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Effects of initial GnRH removal from 5-d CO-Synch + CIDR protocol on ovarian parameters and fixed timed artificial insemination pregnancy rates in beef cattle

Tyler Grussing
Iowa State University

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Effects of initial GnRH removal from 5-d CO-Synch + CIDR protocol on ovarian parameters and fixed timed artificial insemination pregnancy rates in beef cattle

by

Tyler Grussing

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Animal Physiology (Reproductive Physiology)

Program of Study Committee:
Patrick Gunn, Co-major Professor
Curtis Youngs, Co-major Professor
Grant Dewell

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>NOMENCLATURE</td>
<td>vii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>LITERATURE REVIEW</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Endocrinology</td>
<td>8</td>
</tr>
<tr>
<td>2.2.1 Gonadotropin-releasing hormone</td>
<td>8</td>
</tr>
<tr>
<td>2.2.2 Pituitary hormones</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2.1 Follicle stimulating hormone</td>
<td>11</td>
</tr>
<tr>
<td>2.2.2.2 Luteinizing hormone</td>
<td>13</td>
</tr>
<tr>
<td>2.2.3 Steroid hormones</td>
<td>14</td>
</tr>
<tr>
<td>2.2.3.1 Estradiol</td>
<td>15</td>
</tr>
<tr>
<td>2.2.3.2 Progesterone</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Estrous Cycle</td>
<td>19</td>
</tr>
<tr>
<td>2.3.1 Overview</td>
<td>19</td>
</tr>
<tr>
<td>2.3.2 Follicular phase</td>
<td>20</td>
</tr>
<tr>
<td>2.3.3 Luteal phase</td>
<td>21</td>
</tr>
<tr>
<td>2.3.4 Corpus luteum</td>
<td>22</td>
</tr>
<tr>
<td>2.3.4.1 Luteinization</td>
<td>22</td>
</tr>
<tr>
<td>2.3.4.2 Steroidogenesis</td>
<td>23</td>
</tr>
<tr>
<td>2.3.4.3 Luteolysis</td>
<td>24</td>
</tr>
<tr>
<td>2.4 Follicular Development</td>
<td>25</td>
</tr>
<tr>
<td>2.4.1 Ovarian reserve</td>
<td>25</td>
</tr>
<tr>
<td>2.4.2 Follicular classification</td>
<td>26</td>
</tr>
<tr>
<td>2.4.3 Folliculogenesis</td>
<td>27</td>
</tr>
<tr>
<td>2.4.4 Follicular wave characteristics</td>
<td>31</td>
</tr>
<tr>
<td>2.5 Estrous Synchronization</td>
<td>33</td>
</tr>
<tr>
<td>2.5.1 History and protocol development</td>
<td>33</td>
</tr>
<tr>
<td>2.5.1.1 Progesterone</td>
<td>33</td>
</tr>
<tr>
<td>2.5.1.2 Prostaglandin F2α and gonadotropin releasing hormone</td>
<td>37</td>
</tr>
</tbody>
</table>
2.5.1.3 Multiple hormone protocols ........................................ 39
2.5.1.4 Controlled internal drug release protocols ....................... 42
2.5.2 Recommended protocols ............................................... 45
  2.5.2.1 Heat detection protocols ......................................... 45
  2.5.2.2 Heat detection & timed artificial insemination protocols .... 47
  2.5.2.3 Fixed timed artificial insemination protocols ................. 48
2.6 5-d CO-Synch + CIDR Protocol ........................................ 50
  2.6.1 Development ....................................................... 50
  2.6.2 Modifications ..................................................... 53
2.7 Statement of the Problem ................................................. 55

CHAPTER 3. OVARIAN FUNCTION IN BEEF COWS WHOSE OVULATION WAS
SYNCHRONIZED USING THE 5-DAY CO-SYNCH + CIDR PROTOCOL
WITH AND WITHOUT GNRH AT CIDR INSERTION .......................... 57
  3.1 Abstract .......................................................................... 57
  3.2 Introduction ...................................................................... 58
  3.3 Materials and Methods ................................................... 60
    3.3.1 General ..................................................................... 60
    3.3.2 Animals and treatments .............................................. 60
    3.3.3 Performance characterization ...................................... 61
    3.3.4 Experimental design ............................................... 61
    3.3.5 Ovarian characterization ............................................ 62
    3.3.6 Plasma steroid hormone analyses ................................ 63
    3.3.7 Statistical analysis .................................................. 64
  3.4 Results ............................................................................. 65
    3.4.1 Age and performance characteristics ........................... 65
    3.4.2 Ovarian parameters .................................................. 65
    3.4.3 Plasma steroid hormone analysis ................................ 66
  3.5 Discussion ....................................................................... 68
    3.5.1 General ..................................................................... 68
    3.5.2 Ovarian parameters .................................................. 70
    3.5.3 Plasma steroid hormone analysis ................................ 72
    3.5.4 Conclusion ............................................................. 74

CHAPTER 4. EFFECT OF GNRH ADMINISTRATION AT THE ONSET OF THE 5 DAY
CO-SYNCH + CIDR PROTOCOL IN SUCKLED BEEF COWS ................. 79
  4.1 Abstract .......................................................................... 79
  4.2 Introduction ...................................................................... 80
  4.3 Materials and Methods ................................................... 82
    4.3.1 General ..................................................................... 82
    4.3.2 Animals and treatments .............................................. 82
    4.3.3 Experimental design ............................................... 83
    4.3.4 Plasma steroid hormone analysis ................................. 84
4.3.5 Ovarian characterization .................................................. 85
4.3.6 Statistical analysis ................................................................. 85

4.4 Results .................................................................................... 86
4.4.1 Age and performance characteristics ........................................ 86
4.4.2 Ovarian parameters ................................................................. 87
4.4.3 Fixed timed artificial insemination pregnancy results ................. 88

4.5 Discussion ............................................................................... 89
4.5.1 General ................................................................................. 89
4.5.2 Ovarian parameters ................................................................. 92
4.5.3 Pregnancy results ................................................................. 94
4.5.4 Conclusion ............................................................................ 95

CHAPTER 5. GENERAL DISCUSSION ................................................. 101

LITERATURE CITED .................................................................... 107
LIST OF TABLES

Table 3.1  Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR¹ protocol on age and body condition scores in mature beef cows................................................................. 75

Table 3.2  Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR¹ protocol on ovarian parameters in mature beef cows................................................................................. 76

Table 3.3  Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR¹ protocol on steroid hormone concentrations in mature beef cows............................................................................. 77

Table 4.1  Production characteristics of lactating beef cows stratified by treatment........................................................................................................................................................................ 97

Table 4.2  Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR¹ protocol on activation of estrus detection aid, behavioral estrus and FTAI² pregnancy rates in lactating beef cows. 98

Table 4.3  Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR¹ protocol on ovarian parameters in lactating beef cows.......................................................................................... 99
| Figure 3.1 | Experimental protocol describing GnRH and PGF2α treatments and data collection in mature beef cows for analysis of ovarian parameters and steroid hormone concentrations in the 5-d CO-Synch + controlled internal drug release (CIDR) protocol | 78 |
| Figure 4.1 | Experimental protocol describing GnRH and PGF2α treatments and data collection in lactating beef cows for analysis of fixed-timed artificial insemination (FTAI) pregnancy rates in the 5-d CO-Synch + Controlled Internal Drug Release (CIDR) protocol | 100 |
## NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5dCO</td>
<td>5-d CO-Synch + CIDR protocol</td>
</tr>
<tr>
<td>AI</td>
<td>artificial insemination</td>
</tr>
<tr>
<td>BCS</td>
<td>body condition score</td>
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<tr>
<td>CH</td>
<td>corpus hemorrhagicum</td>
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<tr>
<td>CIDR</td>
<td>controlled internal drug release</td>
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<td>CL</td>
<td>corpus luteum</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<tr>
<td>FTAI</td>
<td>fixed timed artificial insemination</td>
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<tr>
<td>GABA</td>
<td>gamma amino butyric acid</td>
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<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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<td>G1</td>
<td>initial GnRH dose of 5dCO</td>
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<tr>
<td>G2</td>
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</tr>
<tr>
<td>IU</td>
<td>international unit</td>
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<tr>
<td>LH</td>
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<tr>
<td>MAP</td>
<td>medroxyprogesterone acetate</td>
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<tr>
<td>MGA</td>
<td>melengestrol acetate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
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<tr>
<td>Nor</td>
<td>norethandrolone</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>prostaglandin &lt;sup&gt;F&lt;/sup&gt;&lt;sub&gt;2α&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; THAM</td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; tromethamine salt</td>
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<tr>
<td>PRID</td>
<td>Progesterone realizing intravaginal device</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
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<td>standard deviation</td>
</tr>
<tr>
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<td>Syncro-Mate B</td>
</tr>
<tr>
<td>TAI</td>
<td>timed artificial insemination</td>
</tr>
</tbody>
</table>
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ABSTRACT

Removal of the initial GnRH administration within the 5-d CO-Synch + controlled internal drug release (CIDR) protocol (5dCO) on ovarian parameters and fixed timed artificial insemination (FTAI) pregnancy rates was investigated. In the first study, the objective was to evaluate the effects of GnRH removal on ovarian follicle dynamics and steroid hormone concentrations in non-lactating beef cows. Animals received either: 1) standard 5dCO hormone administration including 100 µg of GnRH at CIDR insertion and two concurrent, 25-mg doses of prostaglandin F$_2$α (PGF$_2$α) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent, 25-mg doses of PGF$_2$α at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single, 25-mg dose of PGF$_2$α at CIDR removal (NoG1-1PG). Cows in the NoG1-2PG treatment group displayed elevated peak plasma estradiol concentrations when compared to the NoG1-1PG treatment group with G1-2PG remaining intermediate. However, the remaining steroid hormone concentrations analyzed were not impacted by treatment. Furthermore, the ovarian parameters measured were not different among treatments.

In order to determine if fluctuations in estradiol concentrations observed in the first study would impact fertility, a field study was conducted. The objective of the field study was to evaluate the effect of initial GnRH removal at CIDR insertion on dominant follicle parameters and FTAI pregnancy rates in lactating beef cows. Cows were administered one of three treatments identical to the first study with the exception of
FTAI occurring 72 h following CIDR removal. Although dominant follicle diameter was similar upon CIDR insertion, dominant follicle was greater in the NoG1-2PG treatment group upon CIDR removal. Estrous response prior to FTAI was not different among treatments, but NoG1-1PG did display more advanced (lower) estrous detection aid scores at FTAI. However, FTAI pregnancy rates did not differ among treatments.

In summary, the data from these two studies indicate that removal of the initial GnRH administration in the 5dCO does not negatively affect ovarian parameters or FTAI pregnancy rates. However, differences in dominant follicle diameter and changes in estradiol concentrations, as well as behavioral estrus, prior to FTAI warrant further research.
CHAPTER 1
INTRODUCTION

Reproductive efficiency is one of the largest drivers of profitability within beef cattle operations. One aspect that drives reproductive efficiency is timing of conception within the breeding season. Early conception can help drive profitability in the cow herd by resulting in increased longevity of breeding females and more uniformity with older and heavier calves at weaning (Funston et al., 2017). Multiple technologies are available to improve reproductive efficiency through increasing the number of females that conceive at the beginning of the breeding season (Patterson et al., 2017). Although these technologies exist, not all are cost effective and offer a return on time and money invested to implement them. Therefore, development and promotion of reproductive technologies should be performed with the understanding that return on investment will drive utilization and thus increase profitability on beef cattle operations.

One of these technologies is artificial insemination (AI), which at only 10% utilization (NAHMS, 2009) continues to be a poorly received technology within beef cattle operations in the United States. The main reasons producers do not utilize AI is the shortage of time and labor needed to implement heat detection or estrous synchronization protocols. To combat poor adoption rates of these technologies, multiple ovulation synchronization protocols have been developed to facilitate mass insemination of all females at a single predetermined time called fixed timed artificial insemination (FTAI). Fixed timed-AI allows producers to capture the value of AI, superior
genetics and improved calving distribution, while eliminating the labor-intensive requirements of estrus detection. The beneficial result of these protocols has been conception and pregnancy rates similar to many earlier developed, more time-consuming, estrus detection-based synchronization protocols (Funston et al., 2017; Patterson et al., 2017).

Specifically, the 5-d CO-Synch + controlled internal drug release (CIDR) protocol (5dCO) is a FTAI protocol that has proven efficacious with acceptable pregnancy rates when implemented in both beef and dairy females (Wilson et al., 2007; Bridges et al., 2008; Santos et al., 2010). In addition, research by Bridges et al. (2008) has shown the 5dCO to have advantageous FTAI pregnancy rates in Bos taurus females when compared to the more widely used 7-d CO-Synch + CIDR protocol. The standard 5dCO involves insertion of a CIDR and administration of an initial dose of gonadotropin releasing hormone (GnRH; G1) followed approximately 5 d later by CIDR removal and administration of two doses of prostaglandin F$_2$$\alpha$ (PGF$_2$$\alpha$) approximately 8 h apart. Fixed timed- AI occurs 72 h following removal of CIDR and initial PGF$_2$$\alpha$ administration (Johnson et al., 2013) concurrent with a second administration of GnRH (G2).

The standard 5dCO, however, still lacks utilization among beef producers within the United States due to labor intensity and cost of additional exogenous hormones. Therefore, modifications to decrease handling time or frequency of hormone administration may prove to be advantageous and increase usage among producers. The requirement of two separate, 25-mg doses of PGF$_2$$\alpha$ has recently been highlighted as an area for potential modification of the 5dCO. Kasimanickam et al. (2009) previously
demonstrated that pregnancy rates are detrimentally affected when only a single, 25-mg dose of PGF$_{2\alpha}$ is administered upon CIDR removal when compared to two separate, 25-mg doses of PGF$_{2\alpha}$ administered 7 h apart. The two separate, 25-mg doses of PGF$_{2\alpha}$ are speculated to be required in beef cows to induce luteolysis of the 5-d old accessory corpus luteum (CL) created by the administration of G1 (Bridges et al., 2008; Kasimanickam et al., 2009). However, in heifers, a single, 25-mg dose of PGF$_{2\alpha}$ has proven effective in engaging luteolysis of the 5-d accessory CL; and similar pregnancy rates were achieved upon FTAI when compared to heifers receiving two, separate, 25-mg doses of PGF$_{2\alpha}$ (Rabaglino et al., 2010; Cruppe et al., 2014; Kasimanickam et al., 2014). The difference in response rates of the 5-d accessory CL to a single, 25-mg dose of PGF$_{2\alpha}$ between cows and heifers has been thought to be due to an increased mg/kg of body weight dosing in heifers compared to cows (Bridges et al., 2012). Bridges et al. (2012) went on to further study this hypothesis when two concurrent, 25-mg doses of PGF$_{2\alpha}$ was proven adequate to induce luteolysis of the 5-d accessory CL and produce similar FTAI pregnancy rates in cows when compared to females administered two, separate, 25-mg doses of PGF$_{2\alpha}$ approximately 8 h apart.

The requirement of G1 is the other area within the standard 5dCO where modifications have been researched. The administration of G1 is used to synchronize ovarian follicular growth at the start of the FTAI protocol as to better time and synchronize ovulation for optimal FTAI success. However, in beef cows, the response rate to a single GnRH injection is widely variable and ranges on average from 66 to 70% in both cycling and anestrous females (Geary et al., 2000; Atkins et al., 2010a; Atkins et al.
2010b; Bridges et al., 2014). The failure of response to the initial injection of GnRH has proven to be detrimental to pregnancy rates within the 7-d CO-Synch + CIDR protocol (Bridges et al., 2014) by leading to either smaller, immature dominant follicles (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005; Atkins et al., 2010a; Atkins et al. 2010b) with decreased estradiol and progesterone concentrations or persistent follicles that are compromised in terms of both oocyte competence and steroidogenesis (Bridges et al., 2014).

The design of the standard 5dCO more appropriately accommodates females that do not respond to G1 by shortening the period between G1 and corresponding CIDR removal and PGF$_2\alpha$ administration compared to the 7-d protocol (Bridges et al., 2008; Bridges et al., 2014). Therefore, the oocytes that do not respond to G1 would be younger and more competent. Furthermore, the lengthened proestrus phase is expected to increase estradiol concentrations and thus potentially increase FTAI pregnancy rates (Bridges et al., 2008). However, it remains unknown if failure of response to G1 would have similar effects between the 5dCO and the 7-d CO-Synch + CIDR protocol.

When response to G1 within the standard 5dCO was analyzed in Bos taurus cows, Dias et al. (2014) reported that failure to respond to G1, or the lack of the creation of a new accessory CL, resulted in an increased behavioral estrus response and increased FTAI pregnancy rates when compared to animals that did respond to G1 by creating a new accessory CL. The Dias et al. (2014) research raised the question as to whether or not G1 is actually required within the 5dCO, since females not responding
have increased FTAI pregnancy rates. Thus, the actual requirement of G1 within the 5dCO has been recently highlighted for further review.

In dairy heifers, the elimination of G1 from the standard 5dCO resulted in no difference in FTAI pregnancy rates when compared to the standard 5-d CO-Synch +CIDR protocol (Lima et al., 2011; Kasimanickam et al., 2014). However, in beef heifers, the same contrast of treatments has mixed results. Cruppe et al. (2014) reported similar pregnancy rates in beef heifers, while Kasimanickam et al. (2014) reported that elimination of G1 resulted in decreased pregnancy rates in beef heifers when compared to the standard 5dCO. The inconsistent results, in beef heifers, leave an unanswered question as to the requirement of G1 within the 5dCO. Furthermore, few studies have looked at the requirements of G1 in the 5dCO when applied to beef cows.

While acceptance rates of FTAI protocols remain low in the United States beef herd, continual modification to existing protocols may prove advantageous and increase utilization. The standard 5dCO remains an intensive protocol with high labor requirements and hormonal intervention. Although Bridges et al. (2012) could demonstrate that one time of handling could be eliminated by increasing the initial administration of PGF$_{2a}$ from 25 to 50 mg, further modification may prove advantageous through elimination of the number of hormones administered during application of this protocol. Removal of G1 would not only decrease the amount of pituitary hormone analog required but would also allow for PGF$_{2a}$ to be administered in a single, 25-mg dose thus eliminating the need for two concurrent, 25-mg doses. If G1 is not administered to reset the follicular wave, an accessory CL will not be created at the
initiation of the protocol. Thus, a second, 25-mg dose of PGF$_{2\alpha}$ would not be required to induce luteolysis of a 5-d old accessory CL. Therefore, to potentiate a beneficial modification to the 5dCO, the effect of G1 removal on ovarian parameters and FTAI pregnancy rates in beef cows needs to be determined.
CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The following chapter of this thesis contains a review of literature relating to beef cattle reproduction and the development of estrous and ovulation synchronization programs. More specifically, the hormones and endocrine mechanisms involved in the estrous cycle are initially explained in detail, followed by a review of the estrous cycle, follicular development, and ovulatory wave formation. This is followed by a review of the development of estrous and ovulation synchronization with discussion of each protocol recommended for implementation by the Beef Reproduction Task Force. Further discussion on the specific development of the 5-d CO-Synch + CIDR protocol (5dCO) is followed by modifications sought to improve feasibility and implementation by beef producers. The review finishes with general discussion and the rationale for the research trails presented in the thesis.
2.2 Endocrinology

2.2.1 Gonadotropin-releasing hormone

The discovery of gonadotropin releasing hormone (GnRH) first occurred with the prediction of its existence in the late 1940’s and early 1950’s by G.W. Harris (1950). However, it would be two decades before the prediction came to fruition with the determination of the luteinizing hormone-releasing hormone, the classical name of GnRH, and structure as a decapeptide (Burgus et al., 1972). To date three isoforms of GnRH have been discovered (Fernald and White, 1999), however only the isoforms GnRH I and GnRH II are thought to be implicated in regulating the reproductive behavior in mammals with GnRH I being mainly responsible for gonadotropin release and reproductive function (Chen and Fernald, 2008).

GnRH is synthesized in the hypothalamus by neurons located within the tonic center and surge center (Gorski, 1970) in response to multiple influences. GnRH, at this point in time, is believed to be stimulated by kisspeptin binding the G protein-coupled receptor 54 on the cell membrane and can be modulated by steroid hormones such as estradiol (Pielecka-Fortuna et al., 2008). Inhibition of GnRH can also occur through multiple mechanisms from neurotransmitters (corticotropin releasing hormone, gamma-amino butyric acid and β-endorphins; Ciechanowska et al., 2010) to steroid hormones such as progesterone. The tonic center (ventromedial nucleus and arcuate nucleus) is responsible for GnRH secretion in basal synchronized pulses throughout the luteal phase of the estrous cycle. The synchronized pulsatile releases of GnRH occur
every 30 to 120 minutes (Wang et al., 2010) into the median eminence of the perivascular space and travels through the capillaries into the hypothalmo-hypophyseal portal system (Chabbert-Buffet et al., 2000). Conversely, the surge center (preoptic nucleus, anterior hypothalamic area and suprachiasmatic nucleus) initiates the pre-ovulatory synthesis and release of GnRH. The substantial release of GnRH, corresponding with the end of the follicular phase of the estrous cycle, enters the capillaries and travels into the hypothalmo-hypophyseal portal system as previously explained. The hypothalmo-hypophyseal portal system flows from hypothalamus to the anterior pituitary gland where it acts on gonadotroph cells to stimulate the synthesis and release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH; Wang et al., 2010).

Even though GnRH has multiple separate ligands and receptor types, there is no link between the different isotypes and the specific GnRH receptor that they bind to (Chen and Fernald, 2008). The GnRH receptors located in the pituitary are 7-transmembrane domain receptors (Seaflon et al., 1997; Chabbert-Buffet et al., 2000) that are unique members of the rhodopsin-like G protein-coupled receptor superfamily that in mammals have a relatively short third intracellular loop that lacks the intracellular carboxyl terminus (Wang et al., 2010). Binding of GnRH to its selected receptor initiates activation of the gonadotrophs in two ways: calcium influx and activation of the protein kinase C pathway (Ben-Menaham and Noar, 1994). The biphasic calcium influx initially spikes due to the release internal calcium stores and then is maintained through dependence upon L-type calcium channels (Stojilkovic et al.,
This high calcium influx is responsible for the synthesis of the unique β-subunit of LH (Weck et al., 1998). In contrast, the binding of GnRH activates the G proteins that simulate phospholipase C activity leading to protein kinase C and mitogen-activated protein kinase stimulation (Roberson et al., 1995). The mitogen-activated protein kinase pathway leads in to induction of the α-subunit (Weck et al., 1998) common amongst all gonadotropins.

### 2.2.2 Pituitary hormones

The pituitary plays a critical role of intermediary between the hypothalamus and the gonads in reproductively active animals. As it was previously laid out the pituitary is the site of action of GnRH causing release in a pair of heterodimeric glycoprotein hormones, FSH and LH. Although both hormones are synthesized and secreted under the influence of GnRH they contrast in their actions at the level of the gonad. The specific actions of these hormones will be laid out in detail in the following sections. For generalization purposes, however, FSH is synthesized and secreted more frequently, resulting in shorter storage at the level of the pituitary (Apefelbaum and Taleisnik, 1976). Luteinizing hormone in contrast is more abundantly stored within the anterior pituitary and thus, is synthesized at a lesser frequency but secreted in a greater volume (Muyan et al., 1994). Although contrasts between the two glycoproteins exist, it is important to note that they are both equally important to the function of the normal estrous cycle.
2.2.2.1 Follicle stimulating hormone

Follicle stimulating hormone is a heterodimeric glycoprotein that consists of two subunits, α and β, joined by non-covalent forces. As stated earlier, the α-subunit is common amongst all the glycoprotein hormones (LH, thyrotropin, FSH and human choriogonadotropin) while the β subunit is unique for each of the separate hormones (Ryan et al., 1988).

Synthesis and release of FSH occurs within the gonadotroph cells of the anterior pituitary, however the exact mechanism is not as well understood as its counterpart, LH. Primarily, pulsatile binding of GnRH to its receptor leads to stimulation of the gonadotroph to release FSH and LH, although FSH release has been shown to occur in the absence of GnRH stimulation (Padmanabhan et al., 1997). In addition, further studies have shown evidence that either GnRH isoforms (Padmanabhan et al., 2003) or pulsatility patterns may influence differences in LH and FSH secretion (Vizcarra et al., 1999). Vizcarra et al. (1999) was able to demonstrate that LH secretion increased with increased exogenous GnRH pulsatility, while FSH increased at decreased GnRH pulsatility in nutritionally induced anovulatory, ovariectomized beef cows. Even though the exact mechanism for stimulation is still up for debate, the factors that lead to local and systemic inhibition of FSH release are more understood. The steroid hormone estradiol not only affects the secretion of GnRH but has been shown to have effects upon circulating FSH concentrations as well. In ovariectomized heifers, FSH concentrations can be suppressed by the administration of estradiol (Butler et al., 1983).
Furthermore, another glycoprotein, inhibin has also been shown to negatively affect circulating FSH concentrations when administered to ovariectomized heifers (Beard et al., 1990). Both hormones have been found to be secreted by the dominant follicle during the preovulatory period.

FSH is released from the pituitary and travels through the circulatory system to the gonads where it interacts with its receptor. The FSH receptor is a transmembrane G protein-coupled receptor (Gundermann et al., 1995) that when bound activates the adenylyl cyclase/protein kinase A pathway (Simoni et al., 1997) leading to proliferation of the granulosa cells and increased aromatase activity, eliciting estrogen production, within said cell (Saumande, 1990). The overall effects of FSH binding its receptor in the gonads is extremely important in follicle recruitment and development in the female. Adams et al. (1992) showed that initiation of a new follicle wave would occur approximately 1 to 2 d following a surge in circulatory FSH concentrations. Furthermore, once a follicle is selected for dominance FSH leads to up-regulation of LH receptors on the granulosa cells (Ryle, 1972), allowing for maturation to begin. Therefore, elevated FSH concentrations around the time of antral follicular development and dominance may indicate the ability for FSH to facilitate early follicular development (Sunderland et al., 1994).
2.2.2.2 Luteinizing hormone

Like FSH, LH is a heterodimeric glycoprotein that is comprised of an α-subunit, common to glycoprotein hormones, and a unique β subunit non-covalently bound together (Ryan et al., 1988). Gonadotroph cells of the anterior pituitary are responsible for the synthesis and release of LH in response to increased GnRH pulsatility and amplitude (Vizcarra et al., 1999). Therefore, following the actions of GnRH, circulatory LH concentrations vary depending upon stage of the estrous cycle. The progression of the estrous cycle causes a shift in LH from high frequency and low amplitude during the follicular and early luteal phases to a low frequency and high amplitude during the midluteal period (Rahe et al., 1980). Suppression of LH synthesis and secretion occurs through steroid hormones, specifically progesterone and estradiol, as a negative feedback mechanism. Increasing the circulating plasma levels of progesterone through exogenous use leads to suppressed LH pulsatility as noted in ovariectomized Holstein heifers (Beck et al., 1976). Estradiol, on the other hand, acts through a less understood mechanism to potentiate the actions of gonadotrophs regarding LH. Large single doses of estradiol have been shown to have a negative impact on LH pulsatility for a short period of time (Kesner et al., 1981). Oddly, Kesner et al. (1981) also found that suppression of LH with a bolus of estradiol was followed by surge in LH. This finding of an LH surge is more in line with evidence of estradiol having a positive feedback mechanism to promote LH pulse frequency (Stumpf et al., 1989). During the proestrus phase estradiol concentrations are at their highest and cause a surge of GnRH release (Karsch et al., 1997) as well as prime the pituitary to receive that GnRH (Clarke, 1988).
The sensitized pituitary along with the terminus deletion of the GnRH receptor in the anterior pituitary (Wang et al., 2010) is thought lead to the pre-ovulatory surge of LH.

Luteinizing hormone binds to a G protein-coupled transmembrane receptor (Gundermann et al., 1995) at the level of the gonad. The effects of LH binding to the receptor can be varied depending upon the cell type as well as the stage of the estrous cycle and follicular wave. In the antral follicle, the theca cells are dependent upon LH to be able to further convert the steroid hormone progesterone into androstenedione (Smitz and Cortvrindt, 2002). As the follicle progresses and becomes selected for dominance the granulosa cells develop receptors for LH as well (Campbell et al., 1995). This switch of the follicle to LH dependence is needed for final maturation of the follicle (Driancourt, 2001) as well as ovulation in the presence of the preovulatory surge of LH (Evans et al., 1997). Once ovulation occurs LH stimulation is required for the development of a functional corpus luteum (CL; Peters et al., 1994) and subsequent progesterone secretion from the large and small luteal cells which can also be amplified by LH (Fitz et al., 1982).

2.2.3 Steroid hormones

Steroid hormones play a key role as regulators of multiple physiological process of the body. All steroid hormones contain a steroid nucleus and are derivatives of cholesterol, a lipid molecule. While cholesterol can come from dietary sources, primarily it is formed endogenously by tissues such as the gonads from acetate (Morris and
Chaikoff, 1958). Once created steroid hormones are immediately released to execute their function upon the tissues. Typically, the steroid hormones are broken down into five groups according to their physiological behavior and the receptors to which they bind. The five groups of steroid hormones are: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestins. For reproductive purposes, the most important of these (androgens, estrogens, and progestins) can be formed directly within the gonad.

2.2.3.1 Estradiol

Estradiol, as previously mentioned, is a steroid hormone synthesized and secreted by the granulosa cells of the dominant follicle. The granulosa cell requires the enzyme aromatase to convert testosterone to estradiol, which becomes activated when FSH binds to its receptor within the cell membrane (Saumande, 1990). Thus, one would assume that estradiol secretion would be tied to FSH pulsatility and subsequent effects, however such is not the case. Estradiol secretion has been associated with and found to follow pulsatile releases of LH (Rhodes et al., 1995). Rhodes et al. (1995) went on to show that maximal concentrations of estradiol appear 15 to 30 minutes following the peak pulses of LH.

The mechanisms by which estradiol has its effects upon its target cell can vary greatly depending upon type of cell and location of the bound receptor. Classically steroid hormones are believed to act via intracellular or nuclear receptors that can function as transcription factors, but evidence has been presented for estrogen to also
have plasma membrane receptors that function through nongenomic effects such as second messengers, calcium and nitric oxide (Revankar et al., 2005). Regarding reproductive function, the main effects of estradiol include positive/negative hormonal feedback to the hypothalamus and pituitary (previously discussed), induce behavioral characteristics during estrus (Swanson and Hafs, 1971) and preparation of the uterus for pregnancy.

The timing of onset of behavioral signs of estrus, as well as the specific behaviors displayed can fluctuate from animal to animal (Roelofs et al., 2005). Common behaviors that signify an animal is receptive to breeding are vaginal mucous discharge, restlessness, being mounted but not standing, sniffing vaginal area of other cows, resting chin on other cows, mounting cows and standing heat (Van Eerdenburg et al., 1996). Increased estradiol concentrations with strong signs of behavioral estrus prior to artificial insemination have proven increase pregnancy rates in beef cattle, especially prior to induced ovulation with GnRH administration (Perry et al., 2005; Jinks et al., 2012).

### 2.2.3.2 Progesterone

Progesterone, like estradiol, is a steroid hormone synthesized and secreted by the gonads, specifically the luteal cells of the CL (Fitz et al., 1982) in response to LH binding to its receptor on the cell membrane from d 2 to 12 of the estrous cycle, but does not respond to LH from d 12 to 17 (Peters et al., 1994). During this time, large
luteal cells are responsible for most of the progesterone production due to their ability to produce progesterone without the binding of LH to their receptors (Fitz et al., 1982). Within the luteal cells the activation of the adenylyl cyclase/protein kinase A pathway leads to the conversion of pregnenolone into progesterone via the enzyme 3β-hydroxysteroid dehydrogenase (Koritz, 1964).

Progesterone can have multiple systemic effects throughout the body, including those required for reproduction; modulation of the endometrium in preparation of implantation and the hypothalamic/pituitary hormones (Chabbert-Buffet et al., 2000). Two neuronal receptors have been described in literature, A and B, as well as presence of 7 transmembrane receptors (Brinton et al., 2008). Binding of the ligand to these receptors causes a cascade of events to occur resulting in transcriptional changes at the level of the genome, as well as indirect mechanism of action through opioidergic, cholinergic, and gamma amino butyric acid (GABA) systems. Progesterone effects on the hypothalamus neurons secreting GnRH are thought to work through the latter of the two mechanisms previously mentioned in bovine as they do not have receptors for progesterone (Chabbert-Buffet et al., 2000). Gamma amino butyric acid has been shown to have inhibitory effects on GnRH (Seilicovich et al., 1995) and LH (Gallegos-Sanchez et al., 1996) secretions, thus it was thought to be a possible mechanism for progesterone suppression of GnRH and LH. However, Skinner et al. (1999), has shown that the effects of progesterone on inhibition of GnRH take place through classical nuclear receptors in ovariectomized ewes. Furthermore, Skinner et al. (1998) showed in a previous study that the effects of progesterone on GnRH secretion require priming of the neurons by
estradiol. In that study animals that had no exposure to estradiol for 4 months prior to administration of progesterone resulted in non-suppressed LH concentrations. Animals that did receive estradiol exposure, for as little as two weeks, however did show LH suppression after progesterone administration. Therefore, estradiol and progesterone must work together to appropriately regulate hormonal secretion and inhibition in the hypothalamic-pituitary axis.

At the level of the endometrium, progesterone leads to advances in gene expression to produce energy sources or contributors to histotroph (Forde et al., 2009). One such gene, DGAT$_2$, is important in catalyzing acylcoenzyme A to triglyceride (Cases et al., 2001), a suspected energy source for the developing blastocyst (Ferguson and Leese, 2006). Furthermore, progesterone has been shown to induce glucose transporters within the uterine endometrium of ovine (Gao et al., 2009a; Gao et al., 2009b). Thus, indicating the importance of progesterone for the development and growth of the conceptus. However, between d 7 and 13 of the estrous cycle, down regulation of progesterone receptors occurs within the lumen of the uterus (Kimmins and MacLaren, 2001). This down regulation of receptors also corresponds with an increase in genes associated with cell adhesion (Spencer et al., 2008), allowing for the uterus to further prepare for implantation.
2.3 Estrous Cycle

2.3.1 Overview

The bovine estrous cycle consists of subsequent physiologic dynamics that occur approximately every 14 to 26 d with an average of 21 (Nellor and Cole, 1956). The exact length of the estrous cycle is influenced by many factors including age and breed. However, the number of follicular waves occurring during the estrous cycle seems to have a dominant impact on estrous cycle length (Sirois and Fortune, 1988). Follicular waves tend to last 7 to 10 d in length (Sirois and Fortune, 1988; Sunderland et al., 1994) and can occur two, three, or even up to four times per estrous cycle in *Bos taurus* species (Sirois and Fortune, 1988; Ginther et al., 1989; Sunderland et al. 1994). A large proportion of mature cows have two follicular waves per estrous cycle, while heifers have a higher tendency to have three waves per cycle (Rajamahendran et al., 1984; Townson et al., 2002).

The estrous cycle of the bovine is further broken down into two primary phases: the follicular phase and the luteal phase. Each of these phases are highly influenced by gonadotropins leading to the influence of steroidogenesis, folliculogenesis, ovulation, and CL function at the level of the ovary (Kojima, 2003).
2.3.2 Follicular phase

The follicular phase lasts for approximately 4 to 6 d characterized by the absence of a functional CL and presence of a preovulatory follicle. The follicular phase can be further divided down into proestrus (d 17 to 20) and estrus (d 21 to 1) periods (Sunderland et al., 1994; Forde et al., 2011). The proestrus period begins with the regression of the CL and ensuing decrease in circulating progesterone levels. The resulting effects of a decrease in the circulating concentrations of progesterone allows for removal of the negative feedback mechanism on gonadotropins. Rapid proliferation of the preovulatory follicle occurs during this period, as well as an increase in synthesis of estradiol (Forde et al., 2011). Increasing levels of estradiol production by the preovulatory follicle leads to positive feedback mechanisms at the level of the hypothalamus and pituitary (Stumpf et al., 1989). As previously discussed, the positive feedback of estradiol initiates an increase in LH frequency, allowing for maturation and growth of the preovulatory follicle (Adams et al., 2008). The transition to the estrus period begins as peak estradiol concentrations occur and the female exhibits sexual receptivity. Therefore, proestrus length is highly dependent upon the size of the preovulatory follicle at the time of luteolysis (Sirois and Fortune, 1988).

Behavioral estrus or female sexual receptivity, typically demarcated as d 0 of the estrous cycle, designates the estrus period of the follicular phase. At the time of estrus, or immediately preceding, peak estradiol concentrations are achieved (Chenault et al., 1975). Peak estradiol concentrations lead to priming of the pituitary to GnRH (Clarke,
1988) and release of GnRH from the surge center of the hypothalamus (Karsch et al., 1997). The resulting effects at the level of the pituitary cause a preovulatory surge of LH in the circulatory system approximately 2 to 6 h following onset of behavioral estrus (Chenault et al., 1975). Ovulation of the dominant ovulatory follicle occurs after the LH surge, resulting in the total length of estrus, from behavioral onset to ovulation, to be between 25 and 35 h (Chenault et al., 1975).

2.3.3 Luteal phase

The luteal phase makes up most of the estrous cycle, lasting from 14 to 18 d, and is demarcated by the presence of a functional CL producing progesterone on the ovary. Like the follicular phase the luteal phase can also be broken down into two periods: metestrus (d 2 to 4), and diestrus (d 5 to 17). The metestrus period begins immediately following ovulation as the corpus hemorrhagicum (CH) undergoes the process of luteinization. Following luteinization, luteal weight grows rapidly from d 3 to 6 of the estrous cycle and is closely mimicked by progesterone concentrations in circulating plasma (Erb et al., 1971). Once the CL becomes fully functional the transition from metestrus to diestrus occurs. During this time, peak progesterone concentrations in the circulatory system reach levels greater than 4 ng/mL around d 10 and that level is maintained until luteolysis occurs (Adams et al., 2008). Luteolysis or CL regression occurs in late diestrus, subsequently progesterone levels decrease and transition back to the follicular phase occurs.
2.3.4 Corpus luteum

The CL is the dominant structure on the ovary during the luteal phase (d 2 to 17) of the estrous cycle. Development of the corpus luteum occurs through the process of luteinization and creation of the small and large luteal cells. These luteal cells are influenced by gonadotropins to produce the steroid hormone progesterone that influences preparation of the uterus for pregnancy and negative feedback mechanisms at the level of the hypothalamus and pituitary (Chabbert-Buffet et al., 2000). Finally, regression occurs through the process of luteolysis, where the luteal cells go through apoptosis and secretion of progesterone decreases.

2.3.4.1 Luteinization

According to Smith et al. (1994) luteinization is the transition of a preovulatory follicle, through remodeling and proliferation, into a vascular structure capable of progesterone secretion in large quantities. After rupture of the follicle, the follicular wall invaginates and breakdown of the basement membrane separating the granulosa and theca interna layer occurs allowing for migration of fibroblast, endothelial cells, and theca interna cells into more centralized positions with the developing CL (O’Shea et al., 1980). Granulosa cells undergo hypertrophy to form large luteal cells and become the largest steroidogenic cells of the body, while theca interna cells develop into small luteal cells (O’Shea et al., 1980). Furthermore, it has been proposed that the smaller theca interna cells undergo mitosis and proliferate while the larger granulosa cells show no
evidence of mitosis (Smith et al., 1994; McCracken et al., 1999). Proliferation of the CL occurs rapidly from d 3 to d 6 and is followed by variable growth until reaching maximum size around d 15 of the estrous cycle as indicated by CL weights (Erb et al., 1971).

2.3.4.2 Steroidogenesis

As previously stated in this review, progesterone is synthesized and secreted within the luteal cells of the CL in response to LH binding receptors on the cell wall. Therefore, it makes sense that Schallenberger et al. (1984) was able to show that progesterone is secreted in a pulsatile fashion that highly mimics that of the gonadotropins. Proportionally, small luteal cells express more LH receptors and thus in response to LH binding produce more progesterone (Ursely and Leymarie, 1979; Mamluk et al, 1998). However, overall small luteal cells tend to produce 20 times less total progesterone than large luteal cells (Ursely and Leymarie, 1979) due to the ability of large luteal cells to produce progesterone in the absence of LH binding to its receptors (Fitz et al., 1982). Therefore, large luteal cells are responsible for more than 80% of the progesterone that is produced by the CL (Niswender et al. 1985). This is highly significant in the fact that the number of large luteal cells can greatly influence the total progesterone production achieved by the CL. Since large luteal cells are predominately formed by lutenization of the granulosa cells, larger ovulatory follicles
that contain greater numbers of granulosa cells result in larger concentrations of progesterone production during the following diestrus period (Perry et al., 2005).

2.3.4.3 Luteolysis

If maternal recognition of pregnancy, via a viable conceptus, does not occur in the female, luteolysis ensues. In the absence of interferon-tau release from the conceptus (Bazer et al., 1994), the maternal recognition of pregnancy in bovine, tumor necrosis factor-α stimulates the uterine endometrium to secrete basal levels of the eicosanoid, prostaglandin (PG) F₂α (PGF₂α; Skarzynski et al., 2000). Tumor necrosis factor-α elicits its effects through the phospholipase A₂ pathway in the stromal cells of the endometrium in the mid to late luteal phase (Okuda et al., 2004). Basal or sub-luteolytic PGF₂α secretion from the stromal cells travels to the CL and initiates a luteolytic cascade. Binding of PGF₂α to receptors on the luteal cells causes activation of the phospholipase C pathway and an increase in intracellular Ca²⁺ (Davis et al., 1987). The resulting effect leads to a decrease in progesterone production, vasoconstriction, apoptosis and release of oxytocin (McCracken et al., 1999). Oxytocin released from the luteal cells travels via the countercurrent exchange between the ovarian vein and uterine artery (Hixon and Hansel, 1974) to bind receptors on the endometrium epithelial cells that have been up regulated due to progesterone and estradiol priming during the late luteal and early follicular phases (McCracken et al., 1984). Unlike tumor necrosis factor-α which can elicit effects during the mid-luteal, late luteal, and follicular phases,
oxytocin is only effective in stimulating PGF$_{2\alpha}$ secretion by the uterine endometrium in the follicular phase (Skarzynski et al., 2000). However, upon binding the receptors of the endometrial epithelial cells oxytocin can induce a larger synthesis and pulsatile secretion of PGF$_{2\alpha}$. Furthermore, signals to initiate lysis of the CL are received in the posterior pituitary and subsequent neuroendocrine release of oxytocin occurs (McCraken et al., 1999). The positive feedback mechanism of oxytocin initiating PGF$_{2\alpha}$ secretion and PGF$_{2\alpha}$ initiating oxytocin secretion work in cohesion to accelerate CL regression and a decline in progesterone production.

2.4 Follicular Development

2.4.1 Ovarian reserve

The ovarian reserve in females is comprised of a pool of primordial ovarian follicles that originate from primordial germs cells within the developing embryo. During gonadal development of the early embryo, the primordial germ cells migrate from the yolk sac epithelium through the gut mesentery to establish on the gonadal ridge of the mesonephros, proliferate, and form the pool of primordial follicles (Picton, 2001). The actual number of primordial germ cells that develop is highly variable based upon individual but is estimated to be around $2.1 \times 10^6$ cells at the end of the first trimester (Santos et al., 2013) and quickly goes through apoptosis to decline in number to around 133,000 at birth (Erickson, 1966). Although, the preceding thought has been that the ovarian reserve is not renewable, recent work by Johnson et al. (2005) has shown that
bone marrow stem cells from general circulation can generate post-natal oogenesis in mice. However, whether the ovarian reserve is renewable or not, significant decreases occur after 4 years of age until the near zero state is achieved around 20 years of age in the bovine (Erickson, 1966).

2.4.2 Follicular classification

Differentiation of the primordial germ cells into the oogonia occurs early in the developing embryo. Massive colonization of the fetal ovary with mesonephric cells occurs prior to the initiation of meiosis of the oocytes. The mesonephric cells are thought to form the precursors for follicular cells (Picton, 2001). During this process, oocytes become enclosed in a single squamous cell layer of pre-granulosa cells separated by a basement membrane from stromal cells, which eventually differentiate into theca cells (Orisaka et al., 2009). Oocytes that are not surrounded by the squamous cell granulosa cell layer degenerate, and nests of primordial follicles remain (Picton, 2001). These primordial follicles form the basis for which further differentiation will occur to form the three categories of follicles widely recognized today: primary, secondary, and tertiary, antral or vesicular.

Differentiation of the primordial to primary follicle begins with the transition of the flattened pre-granulosa, or squamous, cells into cuboidal shaped granulosa cells that form a single cell layer around the primordial follicle and increase in number (Braw-Tal and Yossefi, 1997). Braw-Tal and Yossefi (1997) termed this initial growth phase I, with
changes in the shape and number of granulosa cells but diameter of the follicle remaining unchanged. Continued proliferation of the granulosa cells around the oocyte results in a layering formation. Once the oocyte attains two layers of granulosa cells it becomes classified as a secondary follicle (Aerts and Bols, 2010). The increase in granulosa number and layers, up to 6, was found to be high correlated with the growth of the oocyte during what was termed growth phase II by Braw-Tal and Yossefi (1997).

As the follicle transitions to preantral, in their study, they found the oocyte diameter roughly doubled in size and differentiation of theca interna cells began to occur as well as formation of the zona pelucida. With the development of theca interna and granulosa cells a fluid filled vesicle forms, termed antrum, and the transitioning follicle is called a tertiary follicle (Erickson, 1966). As the antrum continues to grow, dominance is achieved and the follicle transitions into a Graffian or preovulatory follicle. The growth of the primordial follicle to ovulatory size has been estimated to take up to 180 d, with the development from the antral stage to preovulatory taking approximately 42 d (Lussier et al., 1987).

2.4.3 Folliculogenesis

Ovarian follicle growth, or folliculogenesis, begins with the initiation of meiosis within the oocytes of the developing fetus. The primary oocyte progresses through meiosis to become arrested within the diplotene stage of the first prophase, where
proliferation of mitochondria and reorganization of the Golgi complex occurs, followed by entry into a quiescent state until puberty (Picton, 2001).

The exact process for reinitiating folliculogenesis is still widely up for debate. However, follicular growth during the quiescent stage appears to be stalled due to inhibitors (Braw-Tal and Yossefi, 1997), suspected to be activin A (Braw-Tal, 2002). Upon the removal of the inhibitor basic fibroblast growth factor, kit ligand, growth differentiation factor 9, and bone morphogenic protein 15 secreted from the oocyte and local stromal/epithelial cells lead to the proliferation of and transition of flat to cuboidal granulosa cells, or growth phase I (Braw-Tal, 2002). Once the cuboidal granulosa cells proliferate to around 40 cells in the largest cross section, growth phase II is initiated (Braw-Tal and Yossefi, 1997) and the granulosa cells begin to express follistatin messenger ribonucleic acid (mRNA) and protein (Braw-Tal, 2002). Although it is suspected that kit ligand and follistatin from the granulosa cells are not the only factors involved (Braw-Tal, 2002) oocyte growth occurs along with continued granulosa cell proliferation in a highly-correlated fashion (Braw-Tal and Yossefi, 1997).

Continued proliferation of the granulosa cells results in multiple layers forming around the oocyte. In addition, rapid growth of the oocyte induces the formation of the zona pellucida (Braw-Tal and Yossefi, 1997) and development of recognizable theca interna cells derived from the interstitial stroma as well as development into layers around the basal lamina occurs (Aerts and Bols, 2010). Up to this point, development of the preantral follicle has been accomplished in the absence of gonadotropin influence.
At the end of the preantral phase the highly vascularized external theca layer can provide the developing follicle with a supply of endocrine factors (Smitz and Cortvridnt, 2002). These endocrine factors, primarily LH and FSH, initiate the production of androgens within the theca interna and granulosa cells. Although the theca interna cells are not LH dependent for survival it is required to produce androstenedione which acts to cytodifferentiate response of the granulosa cell to FSH and promote follicle growth (Smitz and Cortvridnt, 2002). Once the follicles attain 250 granulosa cells (Aerts and Bols, 2010) fluid filled spaces begin to appear within the granulosa cells and eventually coalesce to form a single crescent shaped antrum (Smitz and Cortvridnt, 2002).

It is highly recognized that the antral follicle is dependent upon gonadotropins for further growth and development, however multiple other factors can modulate the action of these gonadotropins. Inhibin, activin, and follistatin are three of these key modulators that are produced within the now differentiated mural and cumulus granulosa cells (Smitz and Cortvridnt, 2002). Inhibin and activin are disulphide-linked glycoproteins that belong to the TGF-β superfamily and while follistatin is a functionally related glycoprotein, it does not belong to the same superfamily (Knight and Glister, 2001). Activin works locally through promoting upregulation of FSH receptors and proliferation of the granulosa cells (Knight and Glister, 2001) but also down regulating steroidogenesis in theca cells (Wrathall and Knight, 1995). Inhibin, on the other hand, works in an opposing way to promote steroidogenesis from the theca cells (Wrathall and Knight, 1995) and, in concert with estrogen systemically, decrease synthesis and
release of FSH from the pituitary (Knight and Glister, 2001). The mechanism of action of follistatin, however, seems to be primarily through high-affinity binding of activin to inhibit steroidogenesis and follicular growth (Knight and Glister, 2001). Therefore, as follicles mature and inhibin production increases (Campbell et al., 1991) these local effects allow for increases in synthesis of androgens by the theca cells and sequential estrogen from granulosa cells.

As the antral follicle continues to grow and transition into a dominant follicle, the granulosa cells develop LH receptors (Xu et al., 1995). This development is an important step, allowing for the continued growth of the follicle while FSH concentrations are declining (Ginther et al., 2003). Furthermore, Ginther et al. (2003) was able to show that circulating LH concentrations may increase around the time of selection (Ginther et al., 2003). The follicle continues to exponentially increase in size during the dominance phase and transcription begins to decline in the nucleus of the oocyte (Hyttel et al., 1997). However, final maturation of the oocyte does not occur until the LH surge. After the LH surge meiosis is resumed and germinal vesicle breakdown occurs (Knight and Glister, 2001). The oocyte progresses through the remaining stages of meiosis I and immediately enters meiosis II where it becomes arrested in the metaphase II stage (Hyttel et al., 1997) until fertilization.
2.4.4 Follicular wave characteristics

Follicular growth in the antral phase occurs in a wave-like pattern in many domestic species such as bovine. As previously stated the number and length of wave that an individual will have is highly dependent upon multiple factors such as age and breed. *Bos taurus* breeds tend to produce 2 to 3 follicular waves between ovulations (Savio et al., 1988; Ginther et al., 1989), while *Bos indicus* are more likely to have 3 to 5 waves between ovulations (Sartori and Barros, 2011). Furthermore, as one would expect, the wavelength of the cycle is also dependent upon the number of cycles per wave. Two wave cows have longer periods of follicular growth, with emergence of waves on d 0 and 10 versus d 0, 9, 16 for two and three wave cows respectively (Ginther et al., 1989). Therefore, the increased wave length results in a longer period of growth and subsequent ovulation of larger follicles that are more mature (Townson et al., 2002).

The wave-like pattern of follicular growth can be broken down into three separate phases: recruitment, selection, and dominance. The recruitment phase begins with the initiation of a follicular wave by an elevation of circulatory FSH (Adams et al., 1992). During this phase, lasting from 1 to 3 d (Draincourt, 2001), a cohort of gonadotropin dependent follicles under 4 mm are stimulated to grow (Adams et al., 1992). These follicles continue to grow under the influence of FSH until the selection phase occurs. To continue into the selection phase the follicles must function to support their own growth through estrogen production and inhibition of the remaining cohort,
resulting in atresia (Savio et al., 1988). The follicular selection phase results in 3 to 6 competent follicle that continue to mature and grow (Fortune et al., 2001). During the selection and remaining dominant phase estradiol and inhibin negative feedback results in lower circulatory FSH concentrations (Adams et al., 1992). Decreased FSH concentrations results in the largest two follicles exhibiting increase growth rates at the end of the selection phase due to decreased FSH requirements (Ginther et al., 2001a) and the development of LH receptors in the granulosa cells (Xu et al., 1995). During this time, any remaining follicles that do not develop LH receptors regress by atresia due to continued inhibition by viable follicles via inhibin and estradiol (Guilbault et al., 1993). Transition to the dominance phase occurs once follicles reach 8.0 to 9.0 mm in diameter (Ginther et al., 1996) and is characterized by the growth of the largest follicle and regression of the remaining subordinate follicles. Although the exact mechanism is still unknown, selection for dominance is thought to be achieved by the first follicle to acquire LH receptors on its granulosa cells during the late selection period (Draincourt, 2001).

As with the growth from recruitment to dominance, the resulting fate of the dominant follicle is highly dependent upon gonadotropin support. In the early to mid-luteal phases of the estrous cycle progesterone production from the CL in cohesion with estradiol exerts a negative feedback on GnRH and LH (Skinner et al., 1998). Without the support of LH, the granulosa cells of the dominant follicle become atretic and growth is inhibited (Ginther et al., 2001b). However, in the absence of the negative feedback of progesterone during the final wave of the estrous cycle the dominant follicle will
become the preovulatory follicle and undergo ovulation (Ginther et al., 1989) and final maturation of the oocyte with the LH surge (Knight and Glister, 2001).

2.5 Estrous Synchronization

2.5.1 History and protocol development

The first real research into manipulation of the estrous cycle in bovine began in early 1940’s when Casida et al. (1943) began injecting sheep pituitary extract into cattle to form a CL on the ovaries. This initial discovery of hormonal influence was quickly followed with studies showing progesterone inhibiting estrous (Christian and Casida, 1948), failure for regression of the CL upon hysterectomy (Wiltbank and Casida, 1956), and regression of the CL in response to oxytocin (Armstrong and Hansel, 1959). These initial studies would lead the way for the next couple of decades for discovery of multiple hormones and the realization that the bovine estrous cycle could be manipulated to benefit reproduction.

2.5.1.1 Progesterone

The first true synchronization protocols came following the discovery of progesterone influence. Willet (1950) was able to report in abstract that a 50% conception rate was achieved following artificial insemination (AI) of dairy heifers after a progesterone-controlled estrus. However, the following studies into using
progesterone followed by insemination were unable to achieve those same results (Trimberger and Hansel, 1955; Nellor and Cole, 1956). Therefore, Nellore and Cole (1956) modified the protocol by using injections of 540 mg emulsified progesterone followed by 750 International Units (IU) of equine gonadotropin, known at the time to cause ovulation, which resulted in 89% of beef heifers exhibiting estrus during a 4 h period 1 d later. These animals were artificially inseminated 48 h after the equine gonadotropin injection resulting in the first recorded timed artificial insemination (TAI) (Nellor and Cole, 1956).

As knowledge on progesterone and its use for manipulation of the estrous cycle grew, synthetics developed and new protocols immerged. Hansel et al. (1961) fed lactating beef cattle 968 mg of medroxyprogesterone acetate (MAP) for 10 consecutive days followed by 500 mg of MAP for the next 10 consecutive days resulting in 91% of the animals detected in estrus or formed a CL during 3 to 5 d after the conclusion of the feeding period. The major downfall of this study, with only using progesterone, remained the lack of adequate pregnancy results (25%) following breeding at first estrus. However, Hansel et al. (1961) helped to firmly establish the use of a synthetic progesterone for inhibition of estrus. Zimbelman (1963) went on to set up dose response studies to determine an effective dose of MAP. He discovered that at doses of 120 to 180 mg in a group feeding scenario, synchronization of ovulation occurred in 94% of beef heifers. Therefore 180 mg fed daily for 18 consecutive days was established as the effective oral dose and The Upjohn Company went on to commercialize the product as Repromix in 1965 (Lauderdale, 2009). However, the product was voluntarily pulled in
1967 due to the expensive price causing a lack of a market among commercial beef producers.

At the same time, successful research continued into alternative oral progesterone synthetics such as chlormadinone acetate (Van Blake et al., 1963; Hansel et al., 1966) and melengestrol acetate (MGA; Zimbelman and Smith, 1966a). Zimbelman and Smith (1966a) found MGA to be extremely potent orally in comparison to MAP, being 300 to 900 times more potent, and an effective alternative. Furthermore, in consecutive papers Zimbelman and Smith (1966a; 1966b) were able to establish 0.4 mg daily as an effective oral dose to inhibit ovulation. However, the initial studies into MGA showed more promise for use as a growth promotant in prepubertal heifers (Bloss et al., 1966; Zimbelman and Smith, 1966b). Bloss et al. (1966) exhibited that the best results for feed efficiency and weight gain were achieved at daily doses between 0.35 and 0.50 mg daily. Therefore, the Food and Drug Administration (FDA) Center for Veterinary Medicine approved the use of MGA in 1968 for increased weight gain, improved feed efficiency, and suppression of estrus in confinement heifers at a rate of 0.25 to 0.5 mg daily (Food and Drug Administration, 1986). MGA use for estrous synchronization continued without approval as it was easily attained under the feedlot approved label. Approval for use in estrous synchronization finally occurred in 1997 (Food and Drug Administration, 1997a).

Even though oral progesterone was effective for suppression of estrus, an alternative for more precise administration was desired. One of the first attempts for an
alternative used subcutaneous polyhydroxy polymer capsules containing norethandrolone (Nor) and achieved an estrus synchronization rate of 81.8% (Curl et al., 1968). In addition, to more precisely control ovulation estrogen was being implemented into synchronization protocols (Lantz et al., 1968) as estrogen acts as a luteolytic agent when administered in the early estrous cycle (Wiltbank et al., 1971). Wiltbank et al. (1971) combined these two concepts using subcutaneous Nor implants for 9 d in the flank and subsequent injections of estradiol valerate at administration to achieve 93% estrus rate. Furthermore, when an injection of estradiol to 17β was added 24 h after implant removal, 98% of cycling heifers exhibited estrus within 48 h period with 100% ovulating in 36 h period (Wiltbank et al., 1971). Later, this same protocol was explored by Wiltbank and Gonzalez-Padilla (1975) and in a series of studies by Spitzer et al. (1978a; 1978b) and Miksch et al. (1978) where they replaced the Nor implant with a norgestomet implant in the ear to achieve satisfactory results. This initial research into the norgestomet implant and later followed by Spitzer et al. (1981) led to development of Syncro-Mate-B (SMB) and its approval for use by the FDA in 1982 (Food and Drug Administration, 1982). The SMB protocol consists of a 6 mg implant of norgestomet for 9 d with and injection of 3 mg norgestomet and 5 mg estradiol valerate at time of implantation (Spitzer et al., 1981).

Even though these products were effective, the desire for a more accurate and easily administrable progesterone source remained. As previously stated, oral progesterone proved hard to precisely dose and the alternative SMB implants had to be removed by surgically incising the ear. Therefore, a third option was developed, the
progesterone releasing intravaginal device (PRID). The PRID was first investigated by Roche (1976b) and was made up of salastic coils impregnated with 10% progesterone. When the PRID was inserted for 12 d with injections of 5 mg of estradiol benzoate and 50 mg of progesterone at insertion, pregnancy rates were like control animals (Roche, 1976a). Furthermore, this same concept was used to develop a controlled internal drug release (CIDR) that was impregnated with 1.38 mg of progesterone (Macmillan and Peterson, 1993) and approved by FDA in 1997 (Food and Drug Administration, 1997b).

2.5.1.2 Prostaglandin F\textsubscript{2α} and gonadotropin releasing hormone

Progesterone was not the only hormone being rapidly developed for estrous synchronization in the early 1970s. By the time of the Nellor and Cole (1956) experiment earlier discussed, as well as stated in the research by Armstrong and Hansel (1959), it was widely known that the pituitary played a crucial role in ovulation and hormonal secretion. Therefore, GnRH as well as LH had been discovered and classified by 1972 (Gorski, 1970; Brugus et al., 1972) and was successful in synchronizing ovulation when used alone (Garverick et al., 1980) and when combined with progesterone (Kaltenbach et al., 1974). However, the real issue remained of how to consistently induce luteolysis of the CL. This was the main topic at the Second Brook Lodge Workshop where PGF\textsubscript{2α} was implicated as a possible initiator of luteolysis (Lauderdale, 2009). With emphasis placed on the need for its discovery PGF\textsubscript{2α} was soon implicated as the luteolytic factor of cattle (Lauderdale, 1972; Rowson et al., 1972; Louis et al., 1973). Lauderdale (1972)
reported that non-pregnant cows returned to estrus in 2 to 4 d following administration of PGF$_{2\alpha}$ tromethamine salt (PGF$_{2\alpha}$ THAM) from d 6 to 16 of the estrous cycle. Furthermore, Rowson (1972) was able to exhibit the analog chloprostenol was effective as well.

Implementation of PGF$_{2\alpha}$ and GnRH into estrous synchronization occurred rapidly. Although GnRH use was limited due to only approximately 66% to 70% of beef cows responding to a single injection (Geary et al., 2000), PGF$_{2\alpha}$ proved quite advantageous. Lauderdale et al. (1974) was one of the first to implement a single injection dose of PGF$_{2\alpha}$ THAM for estrous synchronization and TAI, reporting no difference in pregnancy rates between controls and those bred at estrus or TAI at 72 and 90 h after PGF$_{2\alpha}$ THAM administration. In addition, further studies at the time would go on to establish an effective dose of 25 mg for induction of CL regression (Lauderdale et al. 1977). However, single dose administration of PGF$_{2\alpha}$ remained ineffective in animals that were within the first five days of the estrous cycle as described earlier. Therefore, early programs developed with additional injection of estradiol benzoate 48 h post PGF$_{2\alpha}$ injection (Welch et al., 1975), with one study resulting in increased estrus detection to 90% with estradiol benzoate from 72% without (Peters et al., 1977), or a second dose of PGF$_{2\alpha}$ 11 d following administration of the first PGF$_{2\alpha}$ (Graves et al., 1974). These studies resulted FDA approval for PGF$_{2\alpha}$ to be used for estrous synchronization in either a single dose (Food and Drug Administration, 1981) or consecutive doses 11 to 14 d apart (Food and Drug Administration, 1979).
2.5.1.3 Multiple hormone protocols

Up to this point, most of the protocols developed for estrus synchronization only involved the use of a single hormone to achieve the desired routes. However, optimal enhancement of estrous synchronization is achieved under the influence of multiple hormones used in combination. Implantation of heifers with the SMB implant for 7 d with injection of PGF$_{2\alpha}$ on d 7 at removal resulted in improved estrus synchronization and pregnancy results (Heersche et al., 1974). In addition, when the SMB implant was replaced with feeding MGA for 5 d, PGF$_{2\alpha}$ injection on d 5, similar results were achieved (Lauderdale, 1975). The real advantage in these protocols revolved around the complete removal of progesterone influence at the end of the implant period due to CL regression during the implantation period or PGF$_{2\alpha}$ injection (Odde, 1990).

Combinations of MGA and PGF$_{2\alpha}$ were proving to be effective in estrous synchronization protocols. Beal and Good (1986) were able to show cycling could even be induced successfully in non-cycling cows using this type of protocol. However, it was beginning to become apparent that estrus response could be reduced in cycling cows with both MGA (Beal et al., 1988) and SMB (Brink and Kiracofe, 1988) due to day of the cycle. Therefore, to circumvent issues with decreased pregnancy rates following progesterone treatment, Brown et al. (1988) developed a protocol where MGA was fed for 14 d and PGF$_{2\alpha}$ was administered 16 to 17 d following the last MGA feeding. Moving the PGF$_{2\alpha}$ injection to approximately 17 d after progesterone removal, a time point in late luteal phase, increases efficacy (Lauderdale, 1972). When compared to the SMB
protocol with PGF$_{2\alpha}$ injection upon removal the 14 d MGA protocol increased pregnancy rates with no differences in estrus response (Brown et al., 1988).

Clear advancements were being made with estrous synchronization, however, ovulation synchronization and TAI results remained subpar. Smith et al. (1984) was able to induce a tighter synchrony of estrus by injection of PGF$_{2\alpha}$ 24 h prior to PRID removal. Furthermore, when the same protocol, PRID insert for 7 d with injection of PGF$_{2\alpha}$ on d 6, was compared to two injections of PGF$_{2\alpha}$ 11 d apart, estrus response rate and TAI pregnancy rates were increased significantly in the PRID group (Smith et al., 1984). In addition to PGF$_{2\alpha}$, studies using GnRH to induce ovulation had also shown advantages in TAI. When GnRH was given at either 30 h after progesterone removal with insemination at 6 and 12 h (Roche, 1974) or 18 h (Roche, 1976a) after GnRH injection, TAI pregnancy rates were increased when compared to progesterone alone, use of human chorionic gonadotropin, or estradiol benzoate. While both methods increased TAI pregnancy results, the real advancement came after development of transrectal ultrasonography and understanding of follicular waves. Using transrectal ultrasound resulted in the realization that precise estrus synchronization requires the control of both follicular waves and luteal lifespan (Patterson et al., 2007; Lauderdale, 2009).

Thatcher et al. (1989) was able to further substantiate the fact that estrous synchronization would benefit from both follicular wave and luteal control. Using an injection of GnRH 7 d prior to PGF$_{2\alpha}$ treatment resulted in a reduction of spontaneous estrus between injections, by inducing ovulation or atresia, and a tighter synchrony of
estrus following PGF$_{2\alpha}$ (Thatcher et al., 1989). Implementation of this type of protocol, later known as Select Synch, allowed for a decreased need for estrus detection prior to PGF$_{2\alpha}$ injection without decreasing pregnancy rates (Twagiramunga et al., 1992; Geary et al., 2000). Furthermore, with the addition of a second GnRH injection 48 h after PGF$_{2\alpha}$ injection, synchronization of ovulation into an 8 h window could be effectively achieved (Pursley et al., 1995). This protocol would go on to become known as Ovsynch and was highly validated for TAI among dairy cows but not dairy heifers (Pursley et al., 1997). In addition, fertility was increased when animals were pre-synchronized with two injections of PGF$_{2\alpha}$ or implementing two Ovsynch protocols 7 d apart (Souza et al., 2008). When the Ovsynch was implemented in beef cows, Geary et al. (1998) showed increased pregnancy rates in Ovsynch treated cows compared to SMB treated cows, 54% to 42% respectively. Furthermore, to make the protocol convenient for large scale beef implementation, Geary and Whitter (1997) performed TAI at or 24 h after the second GnRH injection and reported no differences in TAI pregnancy results. The concept of TAI at GnRH injection with the Ovsynch protocol would come to be more accepted in the beef industry and was termed the CO-Synch protocol (Patterson et al., 2007).

The remaining issue of the Ovsynch, Select Synch, or CO-Synch protocols was the lack of progesterone primer to initiate cycling in anestrous or prepubertal animals. Therefore, work into estrus synchronization of heifers remained concentrated on modifications of the MGA protocol. Initial work by Kesler et al. (1996) attempted to decrease the length of time from removal of MGA to PGF$_{2\alpha}$ injection. However, it was
soon discovered that the optimal time for PGF$_{2\alpha}$ injection would be 19 d following MGA removal (Lamb et al., 2000) due to the formation of overly mature, persistent follicles that would lead to decreased pregnancy rates. Although increased pregnancy rates were achieved with administration of PGF$_{2\alpha}$ 19 d following MGA removal (Deutscher, 2000), further modification into the MGA Select protocol by administration of GnRH on d 26 of the protocol resulted in tighter synchronization of estrus 48 to 72 h after PGF$_{2\alpha}$ administration (Wood et al. 2001).

### 2.5.1.4 Controlled internal drug release protocols

The commercialization of the CIDR, vaginal progesterone device, and subsequent validation by Lucy et al. (2001) lead the next wave of development of estrous synchronization protocols, especially in the beef industry. The CIDR was quickly implemented into various protocols already in place to improve synchronization of non-cycling animals. Addition of the CIDR into the CO-Synch protocol from the first GnRH administration to PGF$_{2\alpha}$ administration resulted in increased pregnancy rates of anestrous cows and thus an increase in overall TAI pregnancy rates (Lamb et al., 2001). Further implementation of the CIDR into estrous synchronization by being used with a single PGF$_{2\alpha}$ injection upon removal or with the Select Synch protocol continued to prove that it was an effective addition and resulted in adequate pregnancy results in beef cows (Larson et al., 2006). Furthermore, the addition of a second administration of
GnRH coinciding with AI would lead to the creation of a widely used fixed time AI (FTAI) protocol; 7-d CO-Synch + CIDR protocol.

Using the CIDR to replace MGA in heifer protocols also proved effective. Increased synchrony of estrus and pregnancy rates were achieved with a CIDR Select protocol when compared to MGA Select protocol (Kojima et al., 2004). In addition, the CIDR Select protocol was proven to be equally as effective when a second GnRH injection was given at TAI, 72 h after PGF\textsubscript{2α} administration (Busch et al., 2007). It is important to note that in the CIDR Select protocol GnRH and PGF\textsubscript{2α} administration occur 9 and 16 d after CIDR removal respectively due to decreased time to estrus following CIDR removal (Macmillan and Peterson, 1993). Furthermore, comparison of another set of similar protocols, the 14 d MGA protocol and 14 d CIDR protocol with PGF\textsubscript{2α} administration on d 19 and 16 respectively, resulted increased estrus expression and synchrony in heifers treated with the CIDR protocol while heifers with no difference in pregnancy rates between the two groups were identified (Tauck et al., 2007). When the two long-term CIDR protocols previously mentioned are compared directly, the 14 d CIDR protocol initiates an increased synchronization of estrus and conception to AI after estrus detection (Leitman et al., 2009), as well as increased conception to TAI (Mallory et al., 2011) over the CIDR Select protocol. Thus, suggesting the administration of GnRH is not required for synchronization of heifers within the two similar, long-term CIDR protocols.
More recently, protocols designed to more precisely align with follicular waves and prevent over maturation have been developed. Bridges et al. (2008) hypothesized that decreasing the interval between the initial GnRH and PGF$_{2\alpha}$ as well as length of CIDR insertion within the CO-Synch plus CIDR protocol would enhance estrogen production and increase TAI pregnancy rates. Although the initial experiment showed no differences in pregnancy rates between the two treatments, in follow up experiments Bridges et al. (2008) was able to show increased pregnancy rates in the 5dCO by adjusting TAI from 60 to 72 h after PGF$_{2\alpha}$ administration and CIDR removal. In addition to the 5dCO, modification of the Select Synch protocol with CIDR has occurred to create a 6 d CIDR protocol. In the 6 d CIDR protocol PGF$_{2\alpha}$ is administer 3 d prior CIDR insertion and GnRH injection to pre-synchronize heifers and initiate a new follicular wave at the start of the protocol (Grant et al., 2011). When compared to the Select Synch plus CIDR protocol, the 6 d CIDR protocol increased the percentage of heifers initiating a new wave at CIDR insertion and more synchronized follicle size at CIDR removal, as well as through ovulation resulting in tight synchrony of estrus (Grant et al., 2011).

Split-time AI has also recently been studied to see if TAI pregnancy results could be improved by extending interval from PGF$_{2\alpha}$ injection to TAI an additional 20 h for animals not exhibiting estrus. Improvements in pregnancy rates were seen when insemination was delay for heifers on the 14 d CIDR protocol (Thomas et al., 2014). However, Thomas et al. (2014) was unable to exhibit the same results in beef cows on the 7-d CO-Synch protocol. Modifications to the current protocols continue to occur and
often, as in the case of the 5dCO, 6 d CIDR protocols, or split time AI, increased synchronization may require increased management and handling.

### 2.5.2 Recommended protocols

With the rapid development of multiple protocols, the need arose to establish a common nomenclature and to facilitate transfer of the latest research into information to producers. Based upon these needs two organizations developed: Dairy Cattle Reproduction Council and Beef Reproduction Task Force. These groups consist of industry leaders, educators, researchers, veterinarians, and pharmaceutical representatives. These individuals come together to provide recommendations on reproductive technologies and synchronization protocols for their respective industries. Since this review is primarily focused for beef, the current synchronization protocols will cover those recommended by the Beef Reproduction Task Force and published by Johnson et al. (2013).

#### 2.5.2.1 Heat detection protocols

Animals within the heat detection protocols will display peak estrus approximately 48 to 72 h after PGF$_{2\alpha}$ injection (Lauderdale, 1972). To accurately detect this estrus, heat detection must occur at minimum three times per day for a total
minimum time period of 3 h (Johnson et al., 2013). Animals detected in estrus should be
Al approximately 6 to 12 h post first detected standing estrus.

1 Shot PG. Recommended for heifers. Animals within this protocol should
undergo estrus detection and Al from d 0 through d 12 of the protocol. Animals not
exhibiting estrus by d 5 should receive an injection of PGF$_{2\alpha}$.

Select Synch. Recommended for cows. Animals within this protocol will receive
injections of GnRH on d 0 and PGF$_{2\alpha}$ on d 7. Animals should undergo estrous detection
and Al from d 6 though d 13 of the protocol.

Select Synch + CIDR. Recommended for cows. Animals within this protocol will
receive a GnRH injection and CIDR insertion on d 0 and injection of PGF$_{2\alpha}$ and removal of
CIDR on d 7. Animals in this protocol should undergo estrus detection and Al from d 7
through d 13 of the protocol.

7-d CIDR-PG. Recommended for heifers. Animals within this protocol will receive
a CIDR on d 0, followed by removal and injection of PGF$_{2\alpha}$ on d 7. Animals should
undergo estrus detection and Al from d 7 through d 13 of the protocol.

PG 6-d CIDR. Recommended for cows. Animals within this protocol will receive
PGF$_{2\alpha}$ injection on d 0 followed by GnRH injection and CIDR on d 3. Removal of the CIDR
occurs on d 9 with concurrent injection of PG. Animals should undergo estrus detection
and Al from d 0 through d 3 and d 9 through d 12. Animals undergoing Al prior to CIDR
insertion should discontinue the remainder of the protocol.
MGA-PG. Recommended for heifers. Animals within this protocol will be fed 0.5 mg of MGA from d 1 through d 14, followed by PGF$_{2\alpha}$ injection 19 d later, or on d 33 of the protocol. Animals should undergo estrus detection and AI from d 33 through d 39 of the protocol.

2.5.2.2 Heat detection & timed artificial insemination protocols

Animals within the heat detection and TAI protocols will undergo estrus detection and AI for approximately 3 d, followed by AI of all animals not exhibiting estrus on at a predetermined time point with concurrent injection of GnRH in those animals (Johnson et al., 2013).

Select Synch & TAI. Recommended for cows. Animals within this protocol will receive injections of GnRH on d 0 and PGF$_{2\alpha}$ on d 7. Animals should undergo estrus detection and AI for 72 to 84 h after PGF$_{2\alpha}$ injection followed by TAI and GnRH injection on animals not exhibiting estrus.

Select Synch + CIDR & TAI. Recommended for cows and heifers. Animals within this protocol will receive a GnRH injection and CIDR on d 0 and injection of PGF$_{2\alpha}$ and removal of CIDR on d 7. Animals should undergo estrus detection and AI for 72 to 84 h after PGF$_{2\alpha}$ injection followed by TAI and GnRH injection on animals not exhibiting estrus.
**PG 6-d CIDR & TAI.** Recommended for cows. Animals within this protocol will receive PGF$_{2\alpha}$ injection on d 0 followed by GnRH injection and CIDR insertion on d 3. Removal of the CIDR occurs on d 9 with concurrent injection of PG. Animals should undergo estrus detection and AI from d 0 through d 3 and 72 to 84 h after PGF$_{2\alpha}$ injection followed by TAI and GnRH injection on animals not exhibiting estrus. Animals undergoing AI prior to CIDR insertion should discontinue the remainder of the protocol.

**MGA-PG & TAI.** Recommended for heifers. Animals within this protocol will be fed 0.5 mg of MGA from d 1 through d 14, followed by PGF$_{2\alpha}$ injection 19 d later, or on d 33 of the protocol. Animals should undergo estrus detection and AI for 72 to 84 h after PGF$_{2\alpha}$ injection followed by TAI and GnRH injection on animals not exhibiting estrus.

**14-d CIDR-PG & TAI.** Recommended for heifers. Animals within this protocol will receive a CIDR on d 0 with removal occurring on d 14. Animals will receive and injection of PGF$_{2\alpha}$ 16 d later, or on d 30 of the protocol. Animals should undergo estrus detection and AI for 70 to 74 h after PGF$_{2\alpha}$ injection followed by TAI and GnRH injection on animals not exhibiting estrus.

**2.5.2.3 Fixed timed artificial insemination protocols**

All animals in FTAI protocols will not be heat detected for estrus and will undergo AI insemination at a predetermined time point concurrent with an injection of GnRH (Johnson et al., 2013). All animals subjected to FTAI should be inseminated within
a 3 to 4 h period with the approximate average time of AI being equivalent to the listed time in the protocol.

**7-d CO-Synch + CIDR.** Recommended for cows and heifers. Animals within this protocol will receive a CIDR and injection of GnRH on d 0 followed by removal of the CIDR with concurrent injection of PGF$_{2\alpha}$ on d 7. Cows should undergo FTAI and injection of GnRH 60 to 66 h after injection of PGF$_{2\alpha}$. Heifers should undergo FTAI and injection of GnRH approximately 54 h after injection of PGF$_{2\alpha}$.

**5-d CO-Synch + CIDR.** Recommended for cows. Animals within this protocol will receive a CIDR and injection of GnRH on d 0 followed by removal of the CIDR with concurrent injection of PGF$_{2\alpha}$ on d 5 and a second injection of PGF$_{2\alpha}$ 8 h later. Animals should undergo FTAI and injection of GnRH approximately 72 h after injection of the first PGF$_{2\alpha}$. Interval between first PGF$_{2\alpha}$ injection and FTAI should be shortened to approximately 66 h in *Bos Indicus* animals.

**MGA-PG.** Recommended for heifers. Animals within this protocol will be fed 0.5 mg of MGA from d 1 through d 14, followed by PGF$_{2\alpha}$ injection 19 d later, or on d 33 of the protocol. Animals should FTAI and injection of GnRH approximately 72 h after PGF$_{2\alpha}$ injection.

**14-d CIDR-PG.** Recommended for heifers. Animals within this protocol will receive a CIDR on d 0 with removal occurring on d 14. Animals will receive and injection of PGF$_{2\alpha}$ 16 d later, or on d 30 of the protocol. Animals should undergo FTAI with concurrent injection of GnRH approximately 66 h following PGF$_{2\alpha}$ injection.
2.6 5-d CO-Synch CIDR Protocol

2.6.1 Development

The inclusion of the CIDR and subsequent development of the 7-d CO-Synch + CIDR protocol quickly became the dominant estrous synchronization protocol that was researched, as well as recommended for use by the Beef Reproduction Task Force (Lamb, 2008). However, 40 to 50% of the females subjected to this protocol still failed to conceive to TAI in many of the early studies (Lamb et al. 2001; Martinez et al., 2002; Larson et al., 2006). Therefore, room remained for improvements in the 7-d CO-Synch + CIDR protocol to increase TAI pregnancy results to FTAI.

One of the major drawbacks to any estrous synchronization protocol that uses GnRH on the onset of the protocol is that only 66 to 70% of beef cows have been shown to respond to that initial injection of GnRH (Geary et al., 2000). Animals past d 2 of the estrus cycle that do not respond to the initial injection of GnRH have significantly smaller follicles with less estradiol production at timing of the second injection of GnRH (Atkins et al., 2010a). Smaller follicles and lower estradiol concentrations have been shown to have decreased conception and embryonic survival rates to induced GnRH ovulation when compared to large follicles and spontaneous ovulations (Perry et al., 2005; Mussard et al., 2007). Potential exists that follicles not responding to GnRH may be undergoing atresia resulting in follicular wave turnover later than those that respond to GnRH. Conversely, they may also be immature, lacking LH receptors, and become over maturated prior to the second injection of GnRH (Atkins et al., 2010b). Both
outcomes potentially decrease fertility at time of second GnRH injection and therefore, further research was needed to understand the follicular size and age contributions to fertility.

Based upon a series of results from experiments looking into the effect of follicular size and age, evidence began to develop that the length of proestrus was more influential on conception rates than either of the previous two variables (Bridges et al., 2010). Bridges et al. (2010) went on to show that increasing the length of proestrus in ovulating follicles of the same size lead to increased estradiol concentration prior to TAI, increased progesterone concentrations during the mid-luteal phase of the subsequent estrous cycle, and increased TAI pregnancy rates. Furthermore, at this point it was becoming evident that there was a negative relationship between the duration of dominance of the ovulating follicle and fertility (Bleach et al., 2004; Cerri et al. 2009) as highlighted originally by Townson et al. (2002) when he reported increased conception rates in 3 versus 2 wave cows. Thus, a more ideal protocol would both need to shorten dominance and increase time of proestrus.

Modifications of the 7-d CO-Synch protocol to increase length of proestrus to greater than 66 h proved unrewarding (Dobbins et al., 2006) due to increases in early estrus behavior prior to AI likely caused by increased size and maturity of the dominant follicle. Therefore, a novel approach was required to extend the proestrus phase and shorten dominance, without increasing the maturity of the dominant follicle. Bridges et al. (2008) speculated that a reduction in the interval from the initial injection of GnRH to
PGF$_{2\alpha}$ administration and progestin withdrawal would result in elevated levels of estradiol and decrease the maturity of the ovulated follicle and thus increase TAI pregnancy rates. Comparison of the more recently developed 5 d and established 7-d CO-Synch + CIDR protocols resulted in similar TAI pregnancy rates when inseminated at 60 h following PGF$_{2\alpha}$ administration (Bridges et al., 2008). Bridges et al. (2008) further modified the new protocol and extend proestrus by increasing the time between PGF$_{2\alpha}$ administration and TAI to 72 h resulting in increased pregnancy rates to TAI when compared to the existing 7-d CO-Synch + CIDR protocol; 80.0% vs 66.7% and 56.2% vs 65.3% respectively.

The major drawback to the newly developed protocol was the induction of luteolysis in the accessory CL created with the initial GnRH injection. Since the interval from initial GnRH to PGF$_{2\alpha}$ administration was only 5 d it was widely unknown whether luteolysis was achievable with a single 25 mg dose of PGF$_{2\alpha}$ (Bridges et al., 2008). Therefore, in the initial design Bridges et al. (2008) used two separate 25 mg doses of PGF$_{2\alpha}$ approximately 12 h apart. However, in vivo, PGF$_{2\alpha}$ has been demonstrated to act in a pulsatile fashion approximately 6 to 8 h apart with variability in the duration and magnitude (Silvia et al., 1991). Therefore, when the efficacy of two separate 25 mg doses of PGF$_{2\alpha}$ was shown to be required over a single 25 mg dose an interval of approximately 6 to 7 h was used between administrations of PGF$_{2\alpha}$ (Kasimanickam et al., 2009; Peterson et al., 2011). Therefore, based upon this research, the Beef Reproduction Task Force currently recommends an 8 h interval between PGF$_{2\alpha}$ administrations (Johnson et al., 2013).
2.6.2 Modifications

While acceptable pregnancy rates have been reported with the 5dCO, the necessity of two separated doses of PGF$_{2\alpha}$ leads to increased cost and handling times for producers. New research needed to be performed to make this protocol more convenient and applicable for producers in the field. One way to increase convenience was to consider decreasing number of trips through the chute by administration of two doses of PGF$_{2\alpha}$ concurrently with CIDR removal. More recent work in heifers indicated that a single dose of PGF$_{2\alpha}$ at CIDR removal was adequate to induce luteolysis in both dairy (Rabaglino et al., 2010) and beef heifers (Cruppe et al., 2014; Kasimanickam et al., 2014). Therefore, Bridges et al. (2012) speculated that since dairy heifers receive a higher PGF$_{2\alpha}$ dose per body weight, by increasing the PGF$_{2\alpha}$ dose or administering two 25 mg PGF$_{2\alpha}$ doses simultaneously the same results could be achieved in beef cattle. Bridges et al. (2012) went on to demonstrate that FTAI pregnancy rates and regression of the CL did not differ when two 25 mg doses of PGF$_{2\alpha}$ were administered either simultaneously or 8 h apart. Furthermore, both methods proved more efficacious via FTAI pregnancy rates and CL regression than a single 25 mg dose of PGF$_{2\alpha}$.

More recently discussion has focused on the requirement or need for GnRH on the front end on the 5dCO. The fact remains that any estrous synchronization protocol that uses GnRH on the onset of the protocol, such as the 5dCO, only 66 to 70% of beef cows have been shown to respond to the initial injection of GnRH (Geary et al., 2000). The 5dCO was designed and initial studies showed no difference in estradiol
concentrations following PGF$_{2\alpha}$ administration or follicle size at TAI when response to initial GnRH was compared (Bridges et al., 2014). Therefore, the question was raised if the initial administration of GnRH was required. In dairy heifers, Lima et al. (2011) was able to demonstrate that removal of the initial injection of GnRH resulted in no significant change in TAI pregnancy results. Similarly, Cruppe et al. (2014) found no difference in TAI pregnancy results when the initial injection of GnRH was eliminated in beef heifers. However, this data was contrasting to work done by Kasimanickam et al. (2014), who demonstrated that the initial injection of GnRH resulted in a significant, 8 % increase in TAI pregnancy results. Therefore, a conclusion cannot be made on the requirement of the initial GnRH within the 5dCO in beef heifers at this time.

Analysis of the requirement of the initial GnRH in the 5dCO in beef cows has been limited. Dias et al. (2014) studied the response to the initial GnRH and the effect of response to follicular growth, interval to estrus, and TAI pregnancy rates. Interestingly, although dominant follicle size at TAI and response to the second injection of GnRH was not affected by the response to the initial GnRH injection, TAI estrous response and pregnancy rates were significantly higher (p < 0.05) in the cows that did not respond (65.0 %) versus those that did respond (51.5%) to the initial injection of GnRH (Dias et al., 2014) in the 5dCO. Thus, suggesting that the requirement of the initial GnRH in the 5dCO needs to be evaluated in beef cows.
2.7 Statement of the Problem

The continued development of artificial insemination and estrus synchronization strategies have allowed beef producers to integrate superior genetics. However, even though multiple options are available for implementation, reception and utilization within the United States remains at low levels within the beef sector (NAHMS, 2009). To improve adoption of estrus and ovulation synchronization protocols continued development and improvements of existing protocols as well as creation of new protocols needs to be performed with feasibility of the producer in mind. Therefore, modifications of existing protocols that reduce the number of hormonal interventions and/or frequency and duration of animal handling need to be evaluated.

Currently, research into the 5dCO has indicated that decreases in both hormonal intervention and frequency of animal handling can be achieved. Removal of the initial GnRH injection would not only decrease hormonal intervention, but in decrease handling time and eliminate extra costs to the producer. More specifically, effective response to the initial GnRH injection results in a young, accessory CL at CIDR removal that requires two doses of PGF$_{2\alpha}$. By removing the initial GnRH, the extra PGF$_{2\alpha}$ dose is no longer required to induce CL regression. Therefore, increasing feasibility for the producer by eliminating extra handling time/frequency and additional costs for hormones. Increasing the feasibility of the 5dCO will hopefully facilitate increased adoption and utilization within beef producers in the United States.
The overall goals of the experiments included in this thesis are to determine the impact when no GnRH is administered at CIDR insertion on ovarian parameters and FTAI pregnancy rates. From these goals, application of this modified protocol may be explored within the beef industry. In addition, increased convenience of the FTAI protocol may even further promote the implementation of AI programs in beef cattle.
CHAPTER 3

OVARIAN FUNCTION IN BEEF COWS WHOSE OVULATION WAS SYNCHRONIZED USING THE 5-DAY CO-SYNCH + CIDR PROTOCOL WITH AND WITHOUT GNRRH AT CIDR INSERTION

3.1 Abstract

The objective of this experiment was to evaluate the effects of GnRH at controlled internal drug release (CIDR) insertion in the 5-d CO-Synch + CIDR protocol (5dCO) on ovarian follicular dynamics and circulating steroid hormone concentrations. Non-pregnant, non-lactating beef cows (n = 15) were used in a 3 x 3 Latin square design, with repetitions occurring every 21 d. Animals were assigned to treatment by age and BCS to receive either: 1) standard 5dCO hormone administration including 100 µg of GnRH at CIDR insertion and two concurrent 25-mg doses of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25-mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG). All cows were monitored for behavioral estrus for 72 h after CIDR removal at which time 100 µg of GnRH was administered. Cows underwent transrectal ultrasonography to observe ovarian structures, and coccygeal blood samples were taken for subsequent progesterone and estradiol analyses at CIDR insertion, CIDR removal, final GnRH administration, and d 5 and d 10 post PGF$_{2\alpha}$ administration. An additional blood sample was collected on cows that displayed estrus between PGF$_{2\alpha}$ and the second GnRH administration. Data were
analyzed using the MIXED and GLIMMIX procedures of SAS for the continuous and binary response variables, respectively. Dominant follicle diameter did not differ among treatments at CIDR removal or final GnRH \((P \geq 0.61)\). Percentage of animals whose corpus luteum (CL) regressed in response to PGF\(_{2\alpha}\), estrus detection aid score, and CL volume 10 d following PGF\(_{2\alpha}\) administration did not differ among treatments \((P \geq 0.18)\). Post-ovulation plasma progesterone did not differ among treatments \((P \geq 0.29)\), and plasma estradiol was not different at CIDR removal or final GnRH administration \((P \geq 0.59)\). However, peak plasma estradiol concentrations were greater \((P = 0.01)\) in NoG1-2PG than NoG1-1PG \((5.85 \pm 2.93 \pm 0.55 \text{ pg/mL}, \text{respectively})\), with G1-2PG being intermediate \((3.31 \pm 0.55 \text{ pg/mL})\). In conclusion, follicle and CL growth as well as subsequent progesterone concentrations were unaffected by removal of the initial GnRH in the 5dCO; however, inconsistency of estradiol concentrations merits implementation of a field trial to elucidate impacts of protocol modification on fertility.

### 3.2 Introduction

To better facilitate the utilization of artificial insemination in beef cattle, several ovulation synchronization protocols have been developed to facilitate mass insemination of all females at a predetermined fixed time point [fixed timed artificial insemination (FTAI)]. The 5-d CO-Synch + controlled internal drug release (CIDR) protocol (5dCO) has proven efficacious and produces acceptable pregnancy rates following FTAI in beef and dairy cattle (Wilson et al., 2007; Bridges et al., 2008; Santos et al., 2010). The standard 5dCO involves insertion of a CIDR and administration of an
initial dose of gonadotropin releasing hormone (GnRH; G1) followed approximately 5 d later by CIDR removal and administration of two doses of prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\)) approximately 8 h apart. Fixed timed artificial insemination with a second dose of GnRH (G2) occurs 72 h following removal of CIDR and PGF\(_2\alpha\) administration (Johnson et al., 2013).

Recent work (Dias et al., 2014) has shown that failure of beef cows to respond to G1 in 5dCO has resulted in increased behavioral estrus and FTAI pregnancy rates. Although further research is needed to confirm this initial finding, the removal of G1 has been identified as a potential area of modification of 5dCO. If proven effective, the removal of G1 in 5dCO would be advantageous beyond pregnancy success. Removal of G1 would render the second 25-mg dose of PGF\(_2\alpha\) unnecessary because no corpus luteum (CL) younger than 5 d old would be present to regress. Furthermore, this protocol modification would be more cost-effective, potentially aiding in increased utilization of artificial insemination within the beef sector. Therefore, the objective of this study was to evaluate the effects of G1 removal from 5dCO on ovarian follicular dynamics, circulating preovulatory estradiol and postovulatory progesterone concentrations in postpartum beef cows. We hypothesized that removal of G1 from 5dCO would have no detrimental effects upon ovarian parameters and steroid hormone concentrations in beef cows.
3.3 Material and Methods

3.3.1 General

All protocols, procedures, and animal handling were conducted in a manner approved by the Iowa State University Institutional Animal Care and Use Committee. Research was conducted at Iowa State University Zumwalt Station Research Center in Ames, Iowa from March 2015 to May 2015.

3.3.2 Animals and treatments

Non-pregnant, non-lactating mature Angus and Angus-Simmental beef cows [n=15; age = 7.93 ± 0.13 yr; body condition score (BCS) = 4.8 ± 0.1 (1 = emaciated, 9 = obese, Wagner et al., 1988)] were used to study the effects of G1 removal in 5dCO on ovarian follicular dynamics and circulating steroid concentrations. Animals were stratified and allocated by age and BCS to a 3x3 Latin square design to receive either: 1) standard 5dCO including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG). All animals were given a 100-µg dose of GnRH 72 h after CIDR removal. All animals were housed throughout the treatment periods in a single grass lot with access to water and free choice hay and mineral supplement to minimize environmental and nutritional influences.
3.3.3 Performance characterization

Five d prior to initiation of treatments BCS were assessed; subsequent BCS were taken 5 d prior to initiation of each repetition of the Latin square. Body condition scores were the average of scores assessed by the same two trained investigators at each time point. Final BCS for each repetition was the equivalent to the BCS prior to starting the subsequent repetition. Final BCS was taken on d 16 of the final repetition.

3.3.4 Experimental design

The experimental design for the 3x3 Latin square is illustrated in Figure 3.1. On d 0 a CIDR impregnated with 1.38 grams of progesterone (CIDR; Zoetis Animal Health, New York, NY) was placed in all cows. In addition to CIDR insertion on d 0, animals within treatment G1-2PG (n = 5) received 100 mg GnRH (Factrel; Zoetis Animal Health, New York, NY). After 5 d (d 5), all cows had CIDR removed, and an Estrotect™ heat detection aid (Rockway Inc., Spring Valley, WI) was placed on the tail head of each animal. Also, based upon treatment, animals received either two concurrent 25 mg doses (G1-2PG and NoG1-2PG) or a single 25 mg dose (NoG1-1PG) of PGF$_{2\alpha}$ (Lutalyse, Zoetis Animal Health, New York, NY). Animals were observed for 30 min at 0630 h and 1700 h for behavioral estrus by trained personnel for 72 h following CIDR removal. Estrus detection aid activity was scored (1 = patch completely activated or > 50% activated; 2 = patch partially activated or between 25 and 50% activated with obvious signs of mounting; 3 = no signs of mounting, patch not activated or less than 25 %), and aid was removed at the time of behavioral estrus (aid score < 2 and visual confirmation...
of behavioral estrus) if observed. The remaining animals had estrus detection aid activity recorded and aid was removed 3 d following CIDR removal and concurrent PGF$_{2a}$ administration (d 8). In addition, all animals received 100 mg GnRH on d 8 (72 h after CIDR removal and PGF$_{2a}$ administration). Repetitions 2 and 3 of the Latin square started on d 21, or 13 d following G2 of the preceding repetition.

3.3.5 Ovarian characterization

All animals were subjected to transrectal ultrasonography (Ibex™ Portable Ultrasound, variable MHz linear array transducer, E.I. Medical Imaging, Loveland, CO) for characterization of ovarian structures and ovulatory follicular wave. Ultrasound examinations were performed at approximately 0800 h by the same investigator throughout the experiment on d 0, 5, 8, 10, and 15. All antral follicles ≥ 7 mm in diameter had size and location recorded by drawing representative sketches of each ovary. All recorded follicles were measured using the caliper function of the ultrasound, and diameter of a given follicle was determined via the average of the greatest cross-sectional perpendicular measurements. Dominant follicle was designated as the largest growing follicle of the cohort, and the secondary follicle was the second largest growing follicle within the same follicle cohort. Response to GnRH or resetting of the follicular wave was determined by the ovulation, loss of, or consistent decrease in size of the dominant follicle that existed at initiation of treatment.

Corpora lutea locations were recorded in the same manner previously described for ovarian follicles. Total CL volume was calculated using the caliper function of the
ultrasound to determine cross-sectional perpendicular measurements and a formula for rotary ellipsoid $V = \frac{4}{3} \pi ab^2$ (where $a =$ longitudinal axis and $b =$ transverse axis). If a lacuna was present, the volume of the lacuna was calculated via the rotary ellipsoid equation and its area was subtracted from the total CL volume to determine volume of total luteal tissue.

### 3.3.6 Plasma steroid hormone analysis

Blood samples were collected throughout the experiment for evaluation of steroid hormone (estradiol 17-β and progesterone) concentrations. Blood samples were collected on day of CIDR insertion (d 0), day of CIDR removal (d 5), day of G2 administration (d 8), and at three strategic time points to assess luteal tissue growth and function (d 10, d 12, and d 15). Furthermore, an additional blood sample was collected on any animal that displayed behavioral estrus between PGF$_{2\alpha}$ and G2 administration (at time of such behavior was exhibited). Samples were collected via coccygeal venipuncture in 6 mL ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (10.8 mg EDTA; BD Vacutainer™; Becton, Dickinson and Co., Franklin Lakes, NJ), inverted multiple times to mix, and immediately placed in ice. Samples were centrifuged at 1,750 x g for 25 minutes at 4° C, and plasma was recovered into 5 mL polystyrene tubes and frozen at -20° C for subsequent hormone analyses.

Plasma samples obtained at CIDR insertion, G2 administration, and during presumed luteal growth (days 0, 8, 10, 12, and 15 respectively) were analyzed for progesterone concentrations to determine luteal tissue function. Serum concentrations
of progesterone were assayed via radioimmunoassay (RIA) with methods described by Engel et al. (2008). Average inter-assay CV was 15%, and average intra-assay CV was 5.64%. Plasma collected at CIDR removal (d 5), G2 administration (d 8), and behavioral estrus (d 5-8) was analyzed for estradiol 17-β concentrations via RIA at South Dakota State University using the methodology described by Perry and Perry (2008). All estradiol 17-β concentrations were run in a single assay (intra-assay CV of 3.7%). Peak estradiol concentrations were the highest concentration of estradiol from either the plasma samples collected at behavioral estrus or G2 administration.

3.3.7 Statistical analysis

Binomial data (follicular turnover at G1, CL regression, behavioral estrus, and ovulation of the dominant follicle) were analyzed using the GLIMMIX procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). The remaining performance (BCS) and ovarian parameters (dominant follicle size at CIDR removal, estrus detection aid score, dominant and secondary follicle size at ovulation, CL area, and steroid concentrations) were analyzed using the MIXED procedures of SAS 9.3. The final analytical or statistical model included the fixed effect of treatment, and individual animal served as the experimental unit. Steroid hormone concentration was analyzed on an individual basis due to interest in CL reaching functional status following treatment versus the intent of repeated measures.

An ancillary analysis was conducted within the treatment group G1-2PG on whether or not animals responded to G1 administration (fixed effect), individual animal
was the experimental unit. In addition, a second ancillary analysis was performed to evaluate follicular wave reset at time of G1 administration among all treatments, independent of G1 administration, as a fixed effect and individual animals as the experimental unit.

For all variables statistical significance was declared at \( P \)-value \( \leq 0.05 \), and tendencies were identified at a \( P \)-value \( > 0.05 \) and \( \leq 0.10 \). It should be noted that one animal from repetition 1 treatment 2 had a large hematoma on her right ovary, excluding her ovarian parameters from the dataset; however, performance and hormone data remained in the dataset and were included in the final analysis. Furthermore, in the instances of a double ovulation (\( n=2 \)), only data collected from the largest follicle was included in the dataset and final analysis.

3.4 Results

3.4.1 Age and performance characteristics

Per the experimental design of the study, cow BCS and age did not differ at initiation of the treatments (\( P \geq 0.85 \); Table 3.1). In addition, BCS did not differ at any time point during the study; change in BCS during repetition was not different among treatments at any time point (\( P \geq 0.33 \)).

3.4.2 Ovarian parameters

Ovarian parameter data is displayed in Table 3.2. Dominant follicle diameter did not differ among treatments at either CIDR removal or final GnRH administration (\( P \geq 0 \))
The percentage of animals that regressed their CL in response to PGF$_{2\alpha}$ did not differ among treatments ($P = 0.18$). The number of animals displaying behavioral estrus prior to G2 administration, estrus detection aid score, and interval to behavioral estrus did not differ among treatments ($P \geq 0.27$). Ovulation prior to or in response to G2 was not different among treatments ($P > 0.10$). Furthermore, no differences were identified among treatments for CL volume following PGF$_{2\alpha}$ administration ($P = 0.99$).

Ancillary analyses of response to G1 and resetting of follicular wave (data not shown) revealed no differences in follicular diameter, percentage of animals that regressed their CL in response to PGF$_{2\alpha}$, number of animals displaying behavioral estrus prior to G2 administration, estrus detection aid score, interval to behavioral estrus, ovulation response, or CL volume ($P \geq 0.18$).

### 3.4.3 Plasma steroid hormone analysis

Plasma progesterone concentrations did not differ among treatments at CIDR insertion, time of final GnRH administration, or post ovulation ($P \geq 0.34$; Table 3.3). Plasma estradiol concentrations were not different at CIDR removal or G2 ($P \geq 0.59$; Table 3.3). However, peak plasma estradiol concentrations were greater ($P = 0.04$) in NoG1-2PG than NoG1-1PG (3.50 and 2.14 ± 0.37 pg/mL, respectively), with G1-2PG concentrations being intermediate (2.71 ± 0.37 pg/mL). Furthermore, in samples collected at behavioral estrus, plasma estradiol concentrations were greater ($P < 0.01$) in NoG1-2PG than NoG1-1PG (5.85 and 2.93 ± 0.55 pg/mL, respectively), with G1-2PG being intermediate (3.31 ± 0.55 pg/mL).
Plasma progesterone concentrations did not differ among animals within G1-2PG that either did or did not respond to G1 ($P \geq 0.34$; data not shown). However, animals that did respond to G1 had greater ($P < 0.03$) plasma concentrations of progesterone at time of final GnRH administration than animals that did not respond to G1 (1.39 and $0.79 \pm 0.19$ ng/mL, respectively), and the same was true on d 10 (1.70 and $0.74 \pm 0.27$ ng/mL, respectively). Plasma estradiol concentrations were not different at CIDR removal, or at peak concentration ($P \geq 0.34$; data not shown). However, plasma estradiol concentrations were greater on d 8, or at G2 administration, ($P < 0.01$) in animals that responded to G1 than in those that did not respond to G1 (3.10 and $1.31 \pm 0.40$ pg/mL, respectively).

Plasma progesterone concentrations did not differ among animals that reset their follicular wave at time of CIDR insertion or did not reset their follicular wave at CIDR insertion at any time point prior to or at G2 ($P \geq 0.12$; data not shown). However, plasma progesterone differed on d 10 with animals that reset their follicular wave at CIDR insertion having greater ($P = 0.04$) progesterone concentrations than those that did not reset their follicular wave (1.22 and $0.83 \pm 0.19$ ng/mL, respectively; data not shown). Plasma progesterone concentrations did not differ at any time point following d 12, 4 d after CIDR removal ($P \geq 0.77$; data not shown). Plasma estradiol concentrations did not differ at any time point among animals that did and did not reset their follicular wave at CIDR insertion ($P \geq 0.16$; data not shown).
3.5 Discussion

3.5.1 General

Artificial insemination remains as an underutilized technology in beef cattle operations within the United States. According to the National Animal Health Monitoring System (NAHMS, 2009), only 10% of beef herds currently use artificial insemination. Modifications to existing AI protocols (to make them more user-friendly and more cost effective to implement) may allow for increased adoption of AI within the United States beef herd. The objective of this experiment was to evaluate one such modification to assess its potential impact on ovarian follicular dynamics, circulating preovulatory estradiol concentrations and circulating progesterone concentrations in postpartum, non-lactating beef cows.

Although acceptable pregnancy rates can be achieved with the standard 5dCO, modifications to facilitate greater compliance among beef producers (by decreasing handling time or frequency of hormonal injections) may prove advantageous. More specifically, questions have been raised relative to the necessity of G1 and two separate PGF$_{2\alpha}$ doses in 5dCO protocol. Previous work by Kasimanickam et al. (2009) demonstrated that use of a single 25 mg dose of PGF$_{2\alpha}$ in 5dCO in beef cows resulted in decreased FTAI pregnancy results when compared to two 25 mg doses administered 7 h apart. In heifers, however, a single 25 mg dose of PGF$_{2\alpha}$ was adequate to induce luteolysis, and similar pregnancy rates were achieved when compared to heifers receiving two 25 mg doses of PGF$_{2\alpha}$ (Rabaglino et al., 2010; Cruppe et al., 2014; Kasimanickam et al., 2014). It was speculated that heifers had a better response to a
single dose of PGF$_{2\alpha}$ due to increased mg/kg dosing. Yet, Bridges et al. (2012) showed that a single 50 mg dose of PGF$_{2\alpha}$ was adequate to induce luteolysis and achieve statistically similar FTAI pregnancy rates in cows when compared to two 25 mg doses 8 h apart.

While PGF$_{2\alpha}$ requirements have been definitively identified, the requirement of G1 in 5dCO has not been challenged to date. Gonadotropin releasing hormone is theoretically required to synchronize ovulatory follicular wave initiation; however, response rate of beef cows to GnRH is widely variable in both cycling and anestrus beef cows (Geary et al., 2000; Atkins et al., 2010a; Atkins et al. 2010b; Bridges et al., 2014). Failure of beef cows to respond to G1 resulted in reduced TAI pregnancy rates when using the 7-d CO-Synch protocol (Geary et al., 1998) and decreased estradiol and progesterone concentrations in the 7-d CO-Synch + CIDR protocol (Bridges et al., 2014). Failure to respond to G1 in the 7-d CO-Synch + CIDR protocol results in ovulation of smaller, presumably immature, dominant follicles (Atkins et al., 2010a; Atkins et al., 2010b). These smaller follicles are expected to result in decreased pregnancy rates (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005) with increased late embryonic death and fetal loss (Perry et al., 2005). Alternatively, G1 response failure has also resulted in aged dominant follicles with hindered developmental competence and decreased steroidogenesis (Bridges et al., 2008).

The 5dCO, however, was designed to better accommodate cows not responding to G1 by shortening the overall length of the protocol to create younger, more competent oocytes (when compared to the 7-d CO-Synch + CIDR protocol).
Furthermore, the design of 5dCO allows for a lengthened proestrus (compared with the 7-d CO-Synch + CIDR protocol), which leads to increased estradiol concentrations and higher potential pregnancy rates (Bridges et al., 2008; Bridges et al., 2014). However, it remained unknown how animals would be affected within 5dCO when they did not respond to G1. Therefore, multiple studies were designed to determine the effects of G1 within 5dCO.

When G1 was eliminated from 5dCO, no difference in FTAI pregnancy rates was demonstrated in dairy heifers (Lima et al., 2011; Kasimanickam et al., 2014). However, in beef heifers mixed results were demonstrated with either similar (Cruppe et al., 2014) or decreased (Kasimanickam et al., 2014) pregnancy rates when G1 was eliminated. When response to GnRH was studied in beef cows, Dias et al. (2014) demonstrated that lack of response to G1 resulted in increased estrus response and FTAI pregnancy rates when compared to females that responded to G1. Interestingly, dominant follicle size at FTAI and response to the second GnRH injection was not affected by response to G1 (Dias et al., 2014). These results raised speculation as to the requirement of G1 within 5dCO in mature beef cows.

### 3.5.2 Ovarian parameters

Regardless of treatment in this study, no differences were identified among ovarian parameters. Bridges et al. (2014) speculated that animals responding to GnRH within 5dCO may have accelerated follicular growth rates when progesterone is removed. The increased growth rate should theoretically result in increased estradiol
concentrations when compared to animals not responding and potentially having persistent, over-matured follicles. In the current study, G1 administration was expected to result in increased follicle turnover and subsequent increased follicular growth when compared with treatments lacking G1. Interestingly, these differences were not identified and G1 administration did not result in follicular dynamic changes as expected by the Bridges et al. (2014) hypothesis. Furthermore, as would be expected, when no differences in follicular growth rates were identified among treatments, no differences in behavioral estrus were identified among treatments.

Similar to Dias et al. (2014), no differences were identified in the present study in dominant follicle diameter at time of G2 administration or in ovulation rates either prior to or in response to G2 administration when treatment group G1-2PG was isolated and the effects of response to G1 were analyzed. However, unlike Dias et al. (2014), no differences were found for incidence of behavioral estrus, estrus detection aid score, or interval from CIDR removal to behavioral estrus within the same analysis. As previously stated, the differences in estrous response is hypothesized to be due to the accelerated growth rate and increased estradiol production of smaller diameter ovarian follicles after removal of progesterone due to response to G1 (Bridges et al., 2014). However, data from Dias et al. (2014) indicated the opposite, with animals not responding to GnRH displaying greater prevalence of behavioral estrus and decreased time from CIDR removal to behavioral estrus. This finding of Dias et al. (2014) is also in contrast to the results from this study where no differences were identified in any of those parameters. However, although estrus response rate remains open for debate, based upon the
results from this study and from Dias et al. (2014) it is evident that, on average, G1 response does not affect ovulatory follicle characteristics.

Additional data analysis by whether or not cows reset their follicular wave at CIDR insertion revealed similar results to the first ancillary analysis; no differences among any ovarian parameters. These findings are not surprising considering that, even when G1 is used to initiate a new follicular wave, no differences in dominant follicular characteristics are found. The findings within this ancillary analysis further support the theory that 5dCO was designed to accommodate animals that either maintained their dominant follicle at G1 administration or initiated a new follicular wave (Bridges et al., 2008).

3.5.3 Plasma steroid hormone analysis

It is interesting to note that while there were no differences in dominant follicle size, behavioral estrus or estrus detection aid score, peak estradiol concentrations were different among the treatment groups. Because there were no differences among treatments in ovarian follicular dynamics, it is difficult to identify the causative agent for the increased estradiol concentrations within the NoG1-2PG treatment group. A possible explanation for the difference in estradiol concentrations could be due to a numerically larger (but non-significant) proportion of cows within the NoG1-2PG group exhibiting behavioral estrus than either G1-2PG or NoG1-1PG treatments.

Similarly, when data were analyzed based upon response to G1, estradiol concentrations were higher at G2 administration in animals responding to G1. This
finding is consistent with Bridges et al. (2014) theory that animals responding to G1 would have increased estradiol concentrations due to increased follicular growth. However, the follicular data from the current study do not support this claim as no differences in follicular growth rates were identified. What is of interest is that, although not significant, a larger proportion of females not responding to G1 displayed behavioral estrus and had a decreased interval from CIDR removal to behavioral estrus. This finding contrasts Dias et al. (2014) where animals that did not respond to G1 had an increased proportion of females displaying behavioral estrus with a shortened interval from CIDR removal to behavioral estrus. Thus, the Dias et al. (2014) data would suggest that animals not responding to G1 would have increased estradiol concentrations. In both scenarios, however, the lack of differences in follicular dynamics only allows for speculation as to why differences in estradiol concentrations were identified. Within this study, we hypothesize that animals that did not respond to G1 achieved peak estradiol concentration prior to G2 administration. Therefore, circulatory estradiol concentration was diminished at time of G2 administration.

Similar to the result seen by Bridges et al. (2014) plasma progesterone concentrations did not differ among treatments. Interestingly, when data were further analyzed based upon both response to G1 and initiation of a new follicular wave, animals that started a new wave at G1 had increased progesterone concentrations for up to 2 d following G2. Although, initial progesterone concentrations were different up to 2 d following G2, all treatments reached concentrations at or above the 3.0 ng/ml threshold reported to be the normal range of females 6 d following ovulation (Adams et
al., 2008). Although no animals in the current study underwent FTAI, further research is warranted to determine if elevated progesterone concentrations following G2 compromise pregnancy rates.

3.5.4 Conclusion

The removal of G1 from 5dCO had no detrimental effects on ovarian function in mature beef cows in this study. Hormone profiles in cows not receiving G1 warrant further research. The factor(s) driving increased estradiol concentration in cows from the NoG1-2PG treatment need to be elucidated. Furthermore, the need remains to repeat these treatments and collect fertility and pregnancy data to determine if greater estradiol concentrations impact fertility when G1 is eliminated from the 5-d CO-Synch + CIDR protocol.
Table 3.1. Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR\(^1\) protocol on age and body condition scores in mature beef cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^2)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
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<td></td>
<td>G1-2PG</td>
<td>NoG1-2PG</td>
<td>NoG1-1PG</td>
<td>SEM(^3)</td>
<td>P-Value</td>
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<td>4.79</td>
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<td></td>
</tr>
<tr>
<td>BCS(^4), final</td>
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<td>4.90</td>
<td>4.91</td>
<td>0.07</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>BCS(^4), change</td>
<td>0.14</td>
<td>0.01</td>
<td>0.12</td>
<td>0.07</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Controlled internal drug release

\(^2\) Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2\alpha}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2\alpha}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insert and a single 25 mg dose of PGF\(_{2\alpha}\) at CIDR removal (NoG1-1PG).

\(^3\) n = 45

\(^4\) BCS on scale of 1 to 9 (1= emaciated, 9= obese; Wagner et al, 1988).
Table 3.2. Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR\(^1\) protocol on ovarian parameters in mature beef cows

<table>
<thead>
<tr>
<th>Item</th>
<th>G1-2PG</th>
<th>NoG1-2PG</th>
<th>NoG1-1PG</th>
<th>SEM(^3)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular diameter at CIDR removal, mm</td>
<td>11.2</td>
<td>11.2</td>
<td>10.5</td>
<td>0.62</td>
<td>0.61</td>
</tr>
<tr>
<td>CL regression after PGF(_{2α}), %</td>
<td>57.1</td>
<td>40.0</td>
<td>40.0</td>
<td>0.61</td>
<td>0.18</td>
</tr>
<tr>
<td>Estrus prior to G2(^4), %</td>
<td>33.3</td>
<td>64.3</td>
<td>46.7</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Interval from CIDR removal to behavioral estrus, h</td>
<td>58.0</td>
<td>65.2</td>
<td>65.1</td>
<td>0.18</td>
<td>0.37</td>
</tr>
<tr>
<td>Estrus detection aid score(^5)</td>
<td>2.33</td>
<td>1.93</td>
<td>2.13</td>
<td>0.24</td>
<td>0.52</td>
</tr>
<tr>
<td>Follicular diameter at G2, mm</td>
<td>13.9</td>
<td>14.3</td>
<td>14.5</td>
<td>0.54</td>
<td>0.74</td>
</tr>
<tr>
<td>CL volume, mm(^3)</td>
<td>5.66</td>
<td>5.78</td>
<td>5.75</td>
<td>0.74</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(^1\) Controlled internal drug release

\(^2\) Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2α}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent, 25 mg doses of PGF\(_{2α}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single, 25 mg dose of PGF\(_{2α}\) at CIDR removal (NoG1-1PG).

\(^3\) n = 44

\(^4\) Second GnRH administration that occurs at theoretical fixed timed artificial insemination.

\(^5\) Estrus Detection Aids were scored as follows: 1 = patch completely activated or > 50% activated; 2 = patch partially activated or between 25 and 50% activated with obvious signs of mounting; 3 = no signs of mounting, patch not activated or less than 25% activated
Table 3.3. Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR\(^1\) protocol on steroid hormone concentrations in mature beef cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^2)</th>
<th>SEM(^3)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1-2PG</td>
<td>NoG1-2PG</td>
<td>NoG1-1PG</td>
</tr>
<tr>
<td><strong>Progesterone, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>4.54</td>
<td>4.56</td>
<td>4.10</td>
</tr>
<tr>
<td>Day 8</td>
<td>1.03</td>
<td>1.39</td>
<td>0.96</td>
</tr>
<tr>
<td>Day 10</td>
<td>1.12</td>
<td>1.34</td>
<td>0.85</td>
</tr>
<tr>
<td>Day 12</td>
<td>1.40</td>
<td>2.01</td>
<td>1.54</td>
</tr>
<tr>
<td>Day 15</td>
<td>2.98</td>
<td>3.46</td>
<td>3.38</td>
</tr>
<tr>
<td><strong>Estradiol, pg/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>0.55</td>
<td>0.59</td>
<td>0.51</td>
</tr>
<tr>
<td>Day 8</td>
<td>2.03</td>
<td>2.22</td>
<td>1.71</td>
</tr>
<tr>
<td>Behavioral estrus(^4)</td>
<td>3.31(^{ab})</td>
<td>5.85(^a)</td>
<td>2.93(^b)</td>
</tr>
<tr>
<td>Peak E2(^5)</td>
<td>2.71(^{ab})</td>
<td>3.50(^a)</td>
<td>2.14(^b)</td>
</tr>
</tbody>
</table>

1 Controlled internal drug release
2 Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2α}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2α}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF\(_{2α}\) at CIDR removal (NoG1-1PG).
3 \(n = 45\)
4 Time point between d 5 and d 8 when animals displayed behavioral estrus \((n = 13)\).
5 Peak estradiol concentrations were determined as the highest concentration of estradiol from either the plasma samples collected at behavioral estrus or second GnRH administration.
6 Treatments with similar superscripts do not differ.
Figure 3.1. Experimental protocol describing GnRH and PGF$_{2\alpha}$ treatments and data collection in mature beef cows for analysis of ovarian parameters and steroid hormone concentrations in the 5-d CO-Synch + Controlled Internal Drug Release (CIDR) protocol.

1 Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent, 25 mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single, 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG).

2 Administration of 100 mg GnRH

3 No administration of GnRH

4 Two concurrent 25 mg doses of PGF$_{2\alpha}$

5 One single 25 mg dose of PGF$_{2\alpha}$
CHAPTER 4

EFFECT OF GNRH ADMINISTRATION AT THE ONSET OF THE 5 DAY CO-SYNCH + CIDR PROTOCOL IN SUCKLED BEEF COWS

4.1 Abstract

The objective of this experiment was to evaluate whether or not GnRH administration at controlled internal drug release (CIDR) insertion is needed in the 5-d CO-Synch + CIDR protocol (5dCO). Postpartum suckled beef cows (n = 2159) from 11 herds at 5 universities were assigned by age, body condition score (BCS), and days postpartum to receive either: 1) standard 5dCO hormone administration including 100 µg of GnRH at CIDR insertion and two concurrent 25-mg doses of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25-mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25-mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG). Estrus response between PGF$_{2\alpha}$ administration and fixed timed-AI (FTAI) was determined using estrus detection aids. All cows underwent FTAI 72 h after CIDR removal, concurrent with administration of 100 µg of GnRH. Estrous cyclicity prior to synchronization of ovulation was determined using a combination of 2 blood samples collected 10 d apart in conjunction with estrus detection aids applied approximately 24 d prior to CIDR insertion. Transrectal ultrasonography was performed on a subset of cows (n = 130) in two herds at two locations at both CIDR insertion and removal to record ovarian structures, as well as 31 to 42 d after FTAI to test for pregnancy. Data were analyzed using the MIXED and
GLIMMIX procedures of SAS for continuous and binary response variables, respectively. Herd nested within university was included as a random effect. Total number of ovarian follicles and diameter of the two largest follicles at CIDR insertion were not different \((P \geq 0.34)\). However, the largest follicle at CIDR removal had greater \((P = 0.02)\) diameter in NoG1-2PG than G1-2PG and NoG1-1PG \((13.2, 11.5, \text{and } 12.1 \pm 0.5 \text{ mm, respectively})\). Though estrous response was not different \((P = 0.99)\) prior to FTAI, detection aid activation was more advanced \((P = 0.01)\) in NoG1-1PG than G1-2PG and NoG1-2PG. Pregnancy to FTAI did not differ \((P = 0.66)\) among G1-2PG \((55.4 \%)\), NoG1-2PG \((52.8 \%)\), and NoG1-1PG \((50.5 \%)\) treatments. Cows exhibiting estrus prior to FTAI had greater \((P < 0.001)\) FTAI pregnancy rates \((58.1 \%)\) than those not exhibiting estrus \((39.3 \%)\), and cows that were cyclic at synchronization initiation had greater \((P < 0.001)\) FTAI pregnancy rates \((53.6 \%)\) than non-cyclic cows \((37.1 \%)\). In conclusion, FTAI pregnancy rates were not affected by removal of the initial GnRH in the 5-d CO-Synch + CIDR protocol.

### 4.2 Introduction

Follicular waves are synchronized at the start of fixed-timed artificial insemination (FTAI) protocols with the administration of gonadotropin releasing hormone (GnRH), concurrent with insertion of a controlled internal drug release (CIDR) device. However, the response rate of beef cows to a single GnRH injection is widely variable in both cycling and anestrous beef cows (Atkins et al., 2010a; Atkins et al. 2010b; Bridges et al., 2014; Geary et al., 2000). Failure of beef cows to respond to GnRH
has been detrimental to FTAI pregnancy rates (Geary et al., 1998) and led to decreased estradiol and progesterone concentrations in the 7-d CO-Synch +CIDR protocol (Bridges et al., 2014).

The 5-d CO-Synch + CIDR protocol (5dCO) was designed, in part, to theoretically better accommodate cows not responding to the initial GnRH administration (G1). Shortening the length of time from G1 to CIDR removal and concurrent prostaglandin $F_2\alpha$ (PGF$_{2\alpha}$) administration is speculated to allow for a younger, more developmentally competent oocyte to be present at FTAI and to prevent over-maturation of ovulatory follicles (Bridges et al., 2008; Bridges et al., 2014). When Dias et al. (2014) studied beef cows’ response to G1 in 5dCO, failure of response to G1 administration increased estrous response and FTAI pregnancy rates when compared with females that did respond to G1. However, Cruppe et al. (2014) observed similar FTAI pregnancy rates in beef heifers regardless of G1 administration. These conflicting reports have led to speculation concerning the necessity to administer G1 as part of 5dCO. Accordingly, the objective of this study was to evaluate whether or not G1 administration at CIDR insertion in the 5-d CO-Synch + CIDR protocol is necessary for lactating beef cows. We hypothesized that removal of G1 from the 5dCO would have no detrimental effects upon FTAI pregnancy rates in beef cows.
4.3 Materials and Methods

4.3.1 General

All protocols, procedures, and animal handling were conducted in a manner approved by the Institutional Animal Care and Use Committee of each of five collaborating universities. Research involved in 11 herds across 5 states and was conducted from May 2015 to December 2015.

4.3.2 Animals and treatments

Non-pregnant, postpartum, lactating mature beef cows at 11 locations across 5 universities \( [n = 2159; \text{age} = 5.18 \pm 2.8 \text{ yr}; \text{body condition score (BCS)} = 5.47 \pm 0.76 (1 = \text{emaciated, } 9 = \text{obese, Wagner et al., 1988})] \) were used to study the effects of G1 removal in the 5-d CO-Synch + CIDR protocol on FTAI pregnancy rates. Animals were stratified and allocated within herd by age, BCS, and days postpartum and received either: 1) standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25-mg doses of PGF\(_{2\alpha}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25-mg doses of PGF\(_{2\alpha}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25-mg dose of PGF\(_{2\alpha}\) at CIDR removal (NoG1-1PG). All cows were bred via FTAI at 72 h after CIDR removal with concurrent GnRH (100 µg) administration.
4.3.3 Experimental design

The experimental design is illustrated in Figure 4.1. Estrous detection aids (Estrotec™ heat detection aid; Rockway Inc., Spring Valley, WI) were placed on the tail head of each animal 32 d (d -32) prior to FTAI. Body condition scores were collected on animals 18 d (d -18) prior to FTAI. In addition, a coccygeal blood sample was taken in a subset of cows (n = 280) across 3 locations on d -18 to determine cyclicity. On d -8, estrous detection aids were removed and scores were assigned (1 = patch completely activated or > 50% activated; 2 = patch partially activated or between 25 and 50% activated with obvious signs of mounting; 3 = no signs of mounting, patch not activated or less than 25% activated) and cyclicity was determined (estrus detection aid score ≤ 2).

An intravaginal device impregnated with 1.38 grams of progesterone (CIDR; Zoetis Animal Health, New York, NY) was placed in all cows (d -8), and the same subset of animals previously mentioned had a second coccygeal blood sample taken.

Furthermore, animals within treatment G1-2PG were administered 100 µg GnRH (Factrel; Zoetis Animal Health, New York, NY) concurrent with CIDR insertion. After 5 d (d -3), all CIDRs were removed and, based upon treatment, animals received either two concurrent 25-mg doses (G1-2PG and NoG1-2PG) or a single 25-mg dose (NoG1-1PG) of PGF$_2$α (Lutalyse, Zoetis Animal Health, New York, NY). New estrous detection aids were placed on the tailhead of each animal to monitor estrus activity for 72 h prior to FTAI. Fixed timed-AI occurred approximately 72 h (d 0) following CIDR removal and PGF$_2$α administration. All animals received 100 µg GnRH concurrent with FTAI. Estrous detection aid score was recorded and signs of behavioral estrus determined (estrous
detection aid score ≤ 2) concurrent with FTAI. Pregnancy testing was performed via transrectal ultrasonography (Ibex™ Portable Ultrasound, variable MHz linear array transducer, E.I. Medical Imaging, Loveland, CO) at 32 to 42 d after FTAI. At 42 d following the end of the breeding season, a second pregnancy test was performed to determine final end of season pregnancy rate. Pregnancy was confirmed when a viable fetus was identified.

4.3.4 Plasma steroid hormone analysis

Coccygeal blood samples were collected on d -18 and d -8 on a subset of animals at three locations (n = 280) for assessment of progesterone concentration and pre-synchronization cyclicity status. Samples were collected in 6 mL ethylenediaminetetraacetic (EDTA) vacutainer tubes (10.8 mg EDTA; BD Vacutainer™; Becton, Dickinson and Co., Franklin Lakes, NJ), inverted multiple times, and immediately placed in ice. Samples were centrifuged at 1,750 x g for 25 minutes at 4° C and plasma was recovered into 5 mL polystyrene tubes and frozen at -20° C for subsequent analysis.

Plasma samples were analyzed for progesterone concentrations to determine luteal tissue function and thus cyclicity status. Plasma concentrations of progesterone were analyzed with radioimmunoassay (RIA) via methods described by Engel et al. (2008). The average inter-assay CV was 15%, and the intra-assay CV was 5.64%. Animals were determined to be cyclic if a single sample contained a progesterone concentration ≥ 1.0 ng/ml.
4.3.5 Ovarian characterization

A subset of animals (n = 130) at two herds, across two locations underwent transrectal ultrasonography for characterization of ovarian structures during the ovulatory follicular wave. Ultrasound examinations were performed by trained investigators at each location on days -8 and -3. Diameter and location of follicles ≥ 7 mm in diameter were recorded by drawing representative sketches of each ovary. These follicles were measured using the caliper function of the ultrasound, and diameter was determined as the average of the greatest cross-sectional perpendicular measurements. The largest follicle of the cohort was declared as the dominant follicle, and the secondary follicle was the second largest growing follicle within the same cohort. Corpus luteum (CL) size and location were recorded in the same manner as antral follicles. Response to GnRH (resetting of the follicular wave) was determined by the presence of a new CL on d -3 of the protocol (time of CIDR removal).

4.3.6 Statistical analysis

Binomial response variables (cyclicity, follicular turnover at G1, behavioral estrus, FTAl pregnancy rate, end of breeding season pregnancy rate and within treatment [response to G1]) were analyzed using the GLIMMIX procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). The remaining performance (BCS) and ovarian parameters (dominant and secondary follicle size at CIDR insertion, estrous detection aid score, dominant and secondary follicle size at CIDR removal, and steroid concentrations) were analyzed using the MIXED procedures of SAS 9.3. The final model included the fixed
effect of treatment and herd nested within university as a random effect; individual animal served as the experimental unit.

A first ancillary analysis of all variables was performed to analyze response to G1 administration within the G1-2PG treatment group. Response to G1 was used as a fixed effect, with herd nested within university as a random effect; individual animal served as the experimental unit. A second ancillary analysis was performed to evaluate follicular wave reset at time of CIDR insertion among all treatments, independent of G1 administration. Follicular wave turnover at d -8 was used as a fixed effect, with herd nested within university as a random effect; individual animals served as the experimental unit.

Significance was declared at $P$-value $\leq 0.05$, and tendencies were identified at a $P$-value $> 0.05$ and $\leq 0.10$ for all variables. Cyclicity status prior to synchronization protocol implementation from one university (multiple herds) was excluded from cyclicity data due to lack of coccygeal blood samples or estrous detection aid scores. Furthermore, any animal that lost estrous detection aid patch prior to being scored or did not have coccygeal blood samples taken was excluded from the corresponding cyclicity or behavioral estrus data sets ($n = 589$).

4.4 Results

4.4.1 Age and performance characteristics

Per the experimental design of the study, cow age, BCS, and days postpartum did not differ at initiation of the treatments ($P \geq 0.48$; Table 4.1). Furthermore, ancillary
analysis yielded no differences in age, BCS, and days postpartum between treatment
groups studied ($P \geq 0.20$; data not shown).

### 4.4.2 Ovarian parameters

The percentage of animals that displayed behavioral estrus prior to treatment
initiation (d -8; Fig. 4.1) did not differ among treatment groups ($P \geq 0.85$; Table 4.1).
Diameter of the two largest ovarian follicles did not differ across treatments at CIDR
insertion ($P \geq 0.33$; Table 4.3). In addition, diameter of the second largest follicle was
similar among treatments ($P \geq 0.16$; Table 4.3) at CIDR removal (d -3; Fig. 4.1). However,
the diameter of the largest follicle on d -3 (CIDR removal) was greater ($P = 0.02$; Table
4.3) in NoG1-2PG than in both G1-2PG and NoG1-1PG (13.2, 11.5, and 12.1 ± 0.5 mm,
respectively). The percentage of animals displaying behavioral estrus prior to FTAI did
not differ among treatments ($P = 1.00$; Table 4.2). Intriguingly, estrous detection aid
score (activation) was more advanced (lower; $P = 0.01$; Table 4.2) in NoG1-1PG than in
G1-2PG and NoG1-2PG (1.49, 2.13, and 1.89 ± 0.32, respectively). Yet, FTAI pregnancy
rate were not different ($P = 0.66$; Table 4.2) among treatment groups.

Ancillary analysis of response to G1 in a subset of animals revealed no
differences in estrous detection aid scores or animals that displayed behavioral estrus
prior to treatment ($P \geq 0.35$; data not shown). Diameter of the two largest ovarian
follicles on d -8 was not different between response groups ($P \geq 0.30$; data not shown).
However, the diameter of the dominant follicle and second largest follicle at CIDR
removal (d-3) was greater ($P \leq 0.01$) in animals that did not respond to G1 than in those
that did (12.9 vs 9.6 ± 0.6 mm; and 9.5 vs 7.9 ± 0.5 mm, respectively; data not shown).

Similarly, animals failing to respond to G1 tended ($P = 0.10$) to have an increased percentage of animals displaying behavioral estrus prior to FTAI when compared with animals that did respond (66.7 % vs 40.9 %, respectively; data not shown).

Further ancillary analysis of follicular wave reset at d -8 showed no differences ($P \geq 0.18$) in animals that displayed behavioral estrus prior to treatment or diameter of the two largest follicles at CIDR insertion (data not shown). However, the diameter of the dominant follicle and second largest follicles at CIDR removal (d-3) were greater ($P \leq 0.01$) in animals that did not reset their follicular wave at d -8 than in those that did (13.3 vs 10.4 ± 0.4 mm, respectively; and 10.0 vs 8.3 ± 0.5 mm, respectively; data not shown). Furthermore, animals that did not reset the follicular wave at CIDR insertion had lower (more advanced) estrous detection aid scores ($P \leq 0.01$; 1.4 vs 1.9 ± 0.3, respectively; data not shown) and an increased ($P = 0.02$) percentage of animals displaying behavioral estrus prior to FTAI (75.8 % vs 52.5 %, respectively; data not shown) than animals that did reset their follicular wave.

### 4.4.3 Fixed timed artificial insemination pregnancy results

Fixed time artificial insemination pregnancy rates were affected by cyclicity prior to treatment ($P < 0.001$) and by signs of behavioral estrus prior to FTAI ($P < 0.001$).

Animals that were cyclic at initiation of the synchronization protocol had greater FTAI pregnancy rates than non-cyclic animals (53.6 % vs 37.1 %, respectively; data not shown). In addition, animals that displayed signs of behavioral estrus prior to FTAI had
greater FTAI pregnancy rates than those animals that did not (58.1 % vs 39.3 %, respectively; data not shown). However, pregnancy to FTAI did not differ ($P = 0.66$; Table 4.2) among G1-2PG (55.4 %), NoG1-2PG (52.8 %), and NoG1-1PG (50.5 %) treatments. Furthermore, no differences were identified in FTAI pregnancy rates upon further ancillary analysis of G1 response ($P = 0.35$; data not shown) or follicular wave reset ($P = 0.65$; data not shown).

**4.5 Discussion**

**4.5.1 General**

Artificial insemination of beef cattle within the United States remains an underutilized technology. Less than 10% of beef herds in the United States currently use artificial insemination as a way to increase the genetic value of the offspring produced (NAHMS, 2009). In an effort to increase utilization rates, synchronization protocols have been developed to make artificial insemination more labor efficient. Specifically, FTAI protocols that synchronize ovulation allow mass insemination of beef females. The 5dCO is such a synchronization of ovulation protocol that has proven efficacious and produces acceptable pregnancy rates following FTAI in beef and dairy cattle (Bridges et al., 2008; Santos et al., 2010; Wilson et al., 2007). The standard 5dCO involves insertion of a CIDR and concurrent administration of an initial dose of GnRH followed approximately 5 d later by CIDR removal and administration of two doses of PGF$_{2\alpha}$ 8 h apart. Fixed timed-AI accompanied by a second dose of GnRH occurs 72 h following removal of CIDR and PGF$_{2\alpha}$ administration (Johnson et al., 2013).
The drive to increase utilization of FTAI protocols has led to research on modifications of the 5dCO to decrease frequency of cow handling, number of hormone administrations, or both. Specifically, administration of two PGF$_{2\alpha}$ doses 8 h apart has been a recent focus. Previously, Kasimanickam et al. (2009) demonstrated that two separate, 25-mg doses administered 7 h apart in beef cows resulted in increased FTAI pregnancy rates when compared to a single 25-mg dose in the 5dCO. However, when the same comparison was made in heifers, a single 25-mg dose of PGF$_{2\alpha}$ was adequate to induce luteolysis and yield similar FTAI pregnancy rates as two separate 25-mg doses of PGF$_{2\alpha}$ (Rabaglino et al., 2010; Cruppe et al., 2014; Kasimanickam et al., 2014). The difference in response rates of a single, 25-mg dose of PGF$_{2\alpha}$ from cows to heifers was speculated to result from differences in mg per kg of BW dosing. This hypothesis was bolstered by Bridges et al. (2012) who demonstrated that two concurrent 25 mg (50 mg total) doses of PGF$_{2\alpha}$ was adequate to induce luteolysis of all CL in beef cows and produce similar FTAI pregnancy rates.

Similar to questions regarding PGF$_{2\alpha}$, the administration of GnRH recently has been challenged within 5dCO. Failure of response to G1 led to detrimental effects upon FTAI pregnancy rates within the 7-d CO-Synch + CIDR protocol (Geary et al., 1998). Animals that failed to respond to G1 ovulated smaller, more immature dominant follicles (Atkins et al., 2010a; Atkins et al., 2010b) and cows exhibited decreased estradiol and progesterone concentrations (Bridges et al., 2014). These smaller dominant follicles impede pregnancy rates (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005) and increase late embryonic death and early fetal loss (Perry et al.,
2005). Furthermore, another effect seen in follicles that do not respond to G1 is increased likelihood to become persistent follicles prior to FTAI (Bridges et al., 2008).

The design of 5dCO decreases the time interval from G1 to FTAI to create a younger more developmentally competent oocyte when compared with the 7-d CO-Synch + CIDR protocol (Bridges et al., 2008; Bridges et al., 2014). However, the consequence of failing to respond to G1 on FTAI pregnancy rates within 5dCO remained unknown. Studies were developed in heifers that tested the elimination of G1 to mimic the effects of G1 response failure within 5dCO. No difference in FTAI pregnancy rates was observed in dairy heifers between animals that received or did not receive G1 (Lima et al., 2011; Kasimanickam et al., 2014). However, when the same hypothesis was tested in beef heifers, contrasting results were reported (Cruppe et al., 2014; Kasimanickam et al., 2014). Similar pregnancy rates were reported among animals receiving or not receiving G1 by Cruppe et al. (2014). Kasimanickam et al. (2014) however, reported decreased pregnancy rates in beef heifers when G1 was eliminated from the 5dCO.

To date, only one study has analyzed failure of response to G1 within the 5dCO in beef cows. Dias et al. (2014) demonstrated that failure of beef cows to respond to G1, or the lack of a new CL at CIDR removal within the 5dCO, resulted in increased estrous response and FTAI pregnancy rates when compared with females that responded to G1. Intriguingly, dominant follicle size at FTAI and response to G2 was not affected by response to G1 (Dias et al., 2014). Therefore, failure of beef animals to respond to G1 caused either similar or an increase in FTAI pregnancy rates (Cruppe et al., 2014; Dias et al., 2014). The actual need for G1 has been questioned.
The elimination of G1 from the standard 5dCO could prove advantageous. Not only would removing G1 at the onset of the protocol reduce the amount of pituitary hormone analog required, but also it would eliminate the second 25-mg dose of PGF$_{2\alpha}$, as there would not be a 5-d old accessory CL to lyse (Bridges et al., 2008; Kasimanickam et al., 2009). Therefore, removal of G1 would also allow for a more user friendly and lower cost protocol that could potentially facilitate increased utilization of artificial insemination technology within the beef sector.

4.5.2 Ovarian parameters

In the present study no differences were identified in cyclicity status or ovarian parameters at CIDR insertion among treatment groups. However, upon CIDR removal, diameter of the largest ovarian follicle differed among treatments with NoG1-2PG having a larger dominant follicle than either G1-2PG or NoG1-1PG. Oddly, these data did not correlate to increased estrous aid activation or an increased percentage of females displaying estrous behavior. Instead, estrous detection aid score indicated increased activation in the NoG1-1PG treatment group versus both the G1-2PG and NoG1-2PG treatment groups. This is in contrast to data published by Perry et al. (2005) and Jinks et al. (2012) that indicated larger dominant follicles, upon induced ovulation, have increased estradiol concentrations and should lead to increased behavioral estrus. Thus, it is speculated that the larger dominant follicles within the NoG1-2PG treatment group may have become persistent with diminished steroidogenesis, while follicles of NoG1-1PG cows potentially had more developmentally competent oocytes with potentially
increased growth and steroidogenesis during the proestrus period (Bridges et al., 2008; Bridges et al., 2014).

Interestingly, initial ancillary analysis of response to G1 within the G1-2PG treatment group showed similar results to those noted in the full dataset. Animals not responding to G1 had larger dominant follicles and larger secondary follicles at time of CIDR removal than those animals that did respond to G1. However, in this comparison the increased follicular size did correspond to a tendency for animals not responding to G1 to have increased behavioral estrus prior to G2 administration. This finding is similar to Dias et al. (2014) who reported increased behavioral estrus in animals failing to respond to G1 when compared with animals that did respond. Of interest, however, is that this response is not observed when animals are not administered G1 (i.e., in NoG1-2PG and NoG1-1PG treatments). This highlights that some animals within the NoG1-2PG and NoG1-1PG groups may potentially have benefitted from G1 administration.

Similar to the data just discussed, when the effect of follicular wave reset at CIDR insertion was analyzed, increased follicular diameter was seen in the two largest follicles at CIDR removal of animals that did not reset their follicular wave compared to animals that did reset their wave. Furthermore, the animals that did not reset their follicular wave at CIDR insertion had more advanced estrous detection aid scores with an increased percentage of females also displaying behavioral estrus. The results for the two ancillary analyses presented within this section are more in line with what would be expected from data reported by Perry et al. (2005) and Jinks et al. (2012). Although suspected advantages in ovulatory follicle size are shown from failure to reset the
follicular wave, the ability to identify these animals in an efficient and economical manner at protocol initiation remains elusive. Therefore, further research is warranted to develop an affordable chute-side test to determine G1 response or potential for follicular wave turnover.

4.5.3 Pregnancy results

Considering that the 5-d CO-Synch + CIDR protocol was designed to accommodate animals that either maintained their dominant follicle or initiated a new follicular wave at G1 administration (Bridges et al., 2008), it is not surprising to some that no differences in pregnancy rates were identified among treatment groups in the present study. However, slight numerical differences were identified, likely due to differences in stage of estrous cycle of females within each treatment group. It is probable that a small number of animals in the NoG1-2PG and NoG1-1PG treatment groups would have benefitted from G1 administration or, in the case of the NoG1-1PG treatment group, two concurrent doses of PGF$_{2\alpha}$. Differences in follicular dynamics were identified in the subset of cows, but this did not affect FTAI pregnancy rates. Although NoG1-2PG had larger dominant follicles at CIDR removal and NoG1-1PG had more advanced estrous detection aid scores, neither proved advantageous when pregnancy rates were compared. Between these findings and the numerical differences in pregnancy rates, we are left to speculate if timing of estrus was affected by treatment. The timing of FTAI within this 5dCO was designed to align with animals that responded to G1. When G1 was not administered, we speculate that these animals may have
potentially entered behavioral estrus sooner due to larger and more estrogenic follicles at CIDR removal. Thus, a potential benefit may be had from a shortened window from CIDR removal to FTAI within animals that did not receive G1. Further research is warranted in this area.

In addition to altering timing of FTAI, a second potential for disconnect, and failure of increased estrus response in the NoG1-1PG to result in advantageous FTAI pregnancy rates, may lie in the relatively small subset of cows (n = 130) that were sampled for ovarian parameter assessment. The animals sampled were only within two locations, which makes it difficult to extrapolate across the entire data set. Thus, a need remains to investigate timing of estrus within treatment groups not receiving G1, and an expansion in the sample size of animals analyzed for ovarian dynamics.

The same disconnect noted in regard to follicle diameter and estrus correlation was also identified within the ancillary analyses. The results are in contrast to widely held knowledge that increases in behavioral estrus rates will lead to increased pregnancy rates. This fact was proven within our own data set, as FTAI pregnancy rates were affected by signs of behavioral estrus. Once again, the disconnect may stem from different timing of estrus between groups or the relatively small sample size studied for ovarian parameters within this study.

4.5.4 Conclusion

The removal of G1 from the 5-d CO-Synch + CIDR protocol has no detrimental effects on pregnancy rates in mature lactating beef cows. However, differences in
ovarian follicular dynamics warrant further research in both scale and intensity to
determine the factors driving disconnect between follicle diameter, behavioral estrous
response and FTAl pregnancy rates. Furthermore, the development of a practical chute-
side test to determine follicular wave stage and expected response to G1 may prove
beneficial to synchronization of ovulation protocols that potentially increase FTAl
pregnancy rates.
Table 4.1. Production characteristics of lactating beef cows stratified by treatment

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment1</th>
<th></th>
<th></th>
<th>SEM2</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1-2PG</td>
<td>NoG1-2PG</td>
<td>NoG1-1PG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>4.71</td>
<td>4.71</td>
<td>4.76</td>
<td>0.32</td>
<td>0.93</td>
</tr>
<tr>
<td>BCS3</td>
<td>5.51</td>
<td>5.55</td>
<td>5.56</td>
<td>0.21</td>
<td>0.48</td>
</tr>
<tr>
<td>Days Postpartum, d</td>
<td>71.3</td>
<td>71.9</td>
<td>71.4</td>
<td>2.61</td>
<td>0.88</td>
</tr>
<tr>
<td>Cyclic, %</td>
<td>87.2</td>
<td>85.1</td>
<td>86.3</td>
<td>0.85</td>
<td></td>
</tr>
</tbody>
</table>

1 Treatments included either 1) the standard 5-d CO-Synch + controlled internal drug release (CIDR) protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG).

2 n = 2159

3 BCS on scale of 1 to 9 (1= emaciated, 9= obese; Wagner et al, 1988).
Table 4.2. Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR\(^1\) protocol on activation of estrus detection aid, behavioral estrus and FTAI\(^2\) pregnancy rates in lactating beef cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^2)</th>
<th>SEM(^3)</th>
<th>P-Value(^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1-2PG</td>
<td>NoG1-2PG</td>
<td>NoG1-1PG</td>
</tr>
<tr>
<td>Patch score(^4) at FTAI(^5)</td>
<td>2.13(^b)</td>
<td>1.89(^b)</td>
<td>1.49(^a)</td>
</tr>
<tr>
<td>Behavioral estrus, %</td>
<td>73.8</td>
<td>73.8</td>
<td>71.7</td>
</tr>
<tr>
<td>FTAI pregnancy status, %</td>
<td>55.4</td>
<td>52.8</td>
<td>50.5</td>
</tr>
</tbody>
</table>

\(^1\) Controlled internal drug release
\(^2\) Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2\alpha}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2\alpha}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF\(_{2\alpha}\) at CIDR removal (NoG1-1PG).
\(^3\) n = 2159
\(^4\) Scored on a scale of 1 to 3 (1 = patch completely activated or > 50% activated; 2 = patch partially activated or between 25 and 50% activated with obvious signs of mounting; 3 = no signs of mounting, patch not activated or less than 25% activated).
\(^5\) Fixed-timed artificial insemination
\(^6\) Treatments with similar superscripts do not differ.
**Table 4.3.** Effects of initial GnRH administration or removal in the 5-d CO-Synch + CIDR\(^1\) protocol on ovarian parameters in lactating beef cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment(^2)</th>
<th></th>
<th></th>
<th>SEM(^3)</th>
<th>P-Value(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant follicle diameter at CIDR insertion, mm</td>
<td>G1-2PG</td>
<td>NoG1-2PG</td>
<td>NoG1-1PG</td>
<td>SEM(^3)</td>
<td>P-Value(^4)</td>
</tr>
<tr>
<td></td>
<td>13.1</td>
<td>12.1</td>
<td>12.6</td>
<td>0.85</td>
<td>0.34</td>
</tr>
<tr>
<td>2(^{nd}) Largest follicle diameter at CIDR insertion, mm</td>
<td>8.65</td>
<td>8.44</td>
<td>8.90</td>
<td>1.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Dominant follicle diameter at CIDR removal, mm</td>
<td>11.5(^b)</td>
<td>13.2(^a)</td>
<td>12.1(^b)</td>
<td>0.54</td>
<td>0.02</td>
</tr>
<tr>
<td>2(^{nd}) Largest follicle diameter at CIDR removal, mm</td>
<td>8.75</td>
<td>10.2</td>
<td>9.08</td>
<td>0.63</td>
<td>0.16</td>
</tr>
</tbody>
</table>

\(^1\) Controlled internal drug release
\(^2\) Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2α}\) delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF\(_{2α}\) at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF\(_{2α}\) at CIDR removal (NoG1-1PG).
\(^3\) n = 130
\(^4\) Treatments with similar superscripts do not differ.
Figure 4.1. Experimental protocol describing GnRH and PGF$_{2\alpha}$ treatments and data collection in lactating beef cows for analysis of fixed-timed artificial insemination (FTAI) pregnancy rates in the 5-d CO-Synch + Controlled Internal Drug Release (CIDR) protocol. Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG).

1 Treatments included either 1) the standard 5-d CO-Synch + CIDR protocol including 100 µg of GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ delivered at CIDR removal (G1-2PG), 2) no GnRH at CIDR insertion and two concurrent 25 mg doses of PGF$_{2\alpha}$ at CIDR removal (NoG1-2PG), or 3) no GnRH at CIDR insertion and a single 25 mg dose of PGF$_{2\alpha}$ at CIDR removal (NoG1-1PG).

2 Administration of 100 mg GnRH

3 No administration of GnRH

4 Two concurrent 25 mg doses of PGF$_{2\alpha}$

5 One single 25 mg dose of PGF$_{2\alpha}$
CHAPTER 5
GENERAL DISCUSSION

Modifications to existing fixed timed artificial insemination (FTAI) protocols may lead to increased utilization of artificial insemination (AI) within the beef sector of the United States. Currently, at approximately 10%, AI remains an underutilized technology for the advancement of genetics within the United States beef herd (NAHMS, 2009). Modifications to current ovulation synchronization protocols to make them easier, less labor intensive, and more cost effective to implement, may allow for increased implementation and use. The 5-d CO-Synch + controlled internal drug release (CIDR) protocol (5dCO) is an intensive FTAI protocol that through modifications may become more utilized by beef producers. The standard 5dCO begins with the insertion of a CIDR and concurrent administration of an initial dose of GnRH (G1) followed by CIDR removal and concurrent injection of prostaglandin F\textsubscript{2α} (PGF\textsubscript{2α}) 5 d later. Approximately 8 h following CIDR removal a second dose of PGF\textsubscript{2α} is administered with FTAI and another GnRH (G2) injection occurring approximately 72 h following (Johnson et al., 2013).

Two separate parts of the 5dCO have been highlighted as potential areas where modifications can be made to decrease cost, and potentially labor. The requirement of G1 and the need for two separate, 25-mg doses of PGF\textsubscript{2α} have specifically been identified as areas of potential modification. Bridges et al. (2012) studied the requirement of two separate, 25-mg doses of PGF\textsubscript{2α} 8 h apart by replacing it with two concurrent 25-mg doses of PGF\textsubscript{2α}. Results showed similar FTAI pregnancy rates in cows...
when compared to those whom received two, separate 25, mg doses of PGF$_{2\alpha}$ approximately 8 h apart. However, the second point of emphasis, the requirement of G1, has remained more perplexing in beef animals. Kasimanickam et al. (2014) reported decreased FTAI pregnancy rates in beef heifers when G1 was removed from the standard 5dCO. Yet, Cruppe et al. (2014) reported no differences in FTAI pregnancy rates when heifers either received G1 or were not administered G1. While the effect of removing G1 in beef heifers has delivered inconsistent results, the requirement of G1 in the 5dCO has not previously been studied intensively in beef cows. Dias et al. (2014) demonstrated that G1 may not be needed in cows when specifically evaluating the response to G1 in a 5-d CO-Synch + CIDR protocol. This was shown by beef cows that did not respond to G1 had increased behavioral estrus response and greater FTAI pregnancy rates. The experiments conducted in this thesis were designed to determine the effect of G1 removal from the 5dCO and evaluate effects on ovarian parameters and FTAI pregnancy rates.

The first experiment was designed to study the effects of the removal of G1 during a 5dCO on ovarian parameters and steroid hormone concentrations. As reported in Chapter 3, removal of G1 from the 5dCO had no effect on follicle size at any time point within the study. In addition, no differences were identified in proportion of cows displaying behavioral estrus, interval to behavioral estrus, or ovulation rates to G2. Interestingly, despite the absence of differences in dominant follicle size or behavioral estrus, NoG1-2PG demonstrated greater peak plasma estradiol concentrations.
Previously, Dias et al. (2014) reported that failure to respond to G1 and not producing an accessory corpus luteum (CL) resulted in an increased proportion of females displaying behavioral estrus prior to FTAI, as well as a shortened interval to estrus. However, the data presented in Chapter 3 contrasted those findings as no differences were identified in the ancillary analysis when response to G1 was evaluated as the main effect. Yet, similar to Dias et al. (2014), no differences were identified in dominant follicle size at FTAI when response to G1 was the main effect. Lastly, it should be noted that like the intensive study, steroid hormone concentrations (more specifically estradiol) were significantly different even when no differences were identified in dominant follicle or CL size. It is speculated that the elevation in estradiol concentrations in those animals responding to G1 versus those that did not may be due to females whom did not respond to GnRH displaying behavioral estrus sooner and thus having decreasing concentrations of estradiol at theoretical FTAI. Furthermore, it must also be mentioned that the females not responding to G1 may have developed more persistent follicles. Therefore, these more mature follicles may produce less estradiol, as hypothesized by Bridges et al. (2008).

Further ancillary analysis of the first experiment, Chapter 3, found few differences between animals that either did or did not reset their follicular wave upon initiation of the treatment protocol. The only identifiable difference was an increase in plasma progesterone concentrations for those animals that reset their follicular wave 2 days following theoretical FTAI. This finding was also identified in the previous ancillary analysis, reported in Chapter 3, where females that did respond to GnRH within the G1-
2PG treatment group had elevated plasma progesterone concentrations within the same time frame. However, since no animals underwent FTAI, it is hard to speculate what the effect would be on FTAI pregnancy rates or early embryonic loss. This is especially difficult since all animals reached 1.0 ng/ml plasma progesterone concentrations 4 d following theoretical FTAI.

The second experiment was designed as a follow-up study to Chapter 3 with continuation of ovarian parameter analysis, but more importantly to observe the effect of G1 removal on FTAI pregnancy rates on a larger scale. Therefore, 2159 lactating, multiparous beef cows from 11 herds across 5 universities were combined to create a field study of effects on pregnancy rates. Also, a subset of cows across 3 universities were used to further study the effects of G1 removal from the 5dCO on ovarian parameters in Chapter 4. It is interesting to note that although there were no statistical differences in proportion of animals displaying behavioral estrus, NoG1-1PG did have significantly more advanced (lower) estrous detection aid scores than both G1-2PG and NoG1-2PG. However, the more advanced estrous detection aid scores did not extend to increased FTAI pregnancy rates as there were no differences in FTAI pregnancy among treatments. Although NoG1-1PG had the more advanced estrous detection aid scores (and possibly increased estradiol concentrations), it was NoG1-2PG that had the significantly larger dominant follicles upon CIDR removal when compared to the other treatment groups. Therefore, indicating the larger dominant follicles within the NoG1-2PG treatment group may not have been as estrogenic as the smaller dominant follicles of G1-2PG and NoG1-1PG. This finding is in contrast to the data presented in Chapter 3,
where the NoG1-2PG group had the significantly higher peak estradiol concentrations prior to theoretical FTAI and no differences were identified in dominant follicle diameter at any time point. The differences between the two experiments in this thesis are likely due to sample size with increased statistical power in Chapter 4.

Similar to Dias et al. (2014), females not responding to G1 in Chapter 4 tended to have an increased proportion of females displaying behavioral estrus. This increase in behavioral estrus is likely due to the increased diameter of not only the dominant follicle but also the second largest follicle in females not responding to G1. However, the differences in follicle diameter reported within Chapter 4 were unique as both the data from Chapter 3 and Dias et al. (2014) showed no differences in follicle diameter prior to FTAI in females not responding to G1. Furthermore, it is of interest to note that even though animals not responding to G1 had increased dominant follicle diameter and an increased tendency to display behavioral estrus, it did not correlate to increased FTAI pregnancy rates in this study or as other have reported. The data presented within this thesis and by Dias et al. (2014) does indicate that females that do not respond to G1 administration may potentially benefit through increased follicle diameter, increased behavioral estrus, and potential increased FTAI pregnancy rates. However, this same benefit is not identifiable when G1 is not administered in Chapter 4. Therefore, potential need for research remains in determining why failure to respond to G1 when administered in the 5dCO, has a beneficial effect.

Similar to the ancillary analysis of G1 response from Chapter 3, ancillary analysis of animals in Chapter 4 who either did or did not reset their follicular wave upon CIDR
insertion resulted in increased follicle diameter upon CIDR removal, increased proportion of females displaying behavioral estrus, and more advances estrous detection aid scores. The findings of the ancillary analysis indicate that the 5dCO has added benefits to animals that do not reset their follicular wave; however, this does not correlate to increase FTAI pregnancy rates. It is unique that the increased follicle size and behavioral estrous response rates are not identifiable when G1 is not administered, as in the field study. This would further enhance the argument, presented previously, that failure to respond to G1 had additional benefits on ovarian parameters. Therefore, a need remains for further research to determine the exact effects that failure to respond to G1 has and what leads to the benefits seen upon ovarian parameters versus when no G1 is administered.

Based upon the data presented in this thesis, G1 removal from the 5-d CO-Synch + CIDR protocol does not have detrimental effects upon ovarian parameters or fixed time artificial insemination rates. However, multiple points of interest still remain for further study to potentially increase reproductive efficiency in beef cattle. The development of an economical chute side test to identify follicular wave stage and animals that will or will not need GnRH at the initiation of the 5dCO protocol could prove advantageous through potentially increased FTAI pregnancy rates. In addition, further research is needed to determine why the increased dominant follicle diameter seen within females not responding to G1 administration and increased proportion of behavioral estrus, speculated to indicated increased estradiol concentrations, does not correlate to the expected increased FTAI pregnancy rates.
LITERATURE CITED


and a-b dimer) in morphologically dominant follicles during their growing and regressing phases of development in cattle. Biol. Reprod. 48: 268-276.


