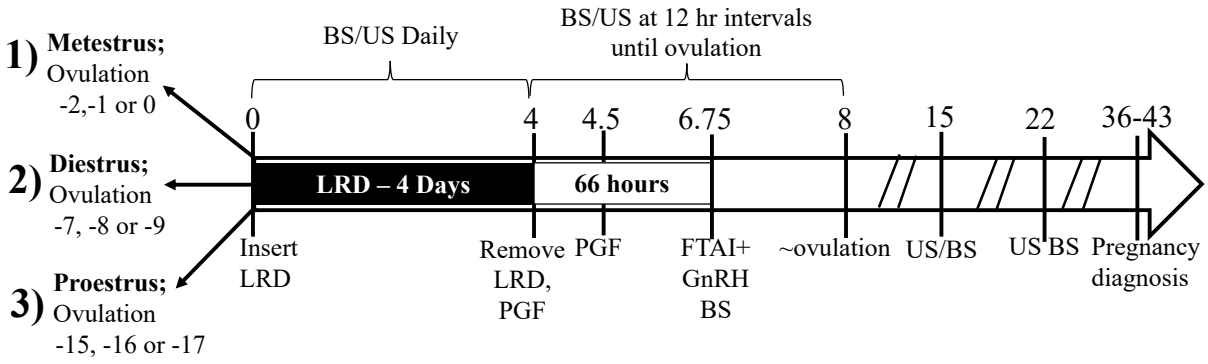

Figure 7. 2**.** Timeline for Experiment 2 to test the effect of initiating a 4-day letrozole releasing device (LRD) regiment during metestrus, diestrus, or proestrus (n=10-11/group). At LRD removal, heifers were given 500 µg cloprostenol (PGF) twice at 12-hour intervals and fixed-time artificial insemination (FTAI) was done concurrently with 100 µg gonadorelin (GnRH) 66 hours after LRD removal. Blood sampling (BS) and transrectal ultrasonography (US) was done daily from Day 0 to 4 and twice daily until ovulation. Following ovulation, US and BS were done 7- and 14 days later.

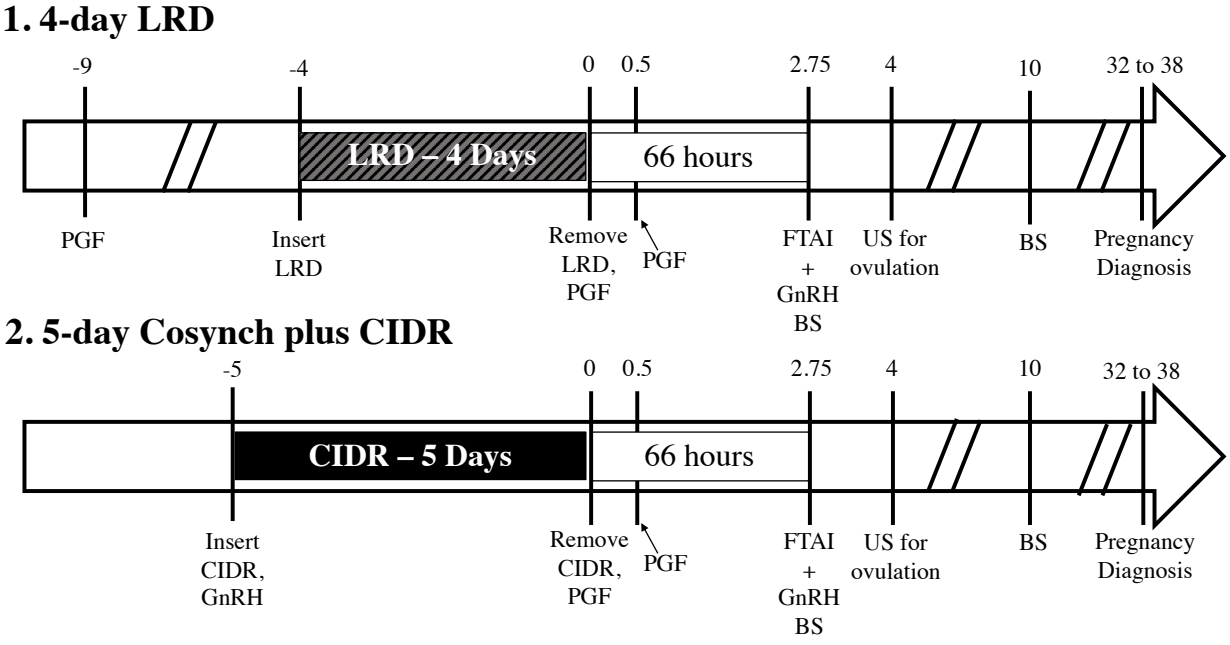

Figure 7. 3**.** Timeline for Experiment 3 comparing a newly developed letrozole releasing device (LRD) protocol vs a conventional 5-day Cosynch plus CIDR protocol for FTAI in beef cattle. To increase the proportion of cattle in the metestrus stage in the LRD protocol, 500 µg cloprostenol (PGF) was given 5 days before insertion of the LRD. In the 5-day Cosynch plus CIDR protocol, 100 µg gonadorelin (GnRH) was given concurrently with CIDR insertion. In both protocols, PGF was given at device removal and 12 hours later and GnRH was given concurrently with fixed-time artificial insemination (FTAI) 66 hours after device removal. A subset of cattle (20/group; 4 groups) had blood sampling done at AI and 6 days after ovulation.

Table 7. 1**.** Estrus and ovulation response (mean ± SEM) in heifers treated with either a letrozole releasing device (LRD) or sham device and given PGF 1x or 2x (Experiment 1).

	LRD		Sham	
Item	PGF 1x	PGF 2x	PGF 1x	PGF 2x
Heifers (n)	6	8	8	8
Onset of estrus (h following PGF)	59.0 ± 5.5	60.4 ± 3.3	63.4 ± 6.9	57.0 ± 4.1
Variation in onset of estrus (residuals)	9.0 ± 3.7	8.2 ± 1.3	12.3 ± 5.2	8.8 ± 2.4
Peak estrus (hrs following PGF)	64.7 ± 5.7	63.9 ± 4.9	67.2 ± 7.6	60.9 ± 4.2
Duration of estrus (h)	16.0 ± 1.7	13.5 ± 1.8	10.4 ± 1.7	15.3 ± 1.6
Total mounts	28.0 ± 5.3	37.3 ± 20	20.8 ± 6.6	35.3 ± 11
Interval to ovulation (hrs following PGF)	104 ± 6.2	99 ± 3.0	105 ± 6.3	96 ± 4.5
Variation in interval to ovulation (residuals)	18.7 ± 5.7[a]	5.3 ± 2.3[b]	13.5 ± 3.7[a]	11.1 ± 3.9[b]
Maximum diameter of the ovulatory follicle (mm)	15.6 ± 0.9	17.9 ± 0.6	16.1 ± 0.9	15.9 ± 0.8

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

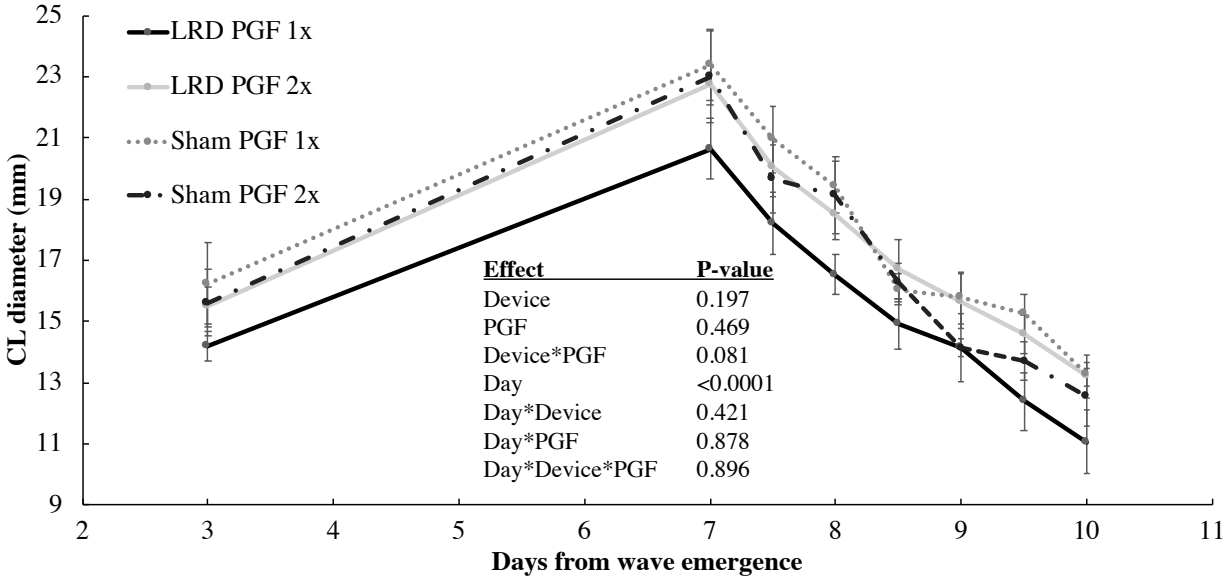

Figure 7. 4**.** Corpus luteum diameter (mean ± SEM) in heifers treated on Day 3 (Day 0 = ovulation) with either a letrozole releasing device (LRD) or a sham device for 4 days. Devices were removed and 500 µg cloprostenol (PGF) was given on Day 7 (PGF 1x) or Day 7 & 7.5 (PGF 2x) in Experiment 1.

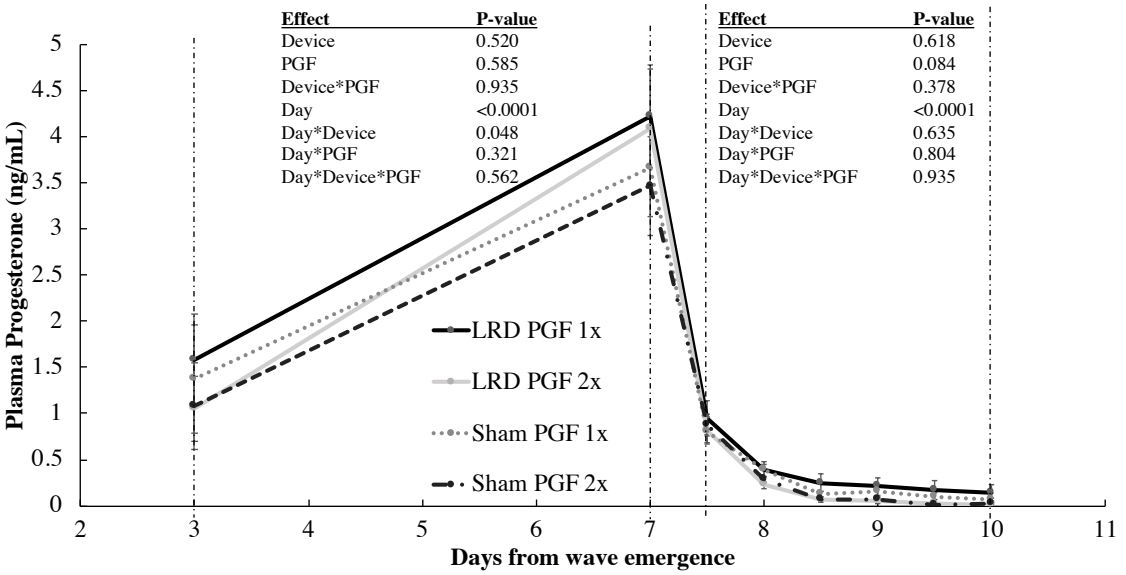

Figure 7. 5**.** Plasma progesterone concentration (mean ± SEM) in heifers treated on Day 3 with either a letrozole releasing device (LRD) or a sham device for 4 days. Devices were removed and 500 µg cloprostenol (PGF) was given on Day 7 (PGF 1x) or Day 7 & 7.5 (PGF 2x). Data were analyzed independently from Day 3 to 7 (treatment period) and Day 7.5 to 10 (luteolytic period) in Experiment 1.

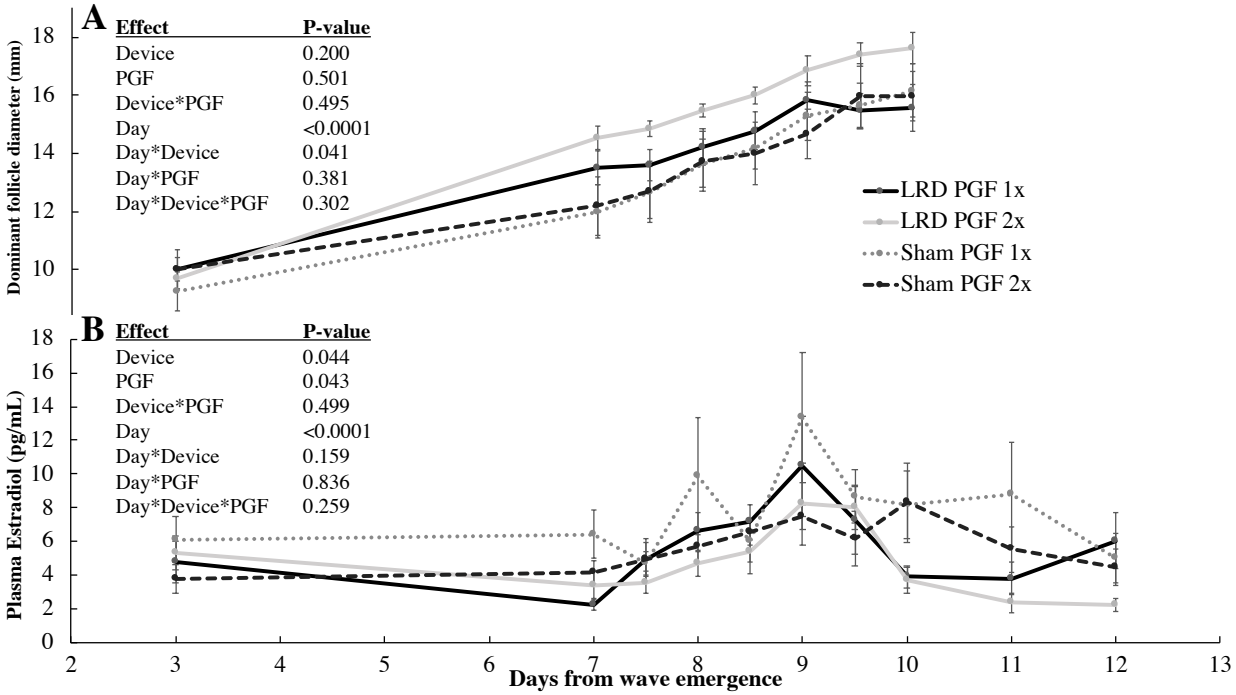

Figure 7. 6**.** Mean (± SEM) dominant follicle diameter (A) and plasma estradiol concentration (B) in heifers treated on Day 3 with either a letrozole releasing device (LRD) or a sham device for 4 days. Devices were removed and 500 µg cloprostenol (PGF) was given on Day 7 (PGF 1x) or Day 7 & 7.5 (PGF 2x); Experiment 1.

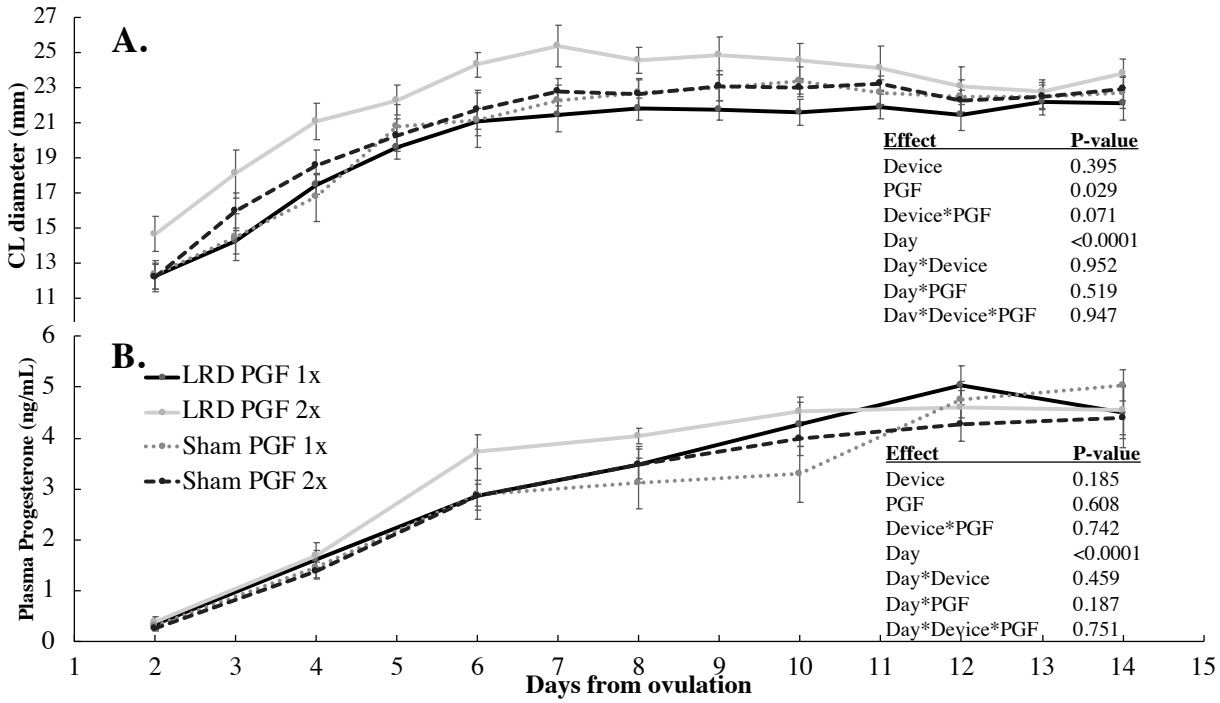

Figure 7. 7**.** Mean (± SEM) corpus luteum diameter (A) and plasma progesterone concentration (B) following post-treatment ovulation in heifers treated with a letrozole-releasing device (LRD) or sham device for 4 days. Devices were removed and 500 µg cloprostenol (PGF) was given on Day 7 (PGF 1x) or Day 7 & 7.5 (PGF 2x). Post treatment ovulation was defined as Day 0. Experiment 1.

Table 7. 2**.** Estrus, ovulation, and pregnancy in heifers in which letrozole treatment was initiated during metestus, diestrus, or proestrus. A portion of estrus and ovulation data are excluded in the proestrus group due to ovulations occurring during or within 24 hours following letrozole treatment. Mean ± SEM; Experiment 2.

	Metestrus	Diestrus	Proestrus
Heifers (n)	11	10	11
Ovulated during or within 24 hrs following LRD	0/11 (0%)[a]	0/10 (0%)[a]	6/11 (55%)[b]
Double ovulation	2/11 (18%)	0/10 (0%)	0/11 (0%)
Interval to estrus (hrs following 1st PGF)	58.7 ± 2.2	56.6 ± 1.3	-
Total mounts	16.5 ± 5.5[a]	33.4 ± 5.5[b]	-
Interval to ovulation (hrs following FTAI/GnRH)	22.4 ± 1.8*	27.6 ± 2.4*	-
Variation in interval to ovulation (residuals)	5.6 ± 0.5	5.8 ± 1.4	-
Dominant follicle diameter at AI (mm)	17.9 ± 0.6	16.6 ± 0.8	-
Pregnancy per AI	10/11 (91%)[a]*	6/10 (60%)[a]*	0/11 (0%)[b]

[ab]Within rows, values with no common superscript are different (P<0.05)
*Values tended to differ (P=0.09).
PGF: 500 µg cloprostenol; AI: Artificial insemination; GnRH: 100 µg gonadorelin

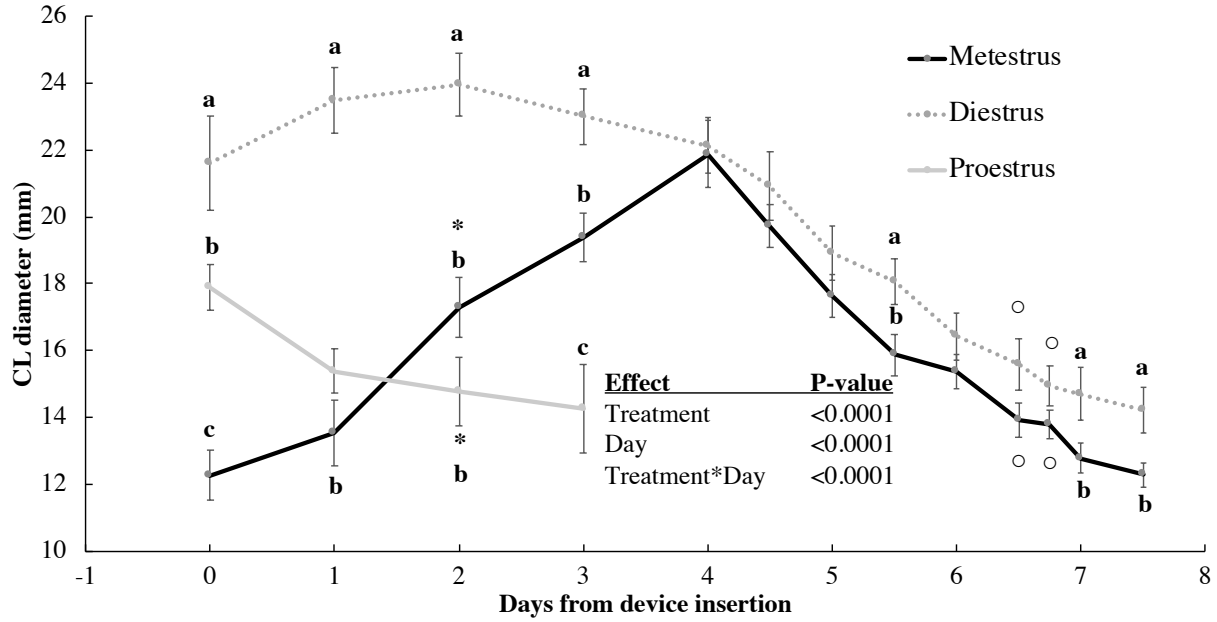

Figure 7. 8**.** Corpus luteum (CL) diameters (mean ± SEM) in heifers in Experiment 2 treated with a letrozole releasing device during metestrus, diestrus, or proestrus. Data for the proestrus group were truncated at Day 3 due to premature ovulations and inconsistency in detection of regressing CL. [abc]Within days, values with no common superscript are different (P<0.05). *Values tended to be different (P=0.06). ◦Values tended to be different (P=0.10).

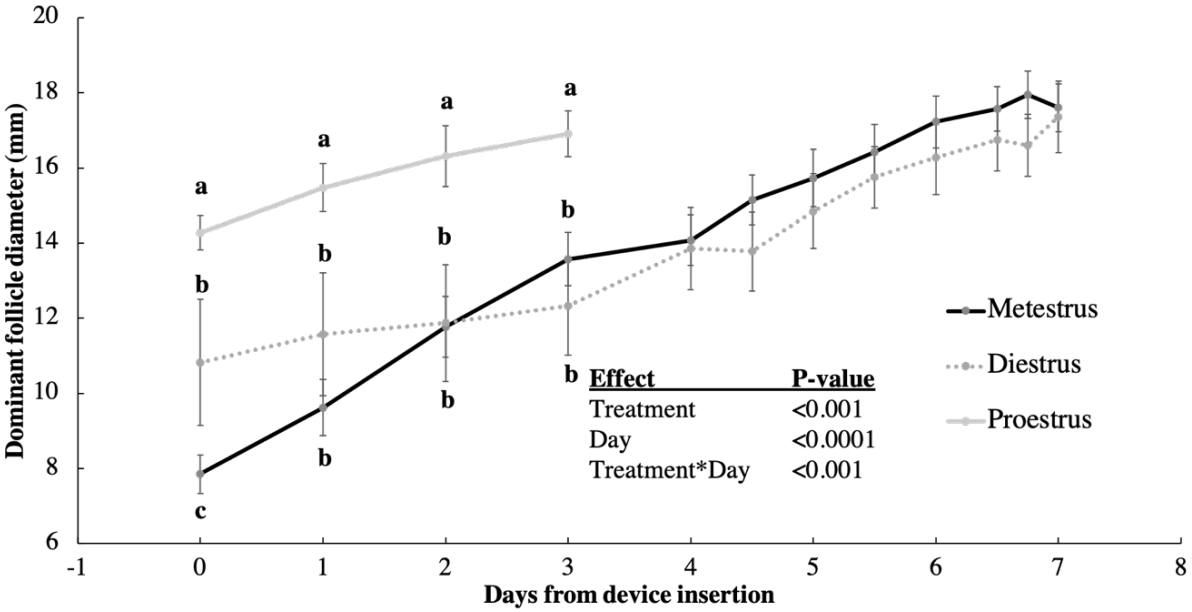

Figure 7. 9**.** Dominant follicle diameter (mean ± SEM) in heifers following treatment with a letrozole releasing device initiated during metestrus, diestrus, or proestrus in Experiment 2. Data for the proestrus group were truncated at Day 3 because of premature ovulations. [abc]Within days, values with no common superscript are different (P<0.05).

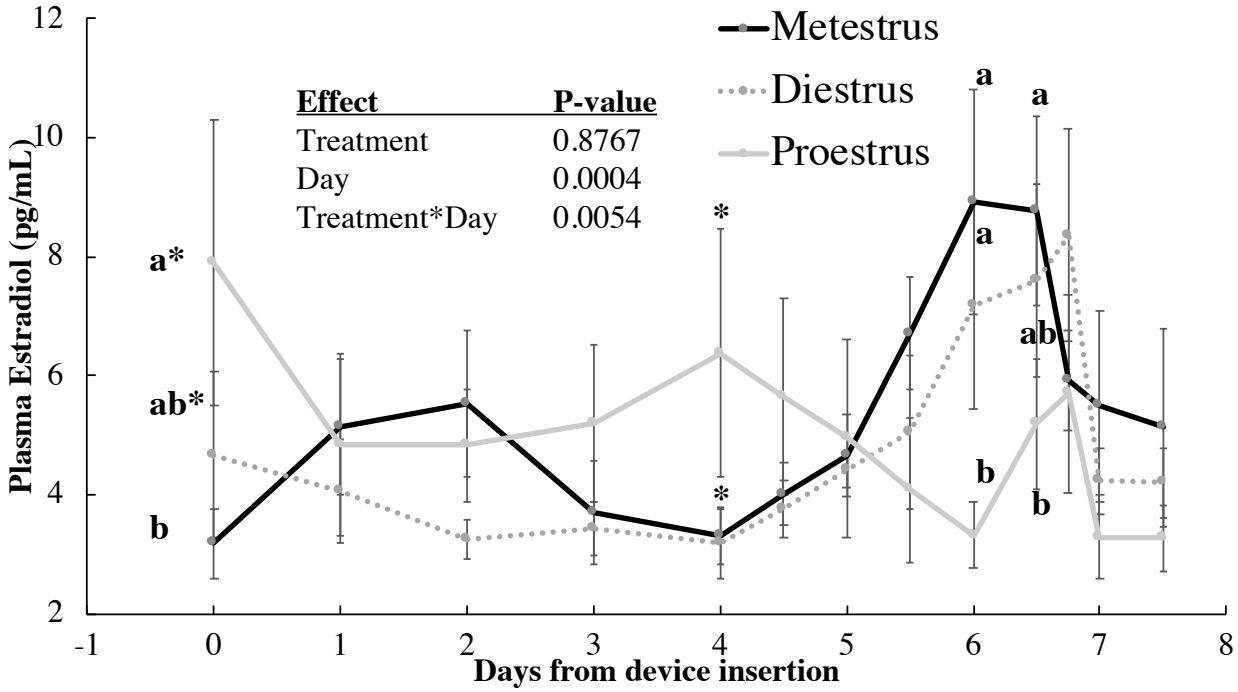

Figure 7. 10**.** Plasma concentrations of estradiol (mean ± SEM) in heifers following letrozole treatment initiated during metestrus, diestrus or proestrus. [abc]Within days, values with no common superscript are different (P<0.05). *Values tended to be different (P=0.09).

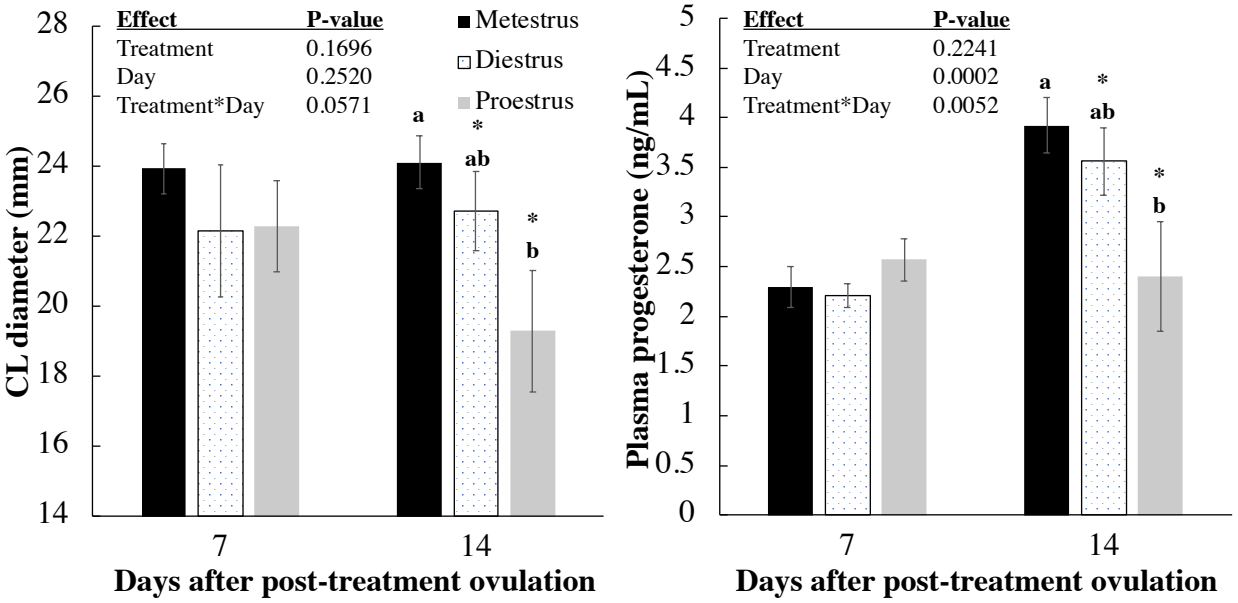

Figure 7. 11**.** Mean (± SEM) corpus luteum (CL) diameter (left) and plasma progesterone (right) 7- and 14-days following post-treatment ovulation in heifers from Experiment 2 treated with a letrozole releasing device during metestrus, diestrus, or proestrus. [ab]Within days, values with no common superscript are different (P<0.05). *Values tended to be different (P=0.07).

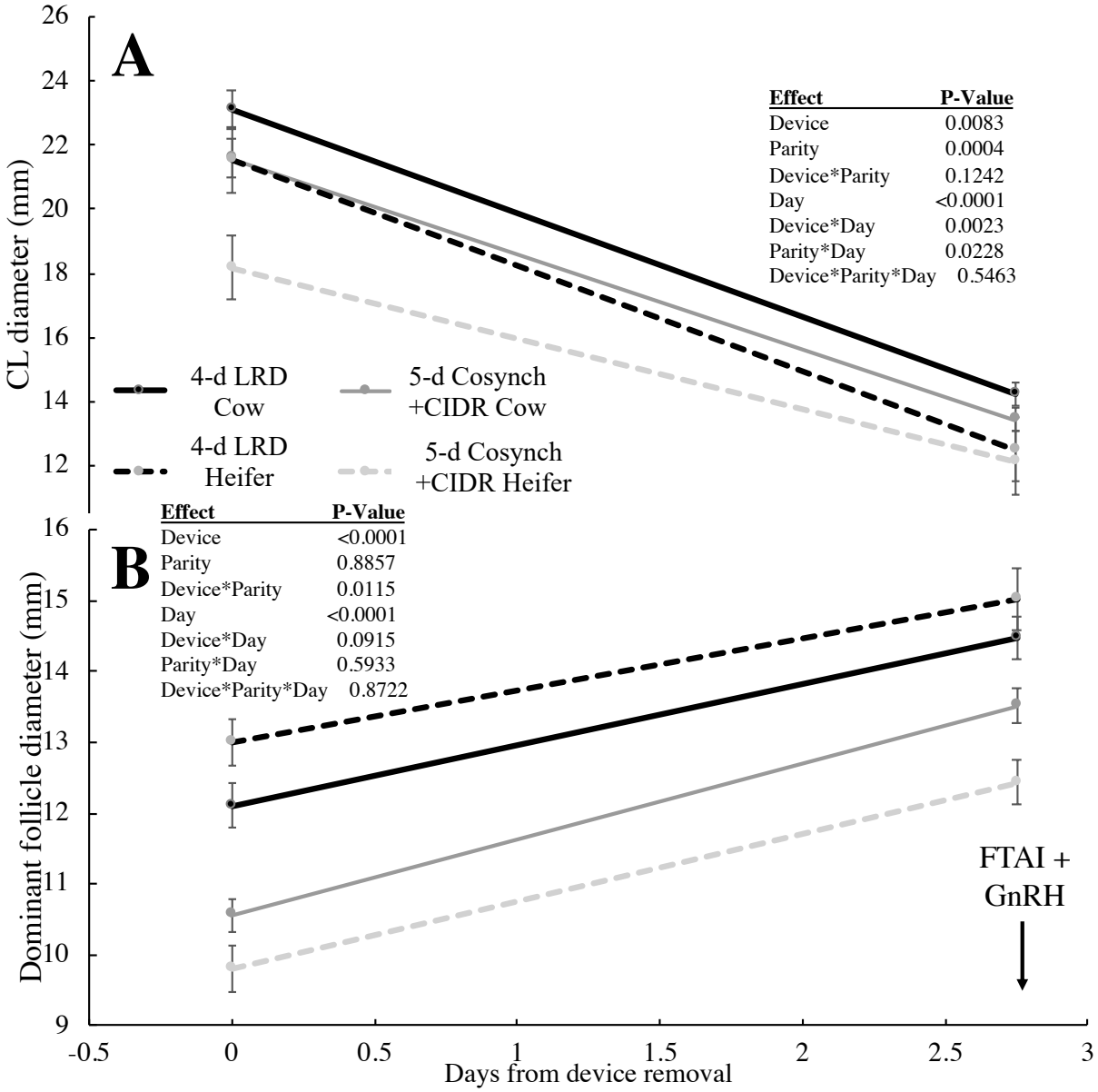

Figure 7. 12**.** Mean (± SEM) corpus luteum (CL) diameter (A) and dominant follicle diameter profiles (B) between heifers (n=69) and cows (n=151) that were treated with either a 4-day letrozole releasing device (LRD) or a 5-day Cosynch plus CIDR protocol. Fixed-time artificial insemination (FTAI) was done concurrently with 100 μg GnRH 66 hours after device removal.

Table 7. 3**.** Endpoints following FTAI comparing a 4-day LRD protocol and a 5-day Cosynch plus CIDR protocol in beef heifers and lactating beef cows. Mean ± SEM

	4-day LRD		5-day Cosynch + CIDR		P-value		
Endpoint	Cow	Heifer	Cow	Heifer	Protocol	Parity	Protocol x Parity
Animals (n)	73	34	78	35	-	-	-
Estrous detection score ≥2 on day of LRD insertion	45/73 (61.6%)	20/34 (58.9%)			-	0.83	-
Ovulated to initial GnRH	-	-	40/78 (51.3%)	14/35 (40%)		0.27	
Ovulation detected before FTAI	1/73 (1.4%)	6/32 (18.8%)	2/78 (2.6%)	7/35 (20.0%)	0.57	<0.001	0.73
Estrous detection score ≥2 at FTAI	30/73 (41.1%)	21/32 (65.6%)	40/78 (51.3%)	31/35 (88.6%)	0.01	<0.001	0.15
Estrous detection score ≥2 at 30 hrs after FTAI	47/73 (64.4%)	23/32 (71.9%)	56/77 (72.7%)	32/35 (91.4%)	0.02	0.04	0.15
Ovulated by 30 hrs after FTAI	65/73 (89.0%)	30/32 (93.8%)	73/78 (93.6%)	33/35 (94.3%)	0.33	0.77	0.94
Double ovulation	12/73 (16.4%)	2/32 (6.3%)	6/78 (7.7%)	0/35 (0%)	0.05	0.05	0.97
CL diameter 6 days after ovulation (mm) [1]	24.4 ± 0.4	21.9 ± 0.6	23.1 ± 0.7	21.5 ± 0.7	0.13	<0.001	0.53
Plasma P4 6 days after ovulation (ng/mL) [1]	2.4 ± 0.3[a]	4.1 ± 0.3[b]	2.2 ± 0.2[a]*	2.8 ± 0.2[a]*	0.004	<0.001	0.04
Pregnancy per AI (P/AI)	34/73 (46.6%)	17/32 (53.1%)	43/78 (55.1%)	23/35 (65.7%)	0.15	0.24	0.76
P/AI estrous score ≥2 at FTAI	23/30 (76.7%)	15/21 (71.4%)	30/40 (75.0%)	22/31 (71.0%)	0.89	0.57	0.94
P/AI estrous score <2 at FTAI	11/43 (25.6%)	2/11 (18.2%)	13/38 (34.2%)	1/4 (25.0%)	0.58	0.55	0.99
P/AI estrous score ≥2 30 hrs after FTAI	30/47 (63.8%)	16/22 (72.7%)	38/56 (67.9%)	22/32 (68.8%)	0.99	0.54	0.62
P/AI estrous score <2 30 hrs after FTAI	4/26 (15.4%)	1/10 (10%)	5/22 (22.7%)	1/3 (33.3%)	0.27	0.98	0.57
P/AI ovulated to initial GnRH			24/40 (60.0%)	8/14 (57.1%)	-	0.78	-
P/AI no ovulation to initial GnRH			19/38 (50.0%)	15/21 (71.4%)	-	0.22	-
P/AI displayed estrus to Presynch [2]	26/45 (57.8%)	15/19 (78.9%)	-	-	-	0.16	-

[ab]Within rows, values with no common superscript are different (P<0.05); P4: progesterone; [1]n=20/group; [2]Estrus detection score ≥2 at the time of LRD insertion; *P=0.06

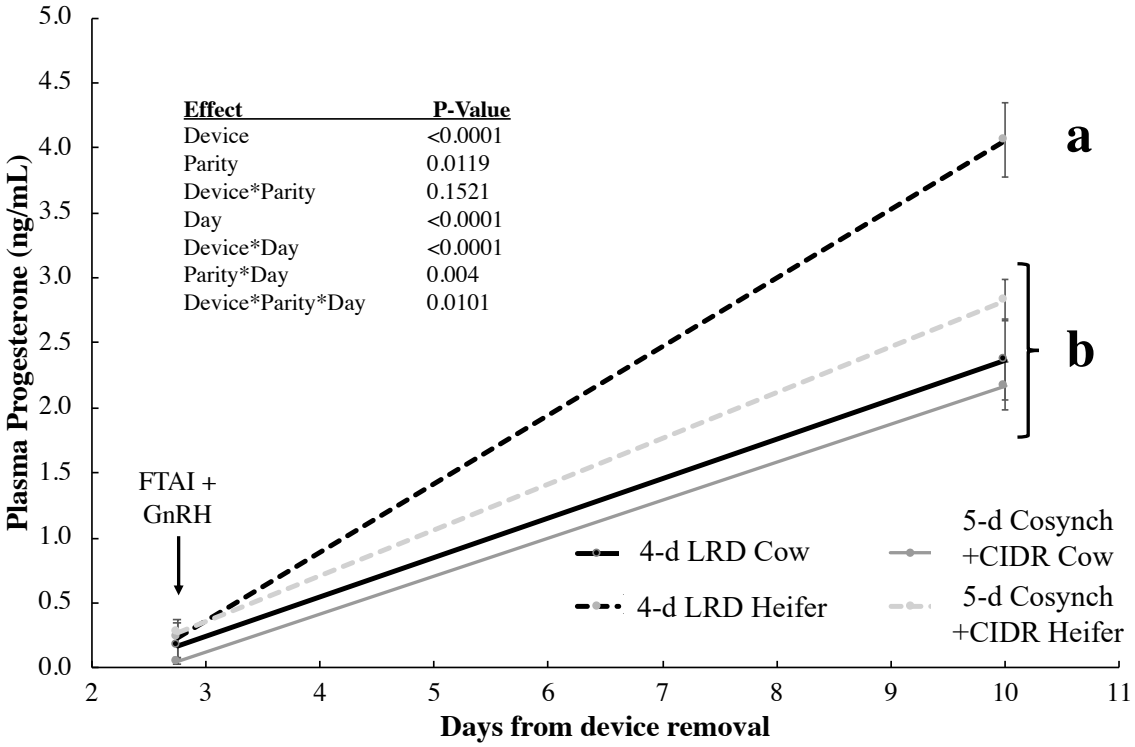

Figure 7. 13**.** Plasma progesterone concentrations (mean ± SEM; n=20/group) from lactating beef cows and beef heifers treated with either a letrozole releasing device (LRD) for 4 days or a 5-day Cosynch plus CIDR protocol. Fixed-time artificial insemination (FTAI) was done concurrently with 100 µg GnRH 66 hours after device removal. [ab]Within days, values with no common superscript are different (P<0.05).

Table 7. 4**.** Effect of sire used for fixed-time artificial insemination in heifers and cows synchronized with either an intravaginal letrozole releasing device (LRD) for 4 days or a 5-day Cosynch plus CIDR protocol.

Heifers	4-d LRD	5-d Cosynch + CIDR	Total
Bull A	8/16 (50%)[ab]	16/18 (88.9%)[a]	24/34 (70.6%) *
Bull B	9/16 (56.3%)[ab]	7/17 (41.2%)[b]	16/33 (48.5%) *
Total	17/32 (53.1%)	23/35 (65.7%)	40/67 (59.7%)

Cows	4-d LRD	5-d Cosynch + CIDR	Total
Bull C	17/36 (47.2%)	23.39 (59.0%)	40/75 (53.3%)
Bull D	17/37 (45.9%)	20/39 (51.3%)	37/76 (48.7%)
Total	34/73 (46.6%)	44/78 (55.1%)	77/151 (51.0%)

[ab]Within rows and columns, values with no common superscript are different (P<0.05).
*Values tended to be different (P=0.06).

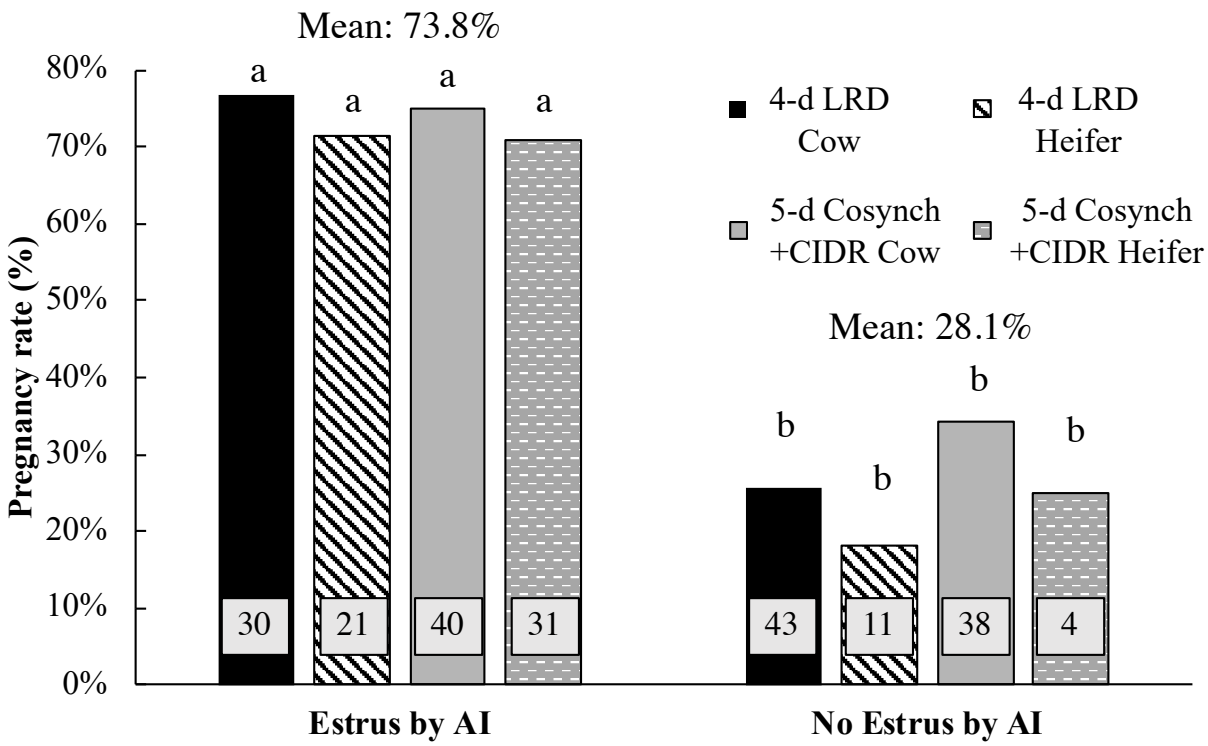

Figure 7. 14**.** Pregnancy per AI following fixed-time artificial insemination (FTAI) from cattle who displayed (estrus detection score ≥2) or did not display estrus (estrus detection score <2) by FTAI. The boxed column numbers indicate the total number of cattle per group. [ab]Within columns, values with no common superscript are different (P<0.0001).

7.5. Discussion

Results of Experiment 1 supported the hypothesis that increasing the rate of luteolysis and extending the period of proestrus minimizes the incidence of short-lived CL. In contrast to our previous studies (Chapter 5), no differences were detected in CL diameter or progesterone profiles during luteolysis. Similarly, subsequent progesterone profiles following post ovulation in Experiment 1 did not differ between LRD- and sham-treated heifers (i.e. normal lifespan CL). We attribute this to 1) no GnRH was given following PGF treatment (i.e. endogenous estradiol led to an endogenous LH surge) and 2) peak estrus and peak plasma estradiol concentrations

occurred at 64 and 72 hours respectively, following PGF (Table 7.1 and Fig. 7.6B). This provides support for our notion that treatment with GnRH in the earlier study (Chapter 6) was given 16 to 24 hours too early and this resulted in a short-lived CL after ovulation. In a previous study, ovariectomized cows were treated with low, medium, or high doses of estradiol for 40 hours to mimic physiological estradiol concentrations at estrus (Mann and Lamming, 2000). Cows in the low and medium estradiol groups had increased oxytocin receptor populations in the endometrium at 4- and 6-days after expression of estrus and increased concentrations of prostaglandin metabolite following an oxytocin challenge compared to cows treated with a high dose of estradiol. These results suggest that when threshold estradiol concentrations are not achieved (i.e., GnRH given before peak estradiol), residual endometrial oxytocin receptors remain, and may then respond to the estradiol produced from the dominant follicle of the first follicular wave increasing PGF production and subsequent luteolysis.

An unexpected outcome in Experiment 2 was the number of heifers that ovulated during LRD placement or within 24 hours of LRD removal in the proestrus group (55%) which was not reported in previous studies. Interestingly, in review of the data of a previous study (Yapura et al., 2016), letrozole treatment did not prevent ovulation during the proestrus and estrus period, where over 40% of heifers ovulated during LRD placement or within 24 hours of LRD removal (data from (Yapura et al., 2016)). Collectively, these data suggest that LRD treatment during the proestrus or estrus period does not suppress LH surge release, even though no signs of estrus were observed. Letrozole treatment may reduce endogenous estradiol concentrations as shown in Fig. 10, but this may not be sufficient to prevent a threshold estradiol concentration to achieve an LH surge. It is interesting that of the six heifers that ovulated during or within 24 hours of letrozole treatment in the present study, two did not show signs of estrus. Similarly, it was shown

recently that oral treatment with a single 20 mg dose of letrozole did not prevent ovulation when given to women with preovulatory follicles (Allaway et al., 2017).

In Experiment 2, the numerically larger dominant follicle at FTAI resulted in a larger CL and increased plasma progesterone 14-days after ovulation in the metestrus group. The positive correlation between ovulatory follicle diameter and both CL diameter- and plasma progesterone following ovulation has been reported in beef cows, but it was concluded that the optimal follicle size for maximizing pregnancy success was 13 to 15 mm (Pfeifer et al., 2012). It is noteworthy that pregnancies resulting from the ovulation of follicles ranging from 15 to 22.5 mm in diameter occurred in the present study in heifers in which letrozole treatment was initiated during the metestrus period. The large ovulatory follicle diameter was attributed to an increase in LH pulsatility (Chapter 5), due to the combination of decreased estradiol (from letrozole) and low progesterone during follicular growth of the first wave dominant follicle (Adams et al., 1992a).

In Experiment 3, FTAI following a newly developed letrozole synchronization protocol resulted in a P/AI that did not differ from the 5-day Cosynch plus CIDR protocol. In this study, a greater proportion of heifers had estrus scores ≥2 on the day of FTAI compared to cows, although approximately 20% of cows showed sign of estrus following FTAI compared to <5% of heifers. The interval from the onset of luteolysis to ovulation was 14 hours longer in lactating Holstein cows than in heifers (Sartori et al., 2004) indicative of slower resumption of estrus in cows. It has also been shown previously that beef cows show estrus 7 to 10 hours later than heifers depending on the follicular stage when PGF was given (King et al., 1982). Therefore, prolonging the interval from PGF to AI past 66 hours may increase the expression of estrus and increase P/AI, especially in cows.

The current study shows one of the highest P/AI following FTAI in cattle that displayed estrus (73.8%). In the present study, P/AI may have been increased even more in cows by delaying FTAI by 6 to 12 hours (i.e. AI at 72 to 78 hours after PGF) as has been reported recently (Macmillan et al., 2020). Evidence for this was the >20% increase in estrus detection score ≥2 between the day of FTAI and the day of ovulation in cows (Table 7.3). In Experiment 3, P/AI in the LRD group did not duplicate what was observed in the diestrus and metestrus groups in Experiment 2 (51/105 [48.6%] vs 16/21 [76.2%]). An explanation for this may be the proportion of cattle that underwent luteolysis to the initial PGF (Presynchronization) and had an estrus detection score ≥2 at the time of LRD insertion (65/107 [60.7%]; Table 3). In cattle with 2 follicular waves (i.e. 20 day interovulatory interval), it is expected that 15/20 (75%) would undergo luteolysis and subsequently ovulate to a single dose of PGF (Kastelic et al., 1990). Therefore, a larger proportion of cattle in the present study were 0- to 5-days post ovulation when the initial PGF was given (42/107 [39.3%]), which may have attributed to the lower estrus response after presynchronization and P/AI.

In summary, PGF given twice at 12-hour intervals on the day of LRD removal resulted in a more synchronous onset of estrus and interval to ovulation in Experiment 1. In Experiment 2, letrozole treatment initiated during metestrus resulted in the greatest CL diameter and plasma progesterone concentrations 14-days following the post-treatment ovulation, as well as the highest P/AI. In Experiment 3, there was no difference in P/AI between a 4-day LRD protocol and the 5-day Cosynch plus CIDR treatment. Future studies should address lengthening the PGF to FTAI interval by 6 to 12 hours to maximize estrus activity, estradiol concentrations, and pregnancy success, especially in lactating beef cows.

Acknowledgments

The authors thank Vetoquinol for their donation of Bioestrovet and Fertiline used in the study. We thank Ky Pohler, Sydney Reese and Gessica Franco at Texas A&M University and Susan Cook, Kim Tran, and Danielle Carriere at the University of Saskatchewan for assistance in running radioimmunoassays. We also thank Chris Jermey, Victoria Wallace and the staff at the University of Saskatchewan's Livestock and Forage Centre of Excellence for assistance in data collection and cattle handling. The study was supported by grants from Alberta Agriculture and Forestry, Venco Animal Health, a subsidiary of Dechra Pharmaceuticals PLC, and the Natural Sciences and Engineering Research Council of Canada.

CHAPTER 8

8. GENERAL DISCUSSION AND CONCLUSION

8.1. General Discussion:

The overall aim of this thesis was to determine the effectiveness of letrozole in controlling ovarian function and to use the knowledge gained to develop ovarian synchronization protocols use in for advanced reproductive techniques in cattle. Our first study was conducted to determine if letrozole could be released from a silicone matrix and have a biological effect on ovarian function (Chapter 2). Then, following the use of an intravaginal letrozole releasing device in lactating dairy cows, letrozole concentrations were quantified in milk for future use in drug depletion studies (Chapter 3). Two trials were subsequently completed to determine if letrozole treatment was advantageous during superovulatory protocols (Chapter 4). To better grasp the effect of letrozole on the local ovarian environment, and the ability of the cumulus-oocyte complex (COC) to undergo maturation and *in vitro* fertilization following letrozole treatment, three experiments were done (Chapter 5). The interval to ovulation and initial fixed-time artificial insemination trials following letrozole synchronization was done next, which was followed by the development of a new letrozole releasing device that increased plasma concentrations of letrozole (Chapter 6). Finally, the interval to estrus and a fixed time artificial insemination field trial using a letrozole-based protocol was done in three experiments (Chapter 7).

The concept of the silicone letrozole releasing device (LRD) developed in Chapter 2, which was used throughout this thesis, was conceived while researching the development of the controlled internal drug release device (CIDR) in cattle (Ogle, 1999). Progesterone, which was

impregnated into the silicone matrix of the CIDR, exhibited qualities that were similar to that of letrozole (i.e., <350 g/mol and lipophilic molecules). To test the original hypothesis that letrozole could be released from a silicone matrix, industrial silicone was purchased and preliminary testing was done by mixing the silicone and letrozole (10% wt/wt drug load) and waiting for the required 24-hour period for it to cure. Initial fears that the letrozole nitrile group would cause cure inhibition i.e., the silicone would never harden were unfounded. Following 24 hours of curing, the silicone plus letrozole matrix hardened and initial testing of release into ethanol was positive. This initial testing with industrial-grade silicone indicated that letrozole did release from the matrix into the ethanol, allowing for initial *in vitro* and *in vivo* testing utilizing medical-grade silicone.

It is speculated that the development of the silicone intravaginal device resulted in decreased variability of manufacturing, drug delivery, and plasma letrozole in comparison to previously designed dip-coat device (Yapura et al., 2015). Unfortunately, one of the pitfalls of the silicone LRD in Chapter 2 was the decrease in plasma letrozole levels in comparison to the dip-coat device (i.e., 80 ng/mL vs >200 ng/mL; (Yapura et al., 2015)). A dose-effect of letrozole was observed in Chapter 2 for dominant follicle diameter, CL diameter, and interwave interval. The original standard surface area LRD exhibited the same surface area compared to the CIDR (120 cm^2), which did not result in sufficient letrozole release to affect plasma estradiol or dominant follicle diameter. Therefore, the surface area was quadrupled by developing a large surface area device by adding 2 mm thick "wings" on the original device, which increased letrozole release. Unfortunately, letrozole plus FSH did not increase the superovulatory response in mature cows (Chapter 4), as observed in women (Garcia-Velasco et al., 2005).

Moreover, in Chapter 5, treatment with our silicone LRD did not affect the mRNA gene expression in granulosa cells obtained from follicles 3 days after wave emergence. In addition, the silicone LRD did not suppress estradiol levels sufficiently to prevent ovulation in the proestrus heifers in Chapter 8. It was hypothesized that the dose of letrozole delivered by the intravaginal device was insufficient to elicit a biological effect. To mitigate this problem and increase plasma letrozole concentrations, giving a dose of intramuscular or intravenous letrozole (Yapura et al., 2014) at the time of LRD insertion was an option which would result in a secondary method of letrozole administration, but this would result in an additional drug application and increased expense. The FDA approval process in 2019 was reported to cost $950,000 per new drug application, and this does not include the costs of studies associated with drug safety, effectiveness, and tissue residue (U.S. FDA, 2018).

One of the negative aspects of using a drug impregnated silicone intravaginal device is high residual drug remaining in the device after use. Past studies in development of the CIDR-B (1.9 grams of progesterone) indicated that a high proportion of progesterone was leftover following removal; therefore, CIDR-B were re-engineered to minimize the thickness of the silicone and subsequently decreased the progesterone load (1.38 grams) to minimize residual progesterone while maintaining similar plasma concentrations (Ogle, 1999). The 15% letrozole large surface area device developed in Chapter 2, contained 5.6 grams of letrozole per device. Therefore, it would have been useful to test residual levels of letrozole after use. Remarkably, following autoclaving, a CIDR 1380 device used once resulted in higher concentrations of initial plasma progesterone concentrations than a new CIDR 1380 (Zuluaga and Williams, 2008; Melo et al., 2018). It was suggested that the autoclaving process modifies the structure of the device or distribution of the drug within the device (Zuluaga and Williams, 2008). Autoclaving a once-

used PRID resulted in a large quantity of crystalline progesterone on the surface, and this was hypothesized to increase progesterone availability (McPhee et al., 1983). Future studies involving LRD reuse are needed. Following their use in Chapter 7, the LRD were sanitized, autoclaved, and could be used in the future for pharmacokinetic studies.

In Chapter 3, lower mean maximum concentrations of plasma letrozole following the intravaginal LRD were observed in lactating Holsteins compared to nulliparous beef heifers in Chapter 2 (i.e., 35 ng/mL vs. 70 ng/mL, respectively). These lower concentrations were most likely due to a combination of a greater body mass of mature Holstein cows (i.e., 680 kg vs. 422 kg, respectively), a decrease in the elimination half-life in lactating Holstein cows compared to nulliparous heifers (i.e., 15 vs. 33 hours, respectively; Yapura et al., 2013), due to an increased metabolism in lactating cows. For the study in Chapter 3, cull, lactating Holstein cows were purchased from local producers. As milk production was low (9-12 kg/day, the elimination half-life was expected to be higher than in high producing dairy cows around the time of first insemination (~40 kg/day). This speculated difference in elimination half-life poses an essential question as to whether letrozole would even have a biological effect in high producing lactating dairy cows. The downfall of the idea of intravaginal dosing, deployed in this thesis, is the inability to increase the dose of letrozole on a per kilogram basis; therefore, the same amount of letrozole is delivered to a 400 kg heifer compared to an 800 kg lactating dairy cow. This dosing is speculated to be the reason why more intravaginal drug delivery systems are not used in food-producing animals. Therefore, to alter the dose of an intravaginal drug, either surface area or the drug load would need to be changed, and this would necessitate more drug testing and costs, as described previously. This dose effect of letrozole was observed in our FTAI studies done in Chapters 6 and 7. Heifers in the LRD group had dominant follicles that were numerically larger

in diameter at the time of AI compared to cows in the LRD group. This likely resulted from increased plasma letrozole concentrations in the heifers, resulting in a greater suppression of plasma estradiol, which in turn increased LH pulsatility (Kinder et al., 1996) resulting in an increased diameter of the dominant follicle.

A fascinating revelation in Chapter 3 was that letrozole was excreted into the milk of lactating dairy cows. This was the first study done in dairy cattle, as all previous studies utilized beef heifers (reviewed in Yapura et al., 2018). In Chapters 6 and 7, both beef heifers and lactating cows were used, indicating nursing calves consumed letrozole. Understanding the effect of letrozole on prepubertal animals is lacking but is essential for better understanding. Letrozole treatment has been used in prepubertal boys with short stature. Following treatment, an increase in stature was observed in comparison to placebo-treated controls. Although an increase in plasma testosterone concentrations was observed in the letrozole group, no advancement of onset to puberty was reported (Hero et al., 2005). The administration of anastrozole, in adolescent, growth hormone deficient boys, slowed the epiphyseal fusion caused by blockage of estrogen synthesis, which resulted in a significant height increase in comparison to the placebo group (Mauras et al., 2008). Therefore, there is potential for calves consuming letrozole-containing milk from their dams to grow at faster rates than their non-treated counterparts.

In Chapter 4, the co-administration of letrozole and FSH did not increase the superstimulatory effect in cows, as observed in women (Mitwally and Casper, 2002). In women, once-daily oral letrozole is given in combination with recombinant FSH commencing on Day 3 of menses (Garcia-Velasco et al., 2005). Day 3 of menses correlates to increasing concentrations of endogenous FSH and the number of antral follicles ≥ 5 mm in women exhibiting both two- and three-follicular waves (Baerwald et al., 2003). Therefore, ovarian stimulation initiated on

Day 3 occurs at the time of, or shortly after, wave emergence regardless of women exhibiting two- or three- follicular waves per menstrual cycle. In Chapters 4 and 5, letrozole was given to cows immediately following the ablation of follicles ≥5 mm (i.e., approximately 1.5 days before wave emergence) for 3.5 to 4 days. Therefore, letrozole treatment may be disruptive to early follicular growth and result in a lower proportion of ovulations (Chapter 4) and the number of follicles aspirated (Chapter 5) following treatment. To resemble letrozole treatment in women, it would be necessary to insert the LRD at the initiation of FSH treatment in cattle (i.e., at the time of wave emergence). Similarly, a superstimulatory response may be favored utilizing a decreasing (i.e., step-down) vs a constant FSH dosage regiment in cattle. Women undergoing ovarian stimulation endure daily transvaginal ultrasonography to monitor ovarian stimulation with FSH. With the combination of letrozole and FSH in women, a lower dosage of FSH is needed to achieve ovarian stimulation in comparison to FSH alone (Goswami et al., 2004). Therefore, the superstimulatory outcome in cattle may be comparable with letrozole plus half the dosage of FSH (LRD + 200 mg FSH) in comparison to a full dosage alone (400 mg FSH), although this speculation assumes that an accumulation of intraovarian androgens upregulate the FSH-receptor in granulosa cells, a phenomenon that was not seen in granulosa cells in Chapter 7. Although a decrease in estradiol in follicular fluid was observed, no testosterone in follicular fluid was assayed in this thesis.

With current knowledge of bovine reproductive endocrinology, the negative effect of letrozole on early follicular growth immediately preceding wave emergence is speculated to stem from two possibilities: 1) increased plasma LH concentrations causing disruption of early follicular development (Chupin et al., 1984) or 2) increased inhibin produced from the growing dominant follicle before, and at the time of selection suppressing endogenous FSH (Kaneko et

al., 1995). Unfortunately, the first hypothesis was not supported in the first experiment in Chapter 4. In that study, an LRD was given at the time of wave emergence (i.e., 1.5 days after follicular ablation) after the number of antral follicles ≥2 mm were counted. The LRD was removed 5 days later, followed by follicular ablation to induce wave emergence following treatment (i.e., plasma letrozole and LH concentrations were elevated at this time). There was no difference in the number of follicles ≥2 mm before and after letrozole treatment, indicative of no effect of elevated LH on early follicle growth. The hypothesis of an increase in follicular inhibin concentrations was not tested directly, but not supported indirectly in this thesis either, as frequent blood sampling done after 4-days of letrozole treatment did not show a difference in plasma FSH concentrations. To understand the effect of letrozole treatment on early follicular development before wave emergence more frequent blood sampling should be done immediately following follicular ablation and LRD insertion to characterize both LH and FSH concentrations.

Generally, when co-stimulation with letrozole and FSH is used in women, the study population utilizes sub-fertile women with a low ovarian reserve. A study done in women with a high antral follicle count indicated no difference in the number of COC recovered between letrozole vs no letrozole treatment. A lower pregnancy rate was achieved with letrozole in this study, which was attributed to increased plasma progesterone on the day of hCG to induce *in vivo* maturation (Yang et al., 2019). Moreover, a large number of randomized controlled trials in women show no difference in the number of oocytes recovered following letrozole treatment (Goswami et al., 2004; Ozmen et al., 2009; Davar et al., 2010) with the exception of one (Verpoest et al., 2006). These studies could be replicated using the bovine model, as the assessment of a low number of antral follicles by ultrasonography correlates to a low ovarian response following superstimulation (Singh et al., 2004). The combination of letrozole and FSH

may present a viable option in poor-responding cattle undergoing superstimulation, but further testing using the bovine model is necessary.

In Chapter 5, there were no differences detected in granulosa cell mRNA gene expression in all genes analyzed between the LRD and sham-treated groups. Although estradiol tended to be lower in both the dominant and subordinate follicle follicular fluid, no significant differences were detected in CYP19A1 gene expression. Using human luteinized granulosa cells cultured *in vitro*, no difference was detected in either estradiol production or CYP19A1 expression until cultured with 285 ng/mL of letrozole (Lu et al., 2012). Therefore, achieving plasma letrozole concentrations of 70 ng/mL in Chapter 5 may not have been sufficient to affect gene expression of targeted genes. Similarly, unlike described in the literature, no difference was detected in the FSHR gene expression between treatment groups in Chapter 5. The hypothesis that letrozole would increase intraovarian androgens and upregulate FSHR gene expression was not supported using the bovine model in Chapters 4 and 5. Although the number of heifers per group (n=8) was low and the high variation in gene expression, the difference is not conclusive. Therefore, another study should be done using a greater number of animals and including new reference genes to decrease variation.

Although a decreased number of COC were recovered per LRD-treated heifer in Chapter 5, a higher proportion of these COC advanced to the metaphase II stage following 24 hours of *in vitro* maturation compared to the sham group. The reason for this is unknown but is likely attributed to LH pulse amplitude. Although not recognized for its role in the growth of follicles <8 mm, LH may play an integral role in early antral follicle development. The LH-receptors have been localized in mural granulosa cells of follicles >9 mm in diameter, but are found in theca interna cells of follicles ≥4 mm in diameter (Xu et al., 1995). It has been postulated in

humans, that LH acts to accelerate apoptosis of granulosa cells from subordinate follicles by downregulating bone morphogenic protein (BMP-4 and -7; (Tajima et al., 2007)). A large proportion of the COC collected in Chapter 5 were from follicles approximately 3-5 mm in diameter. Therefore, the increase in LH pulsatility may have led to a more substantial proportion of small follicles undergoing regression in the letrozole group and a positive relationship exists between early follicular regression and oocyte competence (Vassena et al., 2003). The increase in LH pulsatility before aspiration may also explain the increased proportion of blastocysts from total COC and embryos from compact and cleaved zygotes in the letrozole group following *in vitro* maturation and *in vitro* fertilization. This difference in oocyte quality was also observed in women undergoing ovarian stimulation with both a GnRH-antagonist plus letrozole. Although a lower number of oocytes were recovered from women in that group, a significantly higher fertilization rate following intracytoplasmic sperm injection was shown (Yarali et al., 2009). Therefore, the effect of increased LH pulsatility and whether it is beneficial or determinantal to early follicle growth and subsequent *in vitro* embryo production still requires investigation.

In Chapters 6 and 7, letrozole synchronization protocols were developed for use in FTAI in cattle. Although synchronous ovulation following a 4-day LRD and a 48-hour interval from PGF to GnRH was observed, a lower pregnancy per AI was achieved compared to commonly used GnRH-based synchronization protocol in Chapter 6. This low P/AI was attributed to a shortened interval from PGF to GnRH, not permitting a rebound in circulating estradiol for sperm transport and subsequently causing short lifespan CL. By understanding the interval from PGF to estrus in LRD-treated heifers, we were able to pinpoint when the rebound in estradiol and endogenous the LH surge occurred. Using the electronic estrus detection data, we could then approximate when to perform AI and give GnRH in a FTAI protocol utilizing letrozole

treatment. With an interval of 66 hours from PGF to GnRH/AI, a high P/AI was achieved in both the metestrus and diestrus group in Chapter 7 (>75%) with a tendency for an increase in the metestrus group. Unfortunately, when we applied this protocol in a field trial, the P/AI was 50%, and no difference was shown between a 4-day LRD vs. a 5-day CIDR Cosynch protocol. It was speculated that the decrease in P/AI was attributed to 2 reasons: 1) Approximately 40% of the cattle were in metestrus at the time of PGF presynchronization; therefore, they did not undergo luteolysis (to the initial PGF) followed by spontaneous ovulation, and 2) the PGF to AI interval was hastened by approximately 6 to 12 hours as evidenced by the estrus detection score at the time of AI. Through the use of estrus detection patches in Chapter 7, it was shown that only 60% of cattle given PGF at random stages of the estrous cycle showed signed of estrus within 5 days. Subsequently, 79% of the heifers that showed estrus following presynchronization with PGF before letrozole treatment became pregnant following FTAI, compared to only 58% of cows. Evidence for the 2nd speculation was shown by the increased proportion of cows that displayed estrus following AI/GnRH (~20%). In a previous study, it was shown that beef heifers display estrus significantly earlier than cows following a 7-day CIDR Cosynch protocol (i.e., 50 vs. 54 hours; Rich et al., 2018). Therefore, by extending the interval from PGF to AI/GnRH from 66 to 72 or 78 hours may have resulted in a greater proportion of cows displaying estrus before AI, which would hypothetically increase pregnancy per AI. Another option would be to give PGF 11 to 14 days apart to increase the proportion of cattle responding to the presynchronization treatment.

A viable option that has yet to be investigated is the potential use of letrozole synchronization for fixed-time embryo transfer (FTET) in cattle. Letrozole for FTET may increase P/AI in several different ways. Firstly, letrozole can be used for synchronization of

ovulation and would result in a larger preovulatory follicle, attributed to increased LH in circulation, as shown in Chapter 5. Following letrozole treatment, a larger ovulatory follicle resulted in a CL that produced a higher concentrations of circulating progesterone 6 days after ovulation in Chapter 7. Secondly, letrozole is luteotrophic (Chapter 2), indicating that it can be used to increase CL size and production of progesterone following ovulation. Lastly, and probably the least explored option in cattle, is the effect of letrozole on endometrial thickness. Ovarian stimulation with letrozole, in women, has been shown to increase endometrial thickness in comparison to clomiphene citrate (Atay et al., 2006) due to decreased estradiol at the time of oocyte aspiration. Endometrial thickness at the time of embryo transfer has been shown to be positively correlated to pregnancy success in women (Wu et al., 2014), and in a large retrospective study in Japan with frozen embryo transfer, ovulation induced with letrozole increased pregnancy success (Tatsumi et al., 2017). Ultrasonographic assessment of the appearance of the bovine uterus throughout the estrous cycle in cattle, has been done (Pierson and Ginther, 1987c). In high producing dairy cows (~40 kg/day), endometrial thickness at the time of artificial insemination was positively correlated to pregnancy success, and it was suggested that high estradiol and low progesterone led to increased endometrial thickness (Souza et al., 2011). Unfortunately, there is a lack of information on the thickness of the endometrium at the time of embryo transfer in cattle.

Therefore, utilizing this knowledge on endometrial thickness, it may be speculated that letrozole synchronization would be beneficial for embryo transfer programs, and a synchronization protocol could be developed as depicted in Figure 8.1 below.

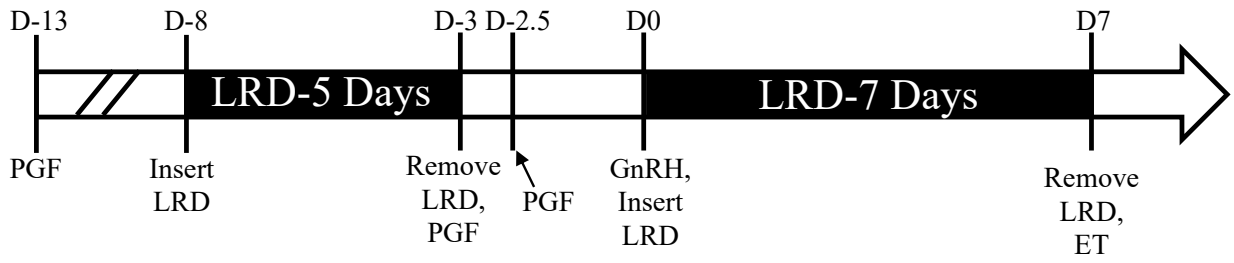

Figure 8. 1**.** A speculated experimental timeline utilizing letrozole for fixed-time embryo transfer in cattle. An LRD is given on Day -8 for 5 days to increase follicular size of the preovulatory follicle. A separate LRD is given on Day 0 for 7 days to increase progesterone production from the corpus luteum and decrease estradiol concentrations, preparing the endometrium for ET on Day 7. PGF: 500 μg cloprostenol; LRD: letrozole releasing device; GnRH: 100 μg gonadorelin; ET: embryo transfer

Using a similar experimental treatment schedule as described in Chapter 7, cattle could be presynchronized with PGF on Day -13 and given an LRD 5 days later (Day -8). By increasing the length of LRD treatment from 4- to 5-days, it is hypothesized that a larger follicle would be present at device removal on Day -3. Similarly, PGF would be given twice 12 hours apart to ensure luteolysis and GnRH would be given 72 hours after device removal (Day 0). At the time of GnRH, an LRD would be inserted for 7 days prior to embryo transfer. This duration of treatment would not impede ovulation (following GnRH), and letrozole's luteotrophic properties would hypothetically increase the size and progesterone secretion of the resultant CL. Moreover, by decreasing estradiol and increasing progesterone, letrozole may increase the receptivity of the endometrium before embryo transfer and overall increase the P/AI following embryo transfer in cattle.

8.2. General Conclusions

8.2.1. Research and development of a silicone letrozole-releasing device to control reproduction in cattle

a) A letrozole-impregnated silicone device provided continuous *in vivo* release of letrozole throughout the 8-day experimental period.

b) A larger surface area letrozole-releasing device resulted in the highest-circulating letrozole concentrations, the largest dominant follicle and most prolonged interwave interval, and the largest CL 7 days post-ovulation.

c) Treatment with the larger surface area letrozole-releasing device decreased plasma estradiol concentrations the most in heifers.

8.2.2. Analysis of letrozole milk residues following intravaginal letrozole treatment in lactating dairy cattle

a) A new method for measuring letrozole concentrations in milk was partially validated by tandem mass spectrometry with a limit of quantification of 5 ng/mL.

b) Following a 4-day intravaginal letrozole regimen, letrozole concentrations tended to be higher in plasma than milk and the maximum concentration of letrozole was higher in plasma than milk.

c) No difference in the interval to ovulation was detected after PGF to induce luteolysis and GnRH-induced ovulation following a 4-day letrozole treatment; the interval to ovulation was more synchronous in the letrozole group.

8.2.3. The bovine model to test the ovarian superovulatory effects of aromatase inhibitors

a) Following ablation-induced wave emergence, a 5-day regimen of intravaginal letrozole treatment did not increase the number of antral follicles at the subsequent wave emergence.

b) A combination of letrozole plus follicle-stimulating hormone (FSH) failed to increase the superovulatory response but lengthened the interval and decreased the variation in the time of onset of estrus.

c) Letrozole plus FSH treatment decreased the proportion of ovulations and the number of ovulations 36 hours after GnRH. Similarly, letrozole decreased the number of quality 1 embryos and tended to decrease the number of total transferable embryos.

d) Letrozole treatment decreased the concentrations of plasma estradiol at the time of the last FSH treatment.

8.2.4. The effect of an aromatase inhibitor on the ovarian-oocyte environment in beef cattle

a) Following 4 days of intravaginal letrozole treatment, follicular fluid concentrations of letrozole were highly correlated and comparable to plasma concentrations. A method for quantifying letrozole in follicular fluid was partially validated by tandem mass spectrometry as part of this experiment.

b) Following letrozole treatment and PGF-induced luteolysis on Day 3 of wave emergence, plasma progesterone concentrations were elevated, and plasma estradiol concentrations

were decreased compared to the sham group. This was indicative of a reduced luteolysis following letrozole treatment.

c) Letrozole treatment tended to decrease both the dominant and subordinate follicle concentrations of estradiol on Day 3 after wave emergence.

d) Letrozole treatment did not affect the expression of genes for steroidogenesis or ovarian function in granulosa cells obtained from both dominant and subordinate follicles 3 days after wave emergence.

e) Following letrozole treatment and frequent blood sampling for 8 hours, LH amplitude and area under the curve increased. No difference was detected for the FSH area under the curve, pulse amplitude, or pulse frequency.

f) Following cumulus-oocyte complex (COC) aspiration done on Day 4 after wave emergence, no difference was detected in the oocyte maturational stage immediately after collection in the letrozole vs. sham group. Following 24 hours of *in vitro* COC maturation, the proportion of metaphase II oocytes was higher in the letrozole treatment group.

g) Letrozole treatment increased the proportion of blastocysts and total embryos following COC aspiration, *in vitro* maturation, and *in vitro* fertilization.

h) Four days of letrozole treatment decreased the total number of follicles aspirated, total COC, and decreased the COC collection rate on 4 days after wave emergence.

8.2.5. Ovulation timing, fixed-time artificial insemination and effect of presynchronization on pregnancy results following aromatase inhibitor treatment in beef cattle

a) Treatment with a letrozole releasing device for 4 days followed by PGF and GnRH at a 48-hour interval resulted in the most synchronous interval to ovulation in lactating beef cows.
b) Letrozole treatment resulted in a lower pregnancy per AI compared to two commonly used synchronization protocols, even though the ovulatory follicle was larger at the time of device removal and AI.
c) Following the development of a new X-LRD, plasma letrozole increased throughout treatment, and the vaginal irritation score was numerically lower compared to a T-LRD.
d) Following presynchronization with PGF 8 days before letrozole treatment, and a PGF to GnRH/AI interval of 48 hours, no difference was detected in P/AI or the proportion of heifers that returned to estrus within 10 days after ovulation compared to heifers given an LRD at a random stage of the estrous cycle.

8.2.6. Estrus timing, corpus luteum function and field trial pregnancy results following aromatase inhibitor treatment in beef cattle

a) There was no effect of a 4-day letrozole treatment initiated 3 days after wave emergence followed by PGF at device removal on the degree of luteolysis (as assessed by CL diameter and plasma progesterone) or the interval to the onset of estrus compared to sham-treated heifers.
b) Giving PGF twice at 12-hour intervals did not increase the degree of luteolysis or the interval to the onset of estrus compared to PGF given once. However, PGF administered twice resulted in a more synchronous interval to the onset of estrus and ovulation.

c) Letrozole treatment initiated during proestrus did not prevent ovulation during device placement or within 24 hours of device removal in heifers compared to letrozole treatment initiated during metestrus or diestrus.

d) Letrozole treatment initiated during metestrus followed by a PGF to GnRH/AI interval of 66 hours resulted in a higher pregnancy per AI compared to treatment initiated during proestrus and tended to be higher than diestrus. Following post-treatment ovulation, treatment initiated during metestrus resulted in a larger CL and higher plasma progesterone 14 days after ovulation compared to treatment initiated during diestrus and proestrus

e) In a field trial with nulliparous beef heifers and multiparous beef cows, a 4-day letrozole treatment with a PGF to GnRH/AI interval of 66 hours, resulted in similar pregnancy per AI compared to a 5-day Cosynch plus CIDR protocol. Following post-treatment ovulation, heifers that received an LRD had higher plasma progesterone levels 7 days after AI.

f) Independent of synchronization, cattle that displayed estrus before AI had a threefold increase in pregnancy per AI.

8.3. Future Studies

Based on the results presented in this thesis, I raise the following questions for future studies with letrozole that may be done:

- Can an increase in plasma letrozole concentrations prevent an endogenous LH surge following luteolysis in cattle?

- What is the magnitude of a GnRH-induced LH and FSH surge following a letrozole-based synchronization? Does long-term letrozole treatment exhaust the pituitary of LH?
- What is the elimination half-life of letrozole in high-producing dairy cows (i.e., >40 kg/day)?
- What is the effect of high physiological LH concentrations at the time of follicle wave emergence? Does letrozole treatment immediately preceding wave emergence affect follicle and oocyte competence of that wave?
- Will an increase in plasma letrozole concentrations increase mRNA FSHR expression and subsequently increase the superovulatory response in cattle?
- Can the use of letrozole decrease the dose of FSH needed for ovarian superstimulation in cattle? Does long-term treatment with letrozole increase intraovarian androgens and increase superstimulatory response?
- Does letrozole treatment increase inhibin production from the dominant follicle compared to non-treated cattle?
- Can letrozole treatment during a time of high circulating progesterone be used to induce ovulation of a dominant follicle?
- What is the impact of suppressed estradiol in prepubertal calves? Can letrozole treatment hasten the onset of puberty?
- Can the luteotrophic properties of letrozole increase the size of the CL and progesterone production during the early luteal phase for improving embryo transfer results?

- What is the effect of letrozole treatment during pregnancy? If abortion does not occur, can letrozole be used for fetal programming using the bovine model?

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