MANIPULATION OF FOLLICLE DEVELOPMENT DURING FSH STIMULATION TO ENHANCE OVULATION RATES IN HOLSTEIN HEIFERS

By

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ABSTRACT

MANIPULATION OF FOLLICLE DEVELOPMENT DURING FSH STIMULATION TO ENHANCE OVULATION RATES IN HOLSTEIN HEIFERS

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The success of superovulation protocols has been greatly limited by the number of embryos recovered for transfer into recipients. Improved protocols have been successful at stimulating a large number of follicles, but struggle in translating this into high numbers of ovulations and thus, embryos. The primary objective of this thesis was to increase ovulation rates in Holstein heifers following superovulation and to characterize the effect of varying pharmaceutical strategies on daily follicle development and numbers of ovulations. Experiment 1 demonstrated that half-doses of FSH did not significantly change the number of ovulations when compared to full doses. Data also indicated that we were able to induce a new cohort of stimulated follicles despite the presence of a dominant follicle (DF). Experiment 2 showed no significant effects of dose timing (once or twice daily) or quality (decreasing vs. non-decreasing) on the number of ovulations. We then investigated the effect of progesterone (P4) manipulation during superstimulation in Experiment 3. Results indicated that a moderate progesterone environment allows for the highest number of ovulations compared to short or long progesterone application groups. Lastly, we re-evaluated half-doses versus full doses of FSH with our newly developed protocol in Experiment 4. Again, there was no difference in ovulation numbers between the groups, indicating that half-doses of FSH are sufficient to induce reliable numbers of ovulations.

This thesis is dedicated to F.D.L. Ahearne 🐾
for diligently keeping me on track and entertained
throughout this scientific process.

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A final, but no less significant, thank you goes out to my family and friends. Your unwavering faith in my abilities was an immense support during this undertaking. A special thank you to the Teeple family who kept me well-fed throughout this process. I am eternally grateful for your patience as I worked through my Masters. Thank you again to everyone who has helped me complete my Master's degree.

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KEY TO ABBREVIATIONS

AETA American Embryo Transfer Association

CIDR controlled internal drug release

CL corpus luteum/corpora lutea

d day(s)

D day of treatment period

DF dominant follicle

eCG equine chorionic gonadotropin

FSH follicle stimulating hormone

GnRH gonadotropin releasing hormone

h hours(s)

LH luteinizing hormone

MHz megahertz

mg milligram

mL milliliter

mm millimeter

min minute(s)

n number

ng nanogram

P_4 progesterone

$PGF_{2\alpha}$ prostaglandin $F_{2\alpha}$

PMSG pregnant mare serum gonadotropin

SEM standard error of the mean

TMR total mixed ration

US ultrasound

CHAPTER 1

INTRODUCTION

Development of Gonadotropins for Stimulation of Multiple Ovulations

One of the hurdles impeding efficient milk production on dairy farms is the lack of high genetic merit cows within herds. Higher genetic merit cows have the capability of producing more milk and are more efficient than their lower genetic merit counterparts (Snijders et al., 2001). More producers are turning to advanced reproductive technologies in an attempt to shorten generation interval and allow quicker improvement of overall herd genetics (Pedersen, 2012; Thomasen, 2015).

The modern embryo transfer industry began to make its mark on the dairy industry in the early 1970's as conveyed by Hasler, 2014, in a historical review of embryo transfer. Since its inception, practitioners have streamlined the collection of embryos from FSH-stimulated high genetic merit heifers or cows; and to transfer them into lower genetic merit recipients. In this manner, a high-quality heifer or cow can produce multiple progeny at a shorter time interval (Dieleman et al., 1993). Unfortunately, these technologies often have low reliability and high associated cost (Hasler et al., 1983). Average number of embryos collected following FSH-stimulation each year in the US is about 5.3 (2018 AETA survey data) per uterine flush in Holstein donors and continues to be unchanged despite over 40 years of research aiming to develop more reliable protocols (Hasler, 2014; Mapletoft, 2002). Embryos collected following superovulation are highly correlated with number of ovulations as estimated from the number of corpora lutea at time of embryo retrieval (Sartori, 2003). Thus, increasing ovulation rate to superovulation programs will make this technology more cost effective and improve its availability to the modern dairy farmer.

Superovulation and embryo transfer protocols depend on the precise combination of ovarian stimulation, coordinated ovulation, atraumatic embryo recovery and transfer into a

synchronized recipient. In order to make protocols successful the initial step in research depended on developing reliable methods for creating multiple embryos in a single treatment period. The role of the pituitary gland on gonadal function was first discovered by Crowe et al. in 1910. Smith et al., 1926, implanted fresh anterior pituitary tissue into mice and enlarged ovaries and superstimulated follicle development in mice and rats. Near the same time, Zondek, 1926, transplanted pituitary glands from cows into immature animals and witnessed an increase in sexual maturity. It was Cole and Hart, 1930, that first discovered pregnant mare serum gonadotropin also known as equine chorionic gonadotropin (PMSG or eCG). Gemzell et al., 1958 were the first to extract follicle stimulating hormone (FSH) for use in stimulating ovarian follicles. During this same period, superovulation in cattle witnessed its initial conception, as early as 1949, when teams began investigating methods to produce multiple embryos from a single treatment period. Umbaugh, 1949, reported the first successful pregnancies from transferred embryos in cattle. He successfully transferred four embryos into recipients, yet all aborted prior to term. Dowling, 1949, also faced challenges when attempting to transfer embryos but delved into the methods of superovulation more deeply by exploring the use of these different gonadotropins. The first live calf was born in 1951 after Willett et al. successfully transferred an embryo from a one-year old Holstein-Shorthorn heifer into a recipient Holstein heifer. Research continued over the following decades, coinciding with the start of the commercial embryo transfer industry in the early 1970's (Mapletoft et al., 2002; Hasler, 2003; Hasler, 2014). Interest in 'exotic' cattle breeds from Europe spurred investigation into reliable techniques for producing multiple embryos at once. This technology, along with the successful recovery of embryos and transfer into recipients, has been utilized to propagate desirable genetics at a shorter generation interval (Dieleman et al., 1993).

Many early studies investigated the use of different gonadotropin preparations for superstimulation protocols. Equine chorionic gonadotropin (eCG or PMSG) is a complex glycoprotein that has both FSH and LH activity (Murphey and Martinuk, 1991). This long-lasting gonadotropin has a 40-h half-life in cattle and causes continued ovarian stimulation (Dieleman et al., 1993). Investigation into reliable superovulation techniques was first performed by Rowson in 1951. In his work, he explored the use of whole pregnant mare serum gonadotropin (PMSG) versus commercially processed powdered pregnant mare serum in the presence of a corpus luteum (CL) or exogenously supplied progesterone. His data suggested that whole PMSG produced a larger percentage of spontaneous ovulations compared to cattle treated with the commercially processed product (42% vs 22%). Newcomb et al., 1979 compared different doses of PMSG preparations (1000 vs 2000 IU) and found an increase in follicle number and ovulations between dosages. In a later study, Gonzalez et al., 1994, compared dose of PMSG (1200, 2400, and 3600 IU) as well as the timing and effect of PMSG antiserum. They found a positive correlation between follicle numbers and dose, yet the number of transferable embryos was highest in the intermediate, 2400 IU, group. The group then investigated the timing of anti-PMSG serum administration, a method previously discovered for diminishing the prolonged ovarian stimulation effects of PMSG (Dieleman et al., 1993). Results indicated that a 60- h interval between $PGF_{2\alpha}$ administration and treatment with 2500 IU of anti-PMSG serum produced the highest number of transferable embryos. Thus, standard protocols utilizing eCG or PMSG involve one treatment of 1500 to 3000 IU via intramuscular injection, followed by a $PGF_{2\alpha}$ injection 48 h later, and 2500 IU anti-PMSG serum 60 h after $PGF_{2\alpha}$.

FSH formulations consisting of either crude pituitary extracts, FSH-P (Armour/ Folltropin), or purified pituitary extracts, NIH-FSH-PI (Folltropin-V), have a shorter half-life of

around 5 h or less (Laster, 1972; Monniaux et al., 1983). Standard protocols involve twice daily treatments of FSH over 4 or 5 d totaling 28 mg of FSH-P or 400 mg of NIH-FSH-PI (Folltropin-V). Optimal dosage determinations were reached after teams discovered a positive correlation between FSH dose and ovulation rate. Gonzalez et al., 1990 and Alkemede et al., 1993, compared the effect of varying doses of LH-reduced Folltropin-V from 100 to 900 mg NIH-FSH-PI. They found that number of ovulations increased with doses up to 400 mg and did not increase at higher dosages. There was not a negative impact of higher than optimal doses on ovulation number or embryo quality. This contrasts with higher doses of FSH-P formulations where the optimal dose has been found to be 28 mg total dosage. Donaldson, 1984, demonstrated that doubling the dose of FSH-P, 60 mg vs 28 mg total dosage, had a negative impact on embryo production. Therefore, LH-reduced FSH products became the preferred formulations above crude pituitary FSH extracts.

There have been conflicting opinions as to which gonadotropin formulation produces the greatest number of ovulations when comparing PMSG and FSH. Both Elsden et al. 1978, and Monniaux et al. 1983, found that animals treated with pituitary FSH extract produced greater numbers of ovulations than eCG/PMSG products. This disagrees with teams that found no differences between numbers of ovulations, or embryos, produced with the different formulations (Alkemade et al., 1993; Goulding et al., 1996; and Mapletoft et al., 1990). Yet, standardized superovulation protocols began to lean towards purified pituitary FSH extracts such as Folltropin-V. This is largely due to the variability of eCG products in their concentrations of FSH and LH (Murphey and Martitnuk, 1991). Although both FSH and LH are required for appropriate follicular development, endogenous LH concentrations appear to be adequate. Many studies have indicated that lower LH concentrations in gonadotropin formulations produce a

better superovulation response (Chuipin et al., 1984; Murphey and Martitnuk, 1991; Murphey et al., 1984). Willmott et al., 1990, investigated the effects of different concentrations of LH in gonadotropin formulations. Results indicated that higher levels of LH have a negative effect on embryo quality in superstimulated cows. Therefore, products such as Folltropin-V, a purified pituitary FSH extract, have become the preferred gonadotropin for superstimulation protocols.

Development of Superovulation Protocols for Embryo Production

Since their inception, superovulation protocols have experienced few advancements and continue to experience wide variability in ovulation rate and numbers of embryos produced. For example, Hasler et al., 1983, reported that, even among healthy donor cows, 14% failed to produce a viable fertilized embryo after superstimulation. This is compared to the approximately 51% failure rate in cattle deemed infertile during the same study. While protocols have been modified and our understanding of reproductive physiology advanced it is apparent that we have yet to maximize the efficiency to superovulation protocols.

It has been well documented that follicular development in cattle occurs in a series of waves. A follicular wave is defined as the synchronous emergence and growth of a cohort of small follicles followed by maturation and selection of a single dominant follicle and the regression of the subordinates (Ginther et al., 1989b; Knopf et al., 1989; Savio et al., 1988). Initial hypotheses of multiple follicular waves were tested via novel early ultrasonographic studies. Savio et al., 1988 followed the growth of follicular development in heifers through two consecutive estrous cycles. Results indicated that 81% of the heifers experienced a three-wave cycle, 15% had two waves and 4% (one heifer) had a single wave. These data agreed with the findings of Knopf et al., 1989, who also observed follicular wave development in heifers, though their team's results indicated 9/10 heifers had two follicular waves.

Many investigations aimed at determining which stage of follicular development is most suitable for initiation of superovulation protocols. Traditional superstimulation protocols begin treatment mid-cycle, between day 8 and 12 of the estrous cycle. It is unclear where the initial convention was developed but, as a deeper understanding of follicular dynamics has emerged, this range approximates with the emergence of the second follicular wave. Time of follicular wave emergence was characterized by Ginther et al., 1989b. His team determined that, in a two-wave estrous cycle follicular wave emergence occurs on day 0 and day 10. In three-wave cycles follicular wave emergence occurs on day 0, 9 and 16. Studies that investigated the timing of superstimulation initiation often mirrored the critical stages of follicular wave emergence. Lindsell et al., 1986 initiated superstimulation protocols with FSH-P on day 3, 6, 9, or 12 of the estrous cycle. More embryos were recovered from the day 9 group with the day 6 and day 12 groups being intermediate. Hypotheses then formed stating that a superior response would occur when protocols were instituted in concert with endogenous follicular wave emergence. Nassar et al., 1993 tested this theory by beginning treatment on Day -1, 0, +1 or +2 of the estrous cycle. A higher response was noted in the Day -1 or 0 groups with those animals exhibiting greater numbers of large follicles and ovulations at the end of the treatment period. These hypotheses were again explored by Fricke et al., 1994 with the theory that initiation of superstimulation early in the estrous cycle, before dominant follicle influence, would result in more consistent and reliable response by donors. Cows treated beginning on Day 2 of their estrous cycle produced the most transferable embryos, yet Day 10 cows also had a consistent response with high numbers of ovulations. These results indicate that treatment at the onset of follicular wave development allows for reliable response to superovulatory protocols.

Arguments have been made for the presence of a dominant follicle (DF) having a negative impact on growth of subordinate follicles during stimulation protocols. Ko et al., 1991 examined this phenomenon by removing the Wave 1 DF via cauterization. Removal of the DF resulted in the emergence of a new follicular wave. In a similar experiment Adams et al., 1993a again removed the DF, this time to observe apparent resurgence of a previously subordinate follicle. The subordinate follicle was able to recover and develop into a new DF. Superovulation protocols aim to overcome this suppression by supplying exogenous FSH influence and inducing multiple follicles to grow to ovulatory size. Guilbault et al, 1991 studied superovulatory responses in heifers stimulated in the presence or absence of a dominant follicle. They observed a decreased super-stimulatory response in the presence of a dominant follicle. Reviews of follicular dynamics indicate that only around 20% of the estrous cycle is favorable for initiation at a time of follicular wave emergence (Mapletoft et al., 2002; Mapletoft and Bo, 2012). These findings suggest a need for intensive management of the estrous cycle for animals intended for superovulatory protocols in order to initiate stimulation at a time when a DF is not present. This greatly increases the reproductive management required by producers interested in adopting advanced reproductive technologies.

Protocol development allowing for stimulation at any part of the estrous cycle, with or without DF influence, would decrease producer input and increase practicality of superstimulation on the modern dairy farm. The primary objective of this thesis was to characterize the effect of varying protocols on superovulatory response from a follicular and ovulatory standpoint. Chapter 2 explores the effect of varying FSH dosage and timing of administration as a method of decreasing producer input. Chapter 3 then delves into the physiologic effects of progesterone modulation in order to increase the proportion of ovulatory

sized follicles that successfully respond to an endogenous LH surge. Success of such protocols will enable producers to utilize superovulation and embryo transfer on a large commercial scale with greater confidence in the success of such endeavors.

CHAPTER 2

MODIFICATION OF SUPEROVULATION PROTOCOLS TO MAXIMIZE OVULATORY RESPONSE IN DAIRY HEIFERS. PART 1: EFFECT OF FSH TIMING AND DOSE

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INTRODUCTION

In order to make reproductive technologies a success, producers and researchers must work together to develop more reliable and cost-effective methods of embryo production. Current superovulation methods can predictably produce large numbers of follicles but continue to struggle in translating high follicle counts into high transferable embryo numbers (Bo and Mapletoft, 2014). A key factor in increasing efficiency of embryo transfer is streamlining the growth and collection of follicles or embryos through modified superovulation protocols. Current protocols call for the administration of FSH, twice daily, in decreasing doses, for four consecutive days. The very high cost of FSH, labor, embryo recovery, laboratory services, and transfer into surrogates keep most producers from devoting much capital to an uncertain venture. Thus, the first step in making embryo transfer a viable option for the average producer is to increase efficiency while decreasing cost.

The success of super stimulation protocols has been limited by the number of embryos produced (Hasler, 2014). Average number of embryos collected following FSH-stimulation each year in the US is about 5.3 (2018 AETA survey data) per uterine flush in Holstein donors and continues to be unchanged despite over 40 years of research aiming to develop more reliable protocols (Hasler, 2014; Mapletoft, 2002). Embryos collected following superovulation are highly correlated with number of ovulations as estimated from the number of corpora lutea at time of embryo retrieval (Sartori, 2003). Thus, improvement in numbers of embryos collected is contingent upon increasing the numbers of dominant follicles that reach the ovulatory pool of follicles and ovulate successfully. Current protocols face challenges in maximizing the number of follicles reaching the ovulatory stage of maturation. There is a paucity of information regarding

the process of follicular growth and atresia during FSH stimulation as well as the influence of stage of the estrous cycle on FSH-stimulation.

The half-life of FSH is approximately five hours in cattle (Laster, 1972; Monniaux et al., 1983). Thus, many studies have utilized twice daily administration of various doses (Gonzalez et al., 1990; Alkemede et al., 1993) of different types of FSH. Doses were often administered in decreasing quantities, at 12-hour intervals, over the treatment period or in a single bolus at the onset of the stimulation period (Garcia and Seidel, 1982; Bergfelt et al., 1997). There is little known about the effects of non-decreasing doses or single day dosing with FSH. A key to maximizing ovulation following FSH stimulation is proper dosing and procedure for administration of FSH, yet few have investigated whether current methods can be improved to increase ovulation rates.

Current stimulation protocols initiate FSH-stimulation with the onset of a new follicular wave. Most FSH-stimulation protocols start between day 8 and 12 of the estrous cycle to coincide with the start of a second follicular wave (Donaldson, 1984; Ginther et al., 1989), or by ablation of all antral follicles which effectively initiates the start of a new wave of follicles (Baracaldo et al., 2000). These protocols require intensive monitoring or manipulation of estrous cycles and are not practical from a logistical perspective.

The objectives of the present studies were to determine the effect of the quantity and timing of FSH dosages on follicle development and ovulation in Holstein heifers. Our hypotheses were: 1) Lower amounts of FSH, ¼ or ½ doses, during a 4 day period will result in similar numbers of new follicles following FSH-stimulation and subsequent ovulations compared to full doses, and 2) numbers of follicles and ovulations will not differ when heifers are treated with non-decreasing amounts of FSH compared to decreasing amounts of FSH during the stimulation period. The model

utilized to test these hypotheses start FSH stimulation following 1st wave deviation of the dominant follicle in Holstein heifers on day 5 of the estrous cycle (Bednar and Pursley, 2000). This potentially removes the confoundedness of variation in the in vivo induced surge of FSH which normally occurs no earlier than day 7 of the estrous cycle in heifers to initiate the second wave of follicular growth (Ireland and Roche, 1983).

MATERIALS AND METHODS

Heifers, housing, feeding and products

Two experiments were conducted between May 15, 2015 and September 1, 2016 on a commercial dairy farm (Nobis Dairy Farm, St. Johns, MI). Heifers in the present study were randomly selected from a group of approximately 200 healthy 11- to 12-month heifers. Heifers were treated with a single intramuscular injection of $PGF_{2\alpha}$ (25 mg dinoprost tromethamine, 5 mL of Lutalyse, Zoetis, Parsippany, NJ). They were then monitored daily from the time of $PGF_{2\alpha}$ for signs of estrus behavior via the herd's SCR activity monitoring system (SCR Engineers Ltd., Netanya, Israel). Day of behavioral estrus was considered day 0 of their estrous cycle. Treatments began on day 5 of the estrous cycle. Heifers were housed in a free stall barn, fed a TMR once daily, and had free access to feed and water. The TMR consisted of corn, wheat and alfalfa silages and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for dairy heifers (NRC, 2001).

All treatments were administered via an injection of a single dose of $PGF_{2\alpha}$ (25 mg dinoprost tromethamine, 5 mL of Lutalyse, Zoetis, Parsippany, NJ) or FSH (Folltropin-V, Vetoquinol, Quebec, Canada) into the semimembranosus or semitendinosus muscles of heifers. Heifers assigned to CIDR administration received a sanitized Eazi-Breed CIDR device (1.38 g progesterone; Zoetis, Parsippany, NJ), previously used for 7 days, via transvaginal insertion.

Blood samples were collected prior to ultrasound examination by puncturing the medial coccygeal artery or vein. Blood was collected into 10 mL Vacutainer clot tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately placed on ice and transported to the laboratory. Serum was separated after blood was centrifuged at 2,000 x g for 20 min. Serum was frozen at -18º C until analysis for P_4. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures described in this manuscript.

Experiment 1 Experimental Design: Effect of FSH dose on follicle recruitment and ovulation

Heifers (n=15) were randomly assigned to receive a 1/4 dose (n=5), 1/2 dose (n=5), or full dose (controls; n=5) of Folltropin V in four equal, once-daily doses beginning on d 5 of the estrous cycle. Each 20 mL vial of Folltropin V contained FSH equivalent to 400 mg NIH-FSH-P1. A full 20 mL vial equates to a full dose of FSH, administered over the treatment period. Used CIDRs were inserted at time of first FSH dose and removed 24 h after final FSH treatment. All treatments, blood sampling and ultrasonography exams took place each morning beginning at 8 AM. Blood samples were collected daily from the onset of FSH administration until the day following ovulation, disappearance of one or more follicle $\geq$ 9 mm, then 3 and 7 days post-ovulation. $PGF_{2\alpha}$ was administered at time of the third FSH treatment in the morning and 12 h later to regress any CL. CIDRs were removed 24 h after final FSH treatment to induce the preovulatory LH surge.

Experiment 2 Experimental Design: Effect of FSH administration protocol on follicle recruitment and ovulation

Heifers were randomly allocated to treatment in a 2 X 2 complete block randomized design in which they were treated once or twice daily with either decreasing or non-decreasing, consistent, doses of FSH. Each heifer received a total of 10 mL Folltropin during the four-day period beginning on d 5 of the estrous cycle. FSH administration was applied as follows; non-decreasing

twice daily heifers received 1.25 mL FSH morning and evening for four days. Non-decreasing once daily heifers received 2.5 mL FSH morning only for four days. Decreasing twice daily heifers received 2.6 mL (D1), 1.3 mL (D2), 0.65 mL (D3), and 0.4 mL (D4) FSH morning and evening for four days. Lastly, the decreasing once daily heifers received 5.2 mL (D1), 2.6 mL (D2), 1.3 mL (D3), and 0.9 mL (D4) FSH morning only for four days. Thus, heifers (n=24) were randomly assigned to the following four groups: non-decreasing twice daily dosing (Non-AM/PM, n=6); non-decreasing once daily dosing (Non-AM, n=6); decreasing twice daily dosing (Dec-AM/PM, n=6), or decreasing once daily dosing (Dec-AM, n=6) groups. Heifers received an initial 5 mL dose of $PGF_{2\alpha}$ on the third day of treatment in the evening and a second $PGF_{2\alpha}$ 12 h later, on the last day of treatment. All heifers received a used CIDR at time of 1st FSH treatment. CIDRs were removed at time of 2nd $PGF_{2\alpha}$, morning of day four of treatment.

Ovarian ultrasonography

Ovarian structures (all visible follicles and corpora lutea) were mapped and measured as previously described by Martins et al. 2011 using a Sonosite MicroMaxx ultrasound machine with a linear array transducer utilizing 10 MHz frequency (Sonosite Inc., Bothell, WA). Each ovary was evaluated using the Cine function to view image frame by frame to count and measure follicles beginning on the 1st day of treatment then daily until ovulation then 3, 5, and 7 days after ovulation. Heifers from the larger pool of available animals without a d 5 CL and DF were not used in the studies, thus resulting in n=15 heifers in Experiment 1 and n=24 heifers in experiment 2. Ovulation after CIDR removal was determined by the disappearance of one or more dominant follicles ($\geq$ 9 mm) previously visualized from the prior day's exam and a new CL two days later, day 3 post-ovulation.

Hormonal assay

Serum concentrations of P_4 were analyzed with a radioactive immunoassay validated by Engel et al., (2008). All sample concentrations were determined in one assay. The interassay CV was 8.15%, the intraassay CV was 4.84% and the sensitivity was 0.2 ng/mL.

Statistical analyses

Statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). Total number of follicles, number of follicles ≥ 9 mm, number of follicles ≥ 9 mm prior to the day of ovulation and number of ovulations are reported as mean ± SEM.

Total number of follicles and number of follicles ≥ 9 mm were analyzed using PROC GLIMMIX. A compound symmetry [cs] covariance structure was applied based on the lower Bayesian information criterion (BIC) score. The model considered the fixed effects of treatment, time and their interaction. The RANDOM statement was used to account for measurements performed over time (days 1, 2, 3, 4 and 5 of treatment). Treatment nested within heifer was included in the SUBJECT option. Significant interactions were sliced utilizing the *% mult* macro of SAS. Multiple comparisons were calculated between treatments and within day following treatment. Single measure variables were analyzed with one-way ANOVA on PROC GLM. Differences in number of ovulations were fitted with a model that included treatment and number of follicles ≥ 9 mm prior to the day of ovulation as fixed effects. Number of follicles ≥ 9 mm prior to the day of ovulation only included treatment in the model. Differences in proportion of ovulations were analyzed via a simple t-test using the Excel data analysis function. Differences in progesterone concentration were analyzed with a one-way ANOVA using the Excel data analysis function.

RESULTS

Experiment 1: Effect of FSH dose on follicle recruitment and ovulation

Heifers treated with varying doses of FSH on d 5 of the estrous cycle, in the presence of a dominant follicle, approximately doubled the number of antral follicles in a 24 h period ($P < 0.001$). Numbers of follicles remained high 2 d after start of FSH. The full dose group had greater ($P<0.05$) numbers of follicles on days 3 through 5 after the start of FSH compared to the ¼ and ½ dose groups (Figure 2.1).

Numbers of follicles ≥ 9 mm in diameter increased after d 3 of the treatment period. There were greater numbers of follicles ≥ 9 mm in diameter in the full dose group compared to the ¼ and ½ dose groups on days 4, 5 and 6 after initiation of FSH ($P < 0.05$). However, there were no differences in numbers of follicles ≥ 9 mm in diameter between the ¼ and ½ dose groups on any given day of treatment ($P > 0.05$) (Figure 2.2).

Ovulation of at least one or more follicle ≥9 mm did not occur in a uniform manner between treatment groups. In the ¼ and ½ dose treatment groups, 40% of heifers (n=2) ovulated on D6 of treatment while 60% of heifers (n=3) ovulated on D7 of treatment period. The full dose group only had a single heifer ovulate on D6 while 80% of heifers (n=4) ovulated on D7 after the onset of treatment (Table 2.1). These findings, although numerically evident, were not significant statistically ($P > 0.05$).

There was an effect of treatment on numbers of follicles ≥ 9 mm in diameter on the day prior to ovulation and numbers of ovulations. Heifers in the full dose group had greater numbers of follicles ≥ 9 mm on the day prior to ovulation compared to the ¼ dose and ½ dose groups (Figure 2.3). Heifers in the ½ dose and full dose groups had greater numbers of ovulations ($P < 0.05$) compared to the ¼ dose group. The ½ dose group had a larger proportion of ovulations when

compared to the ¼ dose or full dose groups. Although there is a numerical difference in proportion of follicles ≥ 9 mm that ovulated the difference was not significant (P > 0.05).

There was no difference between treatments on P4 concentration on any given day (P > 0.05). Progesterone concentrations peaked on D3 of the treatment period at a mean of 5.527 ± 0.170 ng/mL. Levels decreased steadily over the next few days with concentrations of 2.369 ± 0.190 ng/mL on D4 following $PGF_{2\alpha}$ administration and 1.328 ± 0.140 ng/mL on D6, 24 h after removal of CIDR.

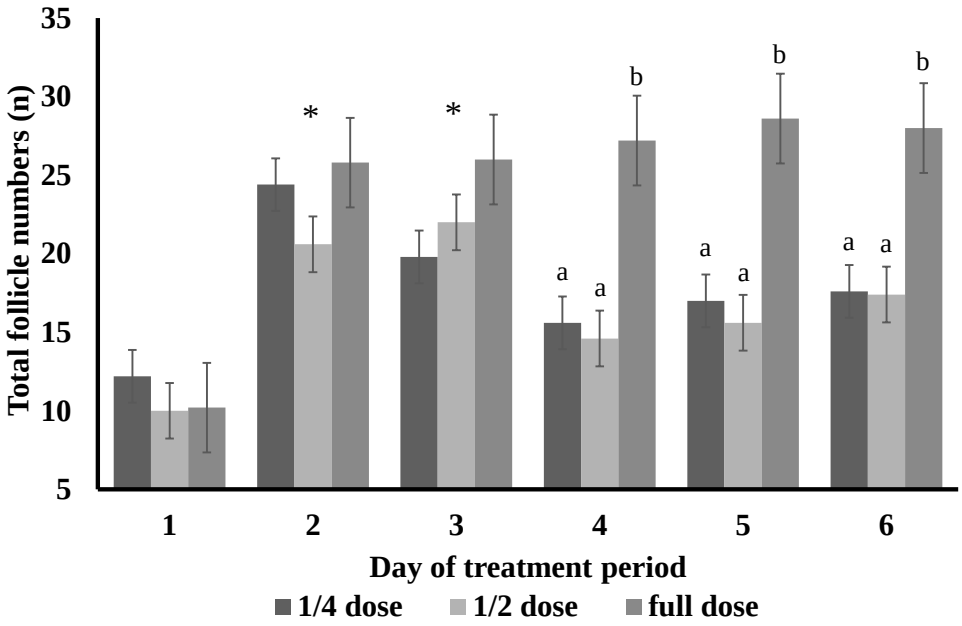

Figure 2.1: Effect of different amounts of FSH during superovulation on total number of follicles on both ovaries in Holstein heifers. (¼ dose, n=5; ½ dose, n=5; full dose, n=5)
* Denotes significant difference between follicle number and numbers on Day 0, P <0.001
a,b Denotes significant difference in follicle number between groups on indicated day of treatment, P <0.05

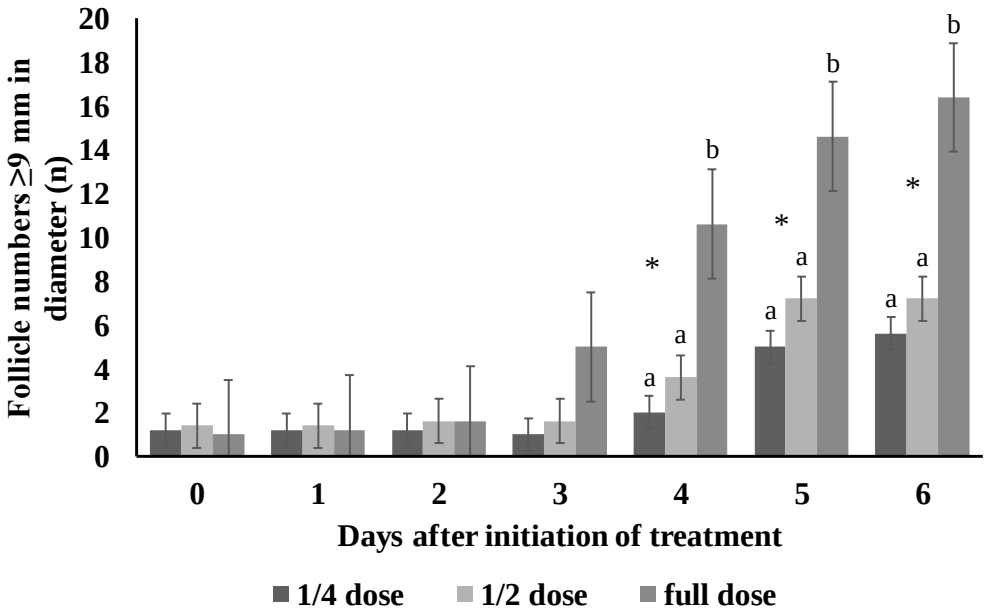

Figure 2.2: Effect of different amounts of FSH during superovulation on ultrasound measurements of total number of follicles ≥9 mm in diameter on both ovaries in Holstein heifers. (¼ dose, n=5; ½ dose, n=5; full dose, n=5)
* Denotes significant difference between follicle number and numbers on Day 1, P <0.001
a,b Denotes significant difference in follicle number between treatments on indicated day of treatment, P <0.05

Group	Day 6	Day 7
¼ dose (n=5)	40% (n=2)	60% (n=3)
½ dose (n=5)	40% (n=2)	60% (n=3)
Full dose (n=5)	20% (n=1)	80% (n=4)

Table 2.1: Effect of treatment on time of ovulation following the initiation of FSH-stimulation in Holstein heifers (n=15) treated with ¼, ½, or full doses of FSH divided into four equal once daily amounts of FSH.
No difference between day of ovulation and treatment group. (P > 0.05)

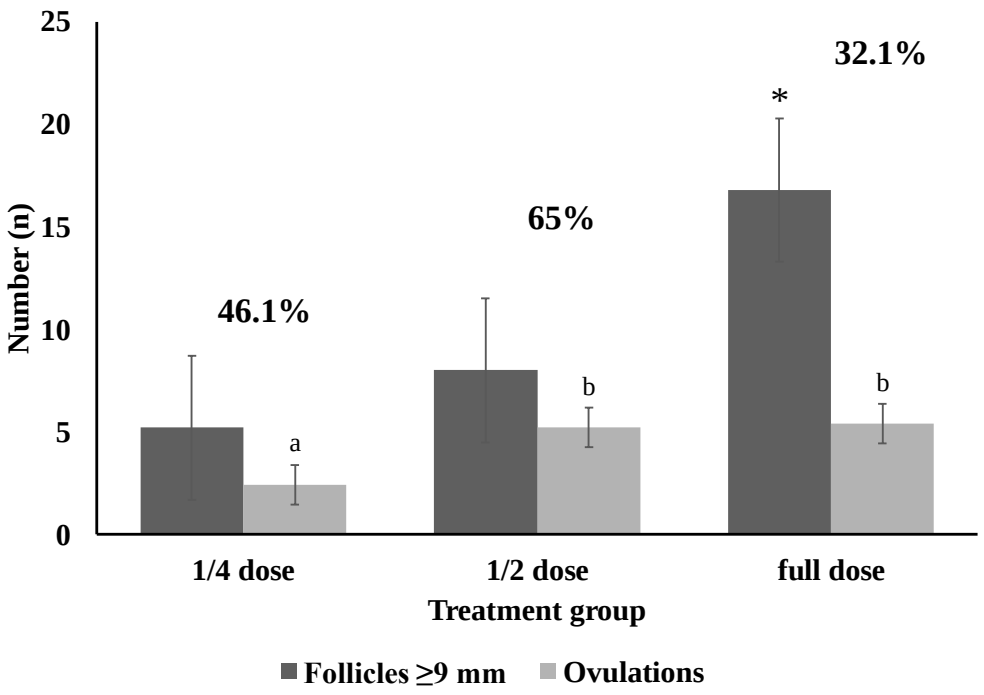

Figure 2.3: Effect of different amounts of FSH during superovulation on ultrasound measurements of follicle numbers ≥ 9mm on day before ovulation and on proportions of ovulations, determined the day after disappearance of at least one follicle ≥ 9 mm in diameter, in Holstein heifers. (¼ dose, n=5; ½ dose, n=5; full dose, n=5)
* Denotes significantly different follicle number ≥ 9mm, P <0.05
a,b Denotes differences in average numbers of ovulations, P <0.05

Experiment 2: Effect of FSH administration protocol on follicle recruitment and ovulation

Similar to the findings in Experiment 1, FSH administration increased (P <0.001) the number of antral follicles significantly within the first 24 hours of treatment despite the presence of a day 5 dominant follicle. Follicle numbers remained elevated for the remainder of the treatment period for all groups (Figure 2.4).

As in Experient 1, numbers of follicles ≥ 9 mm in diameter increased after D4 of the treatment period. There were, however, no differences in numbers of follicles ≥ 9 mm in diameter between the treatment groups on any day of the protocol (P >0.05) (Figure 2.5).

There was no effect of treatment on numbers of follicles ≥ 9 mm in diameter on the day prior to ovulation and numbers of ovulations (P > 0.05) (Figure 2.6). Although, heifers in the Dec-AM/PM group had a tendency (P = 0.09) for greater numbers of ovulations when compared to the other groups.

There was no difference between treatments on P4 concentration on any given day (P > 0.05). Progesterone concentrations peaked on D3 of the treatment period at a mean of 5.773 ± 0.288 ng/mL. Levels decreased steadily over the next few days with concentrations of 3.100 ± 0.080 ng/mL on D4 following first $PGF_{2\alpha}$ administration and 1.323 ± 0.130 ng/mL on D5, 24 h after removal of CIDR.

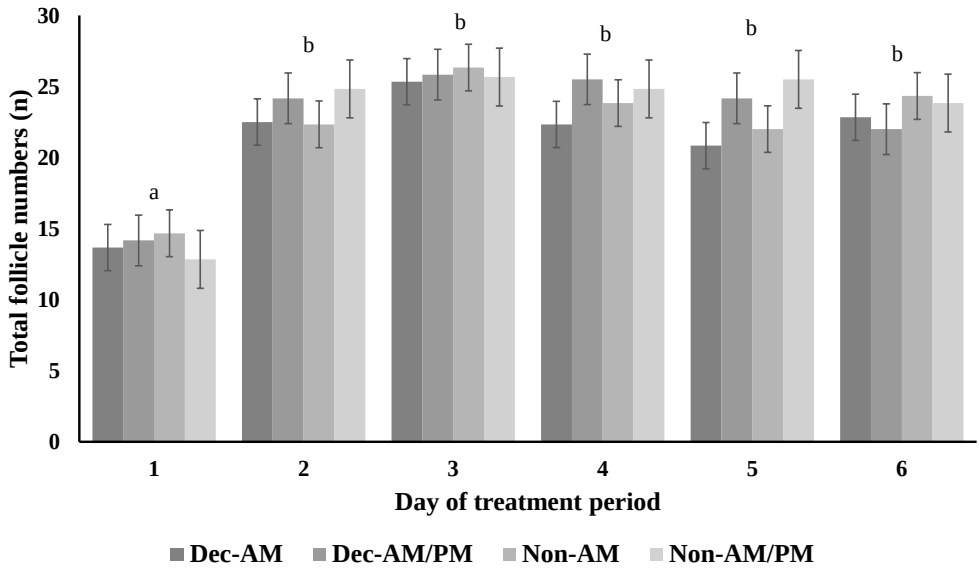

Figure 2.4: Effect of varying administration of FSH on ultrasound measurements of total numbers of follicles on both ovaries measured daily prior to ovulation in Holstein heifers. (Dec-AM, n=6; Dec-AM/PM, n=6; Non-AM, n=6; Non-AM/PM, n=6)
a,b Denotes significant difference in follicle number and day, P <0.05
No differences found between treatments, P >0.05

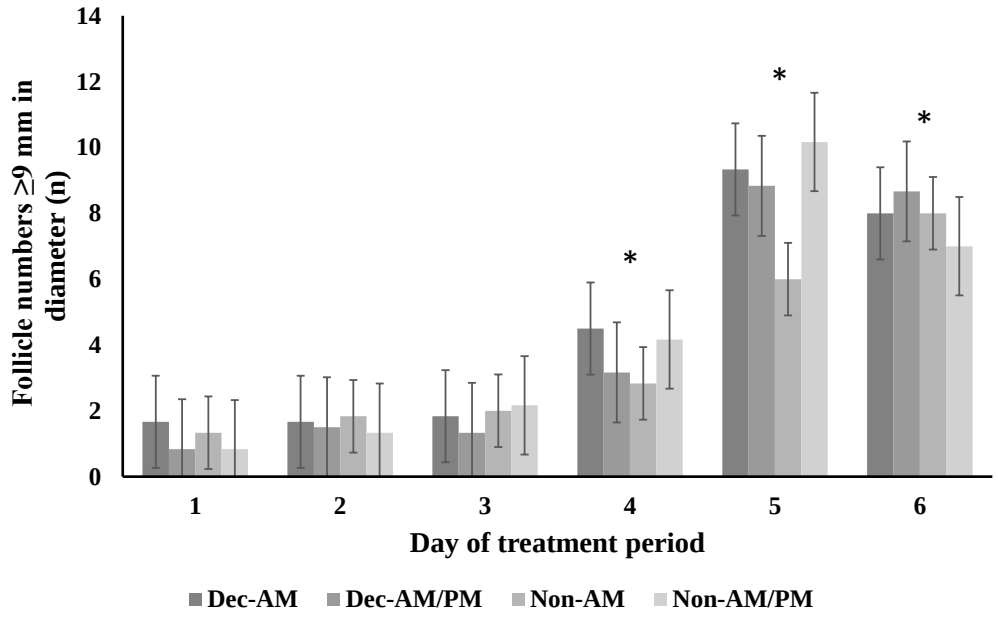

Figure 2.5: Effect of varying administration of FSH on ultrasound measurements of follicles ≥ 9 mm in diameter on both ovaries measured daily prior to ovulation in 11 to 12-month old Holstein heifers. (Dec-AM, n=6; Dec-AM/PM, n=6; Non-AM, n=6; Non-AM/PM, n=6)
* Denotes significant difference between follicle number and day, P <0.05
No differences between treatments, P >0.05

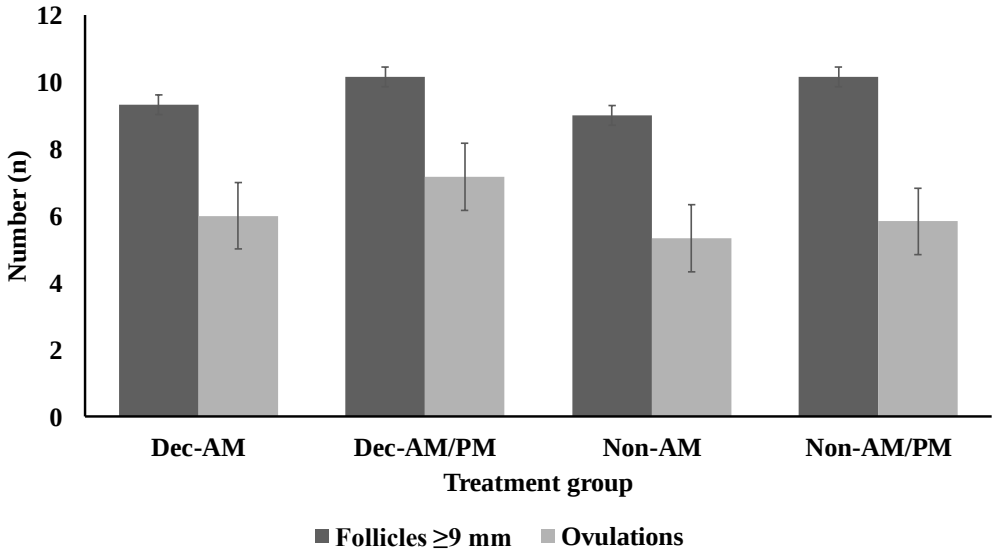

Figure 2.6: Effect of varying administration on ultrasound measurements of follicle numbers ≥ 9 mm in diameter measured on the day before ovulation and on ovulation numbers, determined the day after disappearance of at least one follicle ≥ 9 mm in diameter, in Holstein heifers (n=24). (Dec-AM, n=6; Dec-AM/PM, n=6; Non-AM, n=6; Non-AM/PM, n=6)
No differences in follicle numbers ≥ 9 mm in diameter on the day before ovulation, P >0.05
No differences in numbers of ovulations, P >0.05

DISCUSSION

Our studies aimed to investigate whether a reduction in doses and fewer injections of FSH during superovulation would produce numbers of ovulatory follicles and ovulations similar to higher or more frequent FSH doses. The most significant finding of the present study was that FSH doses could be reduced by at least 50% without a decrease in ovulation rate and in the presence of a first wave DF. When compared with earlier studies (Garcia et al.; 1983; Sreenan and Gosling, 1976; Guilbault et al., 1991) the ovulation numbers produced in these experiments did not vary from previous, spontaneous, ovulation numbers. Earlier studies have reported much larger numbers of ovulations (Guerra et al., 2012) when their team utilized an ovulation aid (Lutropin-

V) to induce final maturation and ovulation of follicles. Some protocols may be enhanced by administration of a GnRH product at the end of the protocol (12-24 hours after CIDR removal). For the purposes of our investigations we allowed for spontaneous ovulations rather than applying an ovulation aid.

Interestingly, a higher proportion of follicles ≥ 9 mm ovulated in both the ¼ dose and ½ dose groups. Potential explanations for this difference in the proportion of large follicles that ovulated in these treatments are: 1) The once daily dose of FSH was likely not sufficient to maintain growth of larger follicles due to the relatively short half-life of FSH (Laster, 1972; Monniaux et al., 1983) especially in the ¼ dose and ½ dose groups. More follicles from heifers in the ½ dose group likely did not make it to the ovulatory pool of follicles (≥ 9 mm in diameter) for this reason. It seems plausible that greater amount of FSH in the full dose group allowed greater numbers of follicles into the ovulatory pool of follicles. But it was not clear as to why more than ½ of those follicles did not ovulate. 2) The likelihood of supraphysiological amounts of estradiol from the increased number of large follicles in the full dose group in combination with 2 to 3 ng/mL of P4 may have caused a portion of the stimulated follicles to become atretic (Kinder et al., 1983). This explanation could also have attenuated formation of LH receptors (Luo et al., 2019) in a portion of these large follicles. It has been shown previously that high progesterone levels during follicle development can have a deleterious effect on follicular growth through the suppression of LH pulses (Adams et al., 1992). But in this case, at the time follicles reached 9 mm in diameter the only progesterone was from a used CIDR device. Levels of progesterone in circulation at this time were between 2 and 3 ng/mL with no difference amongst treatments, making this theory less likely. Lastly, 3) The differences in amount of FSH could have a direct negative effect on a greater proportion of follicles in the ovulatory pool (≥ 9 mm in diameter) on

follicular function via attenuation of LH activity. Gosselin et al., 1995, demonstrated a negative effect on LH amplitude and pulse frequency during superovulation as a function independent of P4 concentrations. The higher doses of FSH, and subsequently elevated estradiol levels, in the full dose group may have modulated LH activity to decrease response of stimulated follicles.

Experiment 2 data support the industry standard of utilizing decreasing FSH doses over the treatment period. It appears that introducing high doses of FSH early in the stimulation protocol, as occurred in the decreasing dose groups, did not increase numbers of new follicles recruited compared to non-decreasing or equal amounts of FSH based upon the similarity in total numbers of follicles. Thus, amount of FSH dose does not appear to influence numbers of follicles recruited, but clearly affects the ability of large numbers of follicles to continue to grow into the ovulatory pool of follicles.

In summary, these data introduce many avenues for further investigation while confirming that a new cohort of follicles can be stimulated in the face of a DF. Experiment 1 confirmed that different amounts of FSH does not affect the numbers of new follicles and but does affect numbers of ovulations. Future experiments would be useful in exploring the effects of progesterone concentrations at specific times during the stimulation period. These findings also suggest that we have not yet maximized the number of ovulations achieved based on the disparity between large stimulated follicles and ovulations achieved. Experiment 2 showed that our hypothesis was correct. There was no statistical difference in the numbers of follicles or ovulations when heifers were treated with non-decreasing amounts of FSH compared to decreasing amounts of FSH during the stimulation period. The tendency for greater numbers ovulations in twice daily decreasing doses suggests this practice is still the most ideal way to administer FSH, but the data are clearly equivocal. It is encouraging that with ½ dosing of FSH we were able to maintain ovulation numbers

on par with industry averages. Further investigation into modified protocols may continue to improve ovulation numbers and reliability of superovulation protocols.

CHAPTER 3

MODIFICATION OF SUPEROVULATION PROTOCOLS TO MAXIMIZE OVULATORY RESPONSE IN DAIRY HEIFERS. PART 2: MANIPULATION OF PROGESTERONE CONCENTRATIONS AT KEY FOLLICULAR DEVELOPMENT POINTS.

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INTRODUCTION

Despite many investigations into the development of reliable superovulation protocols outcomes have continued to produce variable numbers of viable embryos for transfer into recipients (Mapletoft, 2002). This uncertainty influences producer decisions about utilizing these technologies on a larger commercial scale. Without dependable protocols few producers are willing to expend the capitol on an uncertain venture. Previous studies have clearly shown that a large cohort of stimulated follicles can be induced with conventional protocols (Baracaldo et al., 2000; Donaldson, 1984; Roberts and Echternkamp, 1993). Yet, large follicle numbers during stimulation often do not translate into high numbers of ovulations, and even fewer still are successfully fertilized into viable embryos. This disparity between follicle numbers and embryos must first be overcome by ensuring a greater proportion of stimulated follicles reach the ovulatory pool prior to an ovulatory LH surge.

Increasing the consistency and numbers of ovulations following FSH-stimulation is critical to make embryo transfer more efficient and less expensive for cattle producers (Hasler, 2014). Development of strategies to improve ovulation rates following FSH-stimulation is the first step in this process (Bo and Mapletoft, 2014). Data from Bednar and Pursley in 2000, suggested that correct timing of luteolysis or exogenous progesterone removal during superovulation may optimize the number of follicles in the ovulatory pool that respond to the subsequent luteinizing hormone (LH) surge and ovulate. While their results indicated the potential for manipulation of progesterone levels during superovulation, they did not investigate the timing of progesterone removal in regard to ovulation rates. Most studies have utilized numbers of embryos collected following superovulation to compare different FSH stimulation strategies. Yet, few have investigated the development of follicles during FSH stimulation utilizing ultrasound technologies

to gather real time information. Fewer still have characterized changes in follicle growth during manipulation of progesterone in relation to different stages of follicular development and FSH stimulation.

Follicular development in cattle following deviation of the dominant follicle is highly dependent on LH stimulation. Progesterone is a key regulator of LH amplitude and pulse frequency (Goodman and Karsch, 1980; Kinder et al., 1996). Circulating LH prior to deviation may influence acquisition of LH receptors (Luo et al., 2019). PMSG or FSH stimulation for the intention to super-stimulate / -ovulate follicles in cattle appears to reduce LH pulses (Bevers et al., 1989; Price et al., 1999). Thus, some amount of time following the final FSH treatment is needed to allow for an environment with sufficient LH pulses for follicles to mature and ovulate (Nivet et al., 2012). Unfortunately, PMSG or FSH stimulated animals appear to have a shorter interval between cessation of gonadotropin treatment and the final LH surge (Bevers et al., 1989). This phenomenon may decrease the number of ovulatory sized follicles that have time to reach final maturation and respond to the ovulatory LH surge. Correct timing of progesterone removal may help mitigate this problem by restoring LH activity, aiding in the final maturation of follicles before the final LH surge. The objectives of these studies were to 1) determine the effect of timing of the decrease in progesterone on numbers of follicles that reach ≥ 9 mm in diameter and ovulate in Holstein heifers that receive FSH stimulation, and 2) determine the effect of dose of FSH utilizing the most effective strategy for decreasing progesterone during FSH stimulation on numbers of ovulations. We hypothesized that 1) shorter exposure to progesterone supplementation during FSH stimulation will result in higher numbers of ovulations and 2) ½ doses of FSH will be sufficient in maintaining ovulation numbers with the utilization of the most effective progesterone management strategy.

MATERIALS AND METHODS

Heifers, housing, feeding and products

Two experiments were conducted between May 15, 2015 and September 1, 2016 on a commercial dairy farm (Nobis Dairy Farm, St. Johns, MI). Nobis farm maintains a group of approximately 200 11- to 12-month old heifers. The healthy- Holstein heifers used in our studies were uniformly synchronized a single intramuscular injection of $PGF_{2\alpha}$ (25 mg dinoprost tromethamine, 5 mL of Lutalyse, Zoetis, Parsippany, NJ). They were then monitored daily from the time of $PGF_{2\alpha}$ for signs of estrus behavior via the herd's SCR activity monitoring system (SCR Engineers Ltd., Netanya, Israel). Day of behavioral estrus was considered day 0 of their estrous cycle. Treatments began on day 5 of the estrous cycle. Heifers were housed in free stall barns, fed a TMR once daily, and had free access to feed and water. The TMR consisted of corn, wheat and alfalfa silages and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for dairy heifers (NRC, 2001).

All treatments of $PGF_{2\alpha}$ (25 mg dinoprost tromethamine, 5 mL of Lutalyse, Zoetis, Parsippany, NJ) and FSH (400 mg NIH-FSH-P1, Folltropin-V, Vetoquinol, Quebec, Canada) were administered with single dose syringes in semimembranosus or semitendinosus muscles of heifers by trained personnel from our laboratory. Heifers assigned to CIDR administration received an Eazi-Breed CIDR device (1.38 g progesterone; Zoetis, Parsippany, NJ) that had previously been used for 7 d via transvaginal insertion, then sanitized.

Blood samples were collected prior to ultrasound examination by puncturing the medial coccygeal artery or vein. Blood was collected into 10 mL Vacutainer clot tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately placed on ice and transported to the laboratory. Serum was separated after being centrifuged at 2,000 x g for 20 min. and frozen at -18^{o} C until

analysis for P4. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures described in this manuscript.

Experiment 3 Experimental Design: Effect of CIDR application on follicle recruitment and ovulations

Heifers (n=36) were randomly assigned to groups based on whether they received a CIDR and time of $PGF_{2\alpha}$ treatment. CIDR administration was divided between application of a CIDR or no CIDR. Heifers that received a CIDR had it inserted on d5 of the estrous cycle and removed on either D4 (CIDR early, C-E) or D5 (CIDR late, C-L) of treatment. $PGF_{2\alpha}$ administration was divided into early administration (PG-E, AM/PM of D1, and AM of D2) or late administration (PG-L, PM of D3, and AM/PM of D4). Therefore, heifers were evenly divided (n=36) into six treatment groups; PG late-No CIDR (PG-L/C-N), PG early-No CIDR (PG-E/C-N), PG late- CIDR-early (PG-L/C-E), PG early- CIDR-early (PG-E/C-E), PG late-CIDR- late (PG-L/C-L), and PG early- CIDR-late (PG-E/C-L). FSH administration was uniform with each animal receiving a ½ dose of FSH. A full dose is defined as the use of one 20 mL vial of Folltropin V containing FSH equivalent to 400 mg NIH-FSH-P1 during the FSH stimulation period. All heifers received a total of 10 mL FSH, divided into twice daily, decreasing doses, for four days (D1= 2.6 mL x 2, D2= 1.3 mL x 2, D3= 0.7 mL x 2, D4= 0.4 mL x 2).

Experiment 4 Experimental Design: Effect of 1/2 vs full dose FSH on follicle recruitment and ovulations

Heifers (n=16) were randomly assigned to groups based on FSH dose. The first group received a ½ dose of FSH while the second group received a full dose of FSH. A full dose of Folltropin V is equivalent to 400 mg NIH-FSH-P1 in a 20 mL vial. FSH was administered beginning on d 5 of the estrous cycle in decreasing doses, over four consecutive days totaling either

½ (10 mL) or full (20 mL) dosages. Doses were as follows: for the ½ dose group D1= 2.6 mL x 2, D2= 1.3 mL x 2, D3= 0.7 mL x 2, D4= 0.4 mL x 2 and for the full dose group D1= 5.2 mL x 2, D2= 2.6 mL x 2, D3= 1.4 mL x 2, D4= 0.8 mL x 2. Heifers received a CIDR on D1 and had it removed on D4 of the treatment cycle, morning of the last day of FSH treatment. $PGF_{2\alpha}$ was administered in 5 mL doses on D1 of treatment in the morning and evening and the morning of D2, to regress the present CL.

Blood samples were unavailable for one heifer on D1 in the ½ dose group and 2 heifers in the full dose group. Another sample was unavailable on D2 of treatment in the ½ dose group. These variations were accounted for on the specified days during analysis.

Ovarian ultrasonography

Ovarian structures (all visible follicles and corpora lutea) were mapped and measured as previously described by Martins et al. 2011, using a Sonosite MicroMaxx ultrasound machine with a linear array transducer utilizing 10 MHz frequency (Sonosite Inc., Bothell, WA). Each ovary was evaluated using the Cine function to view image frame by frame to count and measure follicles beginning on the 1st day of treatment then daily until ovulation then 3, 5, and 7 days after ovulation. Heifers were confirmed to have ovulated successfully to the $PGF_{2\alpha}$ injection by confirming the presence of a CL on D1 of treatment. Heifers from the larger pool of available animals without a d 5 CL and DF were not used in the studies, thus resulting in n=36 heifers in Experiment 3 and n=16 heifers in Experiment 4. Ovulation after CIDR removal was determined by the disappearance of one or more dominant follicles (≥ 9 mm) previously visualized at prior day's exam and the presence of a new CL 2 d later, day 3 post-ovulation.

Hormonal assay

Serum concentrations of P_4 were analyzed with a radioactive immunoassay validated by Engel et al., (2008). All sample concentrations were determined in one assay. The interassay CV was 8.15%, the intraassay CV was 4.84% and the sensitivity was 0.2 ng/mL.

Statistical analysis

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). Total number of follicles, number of follicles ≥ 9 mm, number of follicles ≥ 9 mm prior to the day of ovulation, number of ovulations and serum progesterone levels are reported as mean ± SEM.

For Experiments 3 and 4, total number of follicles and number of follicles ≥ 9 mm were measured before treatment (Day 1) and continuously measured at days 2, 3, 4 and 5 following treatment. Serum progesterone levels were assessed differently between Experiments 3 and 4. For Experiment 3, circulating progesterone levels were measured at days 1, 2, 3 and 4 in relation to time of treatment administration. Experiment 4 assessed progesterone levels at days 1, 2 and 3 after treatment, at ovulation day (day 5) and 7 days after ovulation. Total number of follicles, number of follicles ≥ 9 mm and serum progesterone levels were analyzed using the GLIMMIX procedure. The model included treatment, time and their interaction as fixed effects. A compound symmetry [cs] covariance structure was applied based on the lower Bayesian information criterion (BIC) score. Time was included within the RANDOM statement to account for measurements performed over time. Treatment nested within heifer was included in the SUBJECT option. Significant interactions were sliced utilizing the *%mult* macro of SAS. Multiple comparisons were calculated between treatments and within day following treatment.

Single measure variables were analyzed with one-way ANOVA on PROC GLM. Differences in number of ovulations were fitted with a model that included treatment and number of follicles ≥ 9 mm prior to the day of ovulation as fixed effects. Differences on number of follicles ≥ 9 mm prior to the day of ovulation only included treatment in the model. Differences in proportion of ovulations were analyzed via a simple t-test using the Excel data analysis function.

RESULTS

Experiment 3: Effect of CIDR application on follicle recruitment and ovulations

All heifers approximately doubled the total number of follicles in a 24 h period following the first FSH dose administration. Groups 1 (PG-L/C-N) and 6 (PG-E/C-L) had the largest number of stimulated follicles numerically throughout the treatment period. Total follicle numbers increased significantly in all groups after the first day (Figure 3.1) ($P < 0.001$).

No differences were present in numbers of follicles ≥ 9 mm on the day before ovulation. Heifers in Groups 1 (PG-L/C-N) and 4 (PG-E/C-E) had the most numbers of ovulations (Figure 3.2) and were not different from group 3 (PG-L/C-E) ($P > 0.05$). Group 2 (PG-E/C-N) had the least number of ovulations and did not differ from Group 5 (PG-L/C-L) ($P > 0.05$). Ovulations in group 5 (PG-L/C-L) and 6 (PG-E/C-L) did not differ ($P > 0.05$) also correlated with Groups 3 (3-PG-L/C-E) and 4 (PG-E/C-E) in a one way ANOVA comparison with Group 3 ($P > 0.05$). Group 2 heifers all ovulated the previously present dominant follicle by D4 of treatment. Three heifers ovulated on D3 of treatment and two heifers ovulated on D4 of treatment. A single heifer in group 2 did not ovulate until D5 of treatment. Group 4 (PG-E/C-E) had a higher ($P < 0.05$) proportion of ovulations compared to groups 2 (PG-E/C-N), 5 (PG-L/C-L), and 6 (PG-E/C-L) (Figure 3.2). The proportions

of ovulations for difference between Groups 1 (PG-L/C-N), 3 (PG-L/C-E) and 4 (PG-E/C-E) were similar ($P > 0.05$).

There was no difference in progesterone concentration between all groups on D1 of the treatment period ($P > 0.05$) except for group 6 which was an elevated outlier that still shared similarity with groups 1, 4 and 5 (Figure 3.3). By Day 2 of treatment groups receiving a CIDR maintained significantly higher levels of progesterone ($P < 0.05$), Groups 3, 4, 5 and 6, than the group that did not receive a CIDR and was administered PG (Group 2). Groups that either received a CIDR and $PGF_{2\alpha}$ (Groups 4 and 6) or maintained their CL (Group 1), thus having only a single source of progesterone, had similar concentrations amongst themselves. Group 2, having no source of progesterone, had significantly lower levels of progesterone than all groups receiving a CIDR (groups 3, 4, 5 and 6). Group 2 was still similar to Group 1 (PG-L/C-N) on Day 2 ($P > 0.05$), but this correlation was no longer evident by Day 3 of treatment. On Day 3 of treatment all groups maintaining a source of progesterone were statistically similar. On Day 4 of treatment all groups receiving a CIDR had similar concentrations of progesterone ($P > 0.05$), Groups 3, 4, 5 and 6. All groups maintaining a single source of progesterone, either CL or CIDR, had similar concentrations of progesterone ($P > 0.05$), Groups 1, 2, 4 and 5. Group 2 now falls into this group, likely due to the fact that all but a one heifer in the group had ovulated the previously present DF by Day 4 of treatment and was beginning to maintain a new CL. By Day 5 of treatment the heifers with CIDR remaining, Groups 5 and 6 maintained similar concentrations of progesterone ($P > 0.05$). While heifers no longer maintaining a source of progesterone were similar with one another, Groups 1, 2, 3 and 4. Interestingly, Group 5, although maintaining numerically higher levels of progesterone compared to the lower progesterone groups did not differ from them on this day. We suspect this correlation is due to the low numbers of heifers in this group and maintain our hypothesis that the

CIDR present is sufficiently maintaining elevated progesterone levels. Progesterone samples were unavailable for one heifer in group five and one heifer in group six and thus they were excluded from the analysis. Single day samples were also unavailable for one heifer in group 5 on day 2, one heifer on day 4 in groups 4 and 6, and one heifer on day 5 in groups 3 and 4.

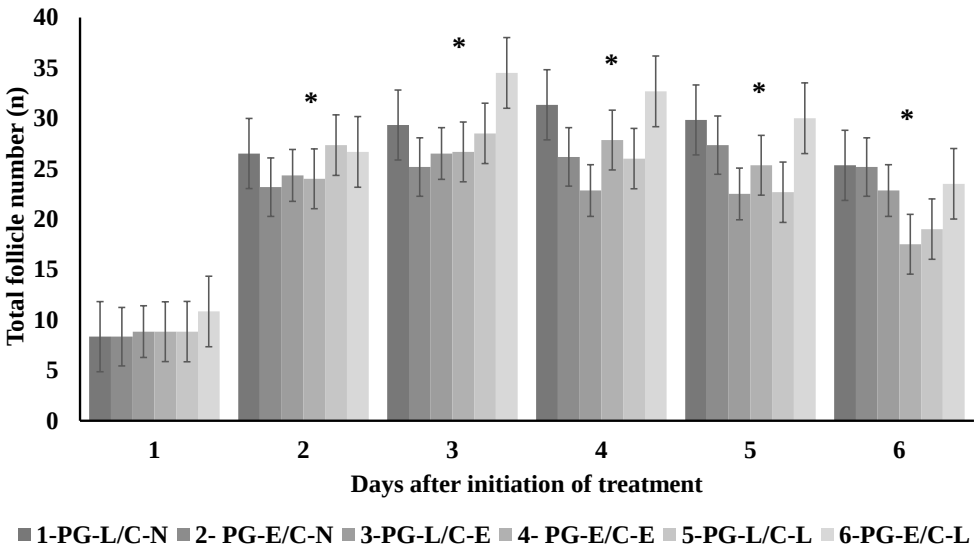

Figure 3.1: Effect of varying CIDR application and PGF2α administration during stimulation with half doses of FSH on ultrasound measurements of total number of follicles on both ovaries.
* Denotes significant difference between follicle number and Day, P <0.001

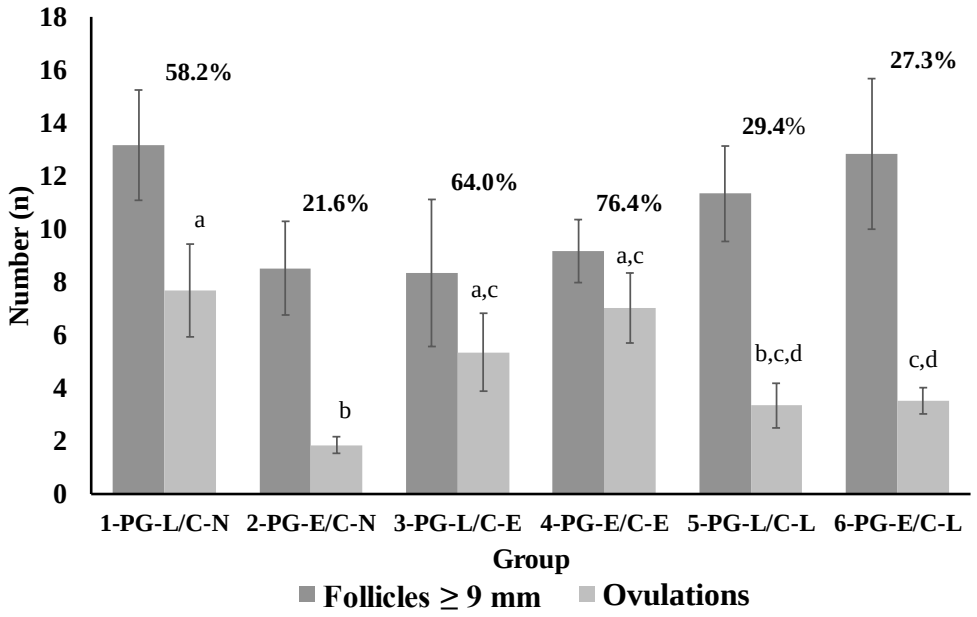

Figure 3.2: Effect of varying CIDR application and PGF2α administration during stimulation with half doses of FSH on proportions of ovulations and follicle numbers ≥ 9mm on day before ovulation.
* Denotes significantly different proportion of ovulations, P <0.05.
a,b,c,d Denotes significantly different numbers of ovulations in a one-way ANOVA, P <0.05. Groups sharing the same denotation were not different.
No difference in the number of follicles on day before ovulation in any group, P >0.05

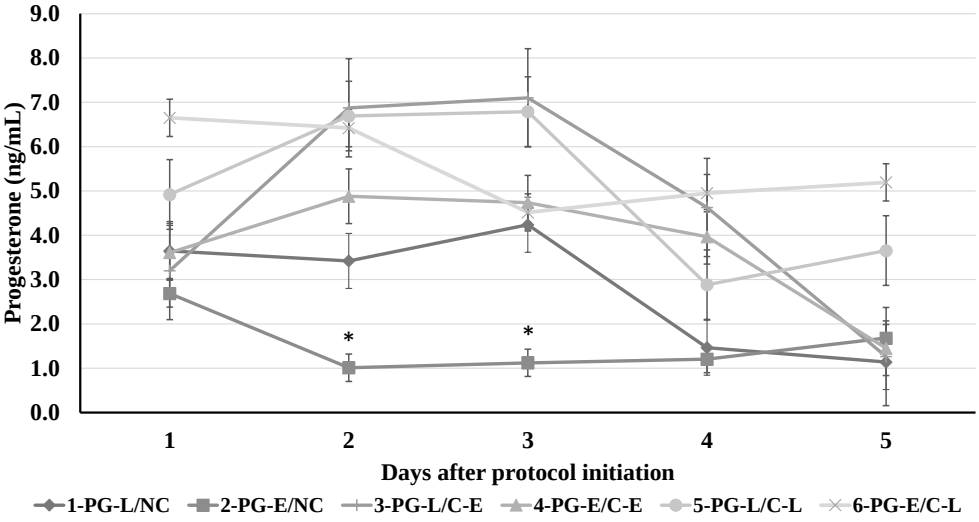

Figure 3.3: Effect of varying CIDR application and PGF2α administration during stimulation with half doses of FSH on progesterone concentrations by day.
* Denotes significantly difference concentration of progesterone on day, P <0.05

Experiment 4: Effect of 1/2 vs full dose on follicle recruitment and ovulations

There was an effect of day between the numbers of follicles on D1 compared to the progressive treatment days (P <0.001). Total number of follicles were similar throughout the treatment period for heifers treated with ½ dose and full dose FSH (Figure 3.4).

Although follicle number ≥ 9 mm on the day before ovulation was higher (P <0.05) in the full compared with the ½ dose group, ovulation number and rate were similar (Figure 3.5). Progesterone concentrations after ovulation were higher (P <0.05) only on day 7 after ovulation for heifers treated with the full compared with ½ dose (Figure 3.6). Samples were unavailable for one heifer on D1 in the ½ dose group and 2 heifers in the full dose group. Another sample was unavailable on D2 of treatment in the ½ dose group. These variations were accounted for on the specified days during analysis.

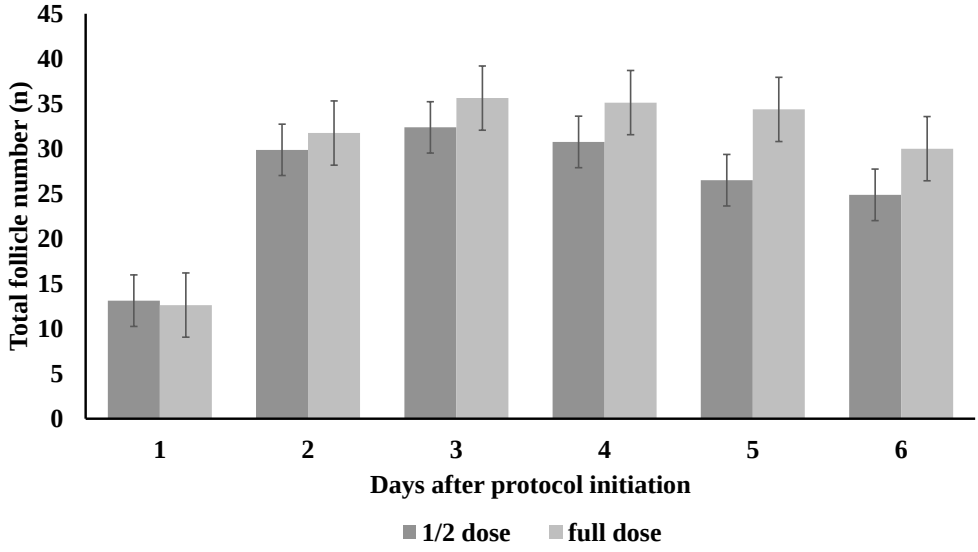

Figure 3.4: Effect of CIDR and ½ or full doses of FSH during superovulation on ultrasound measurements of total number of follicles on both ovaries. Day 1 is the first day of four decreasing amounts for each treatment group (1/2 dose n=8, full dose n=8)
* Denotes significant difference between follicle number and Day 1, P <0.001

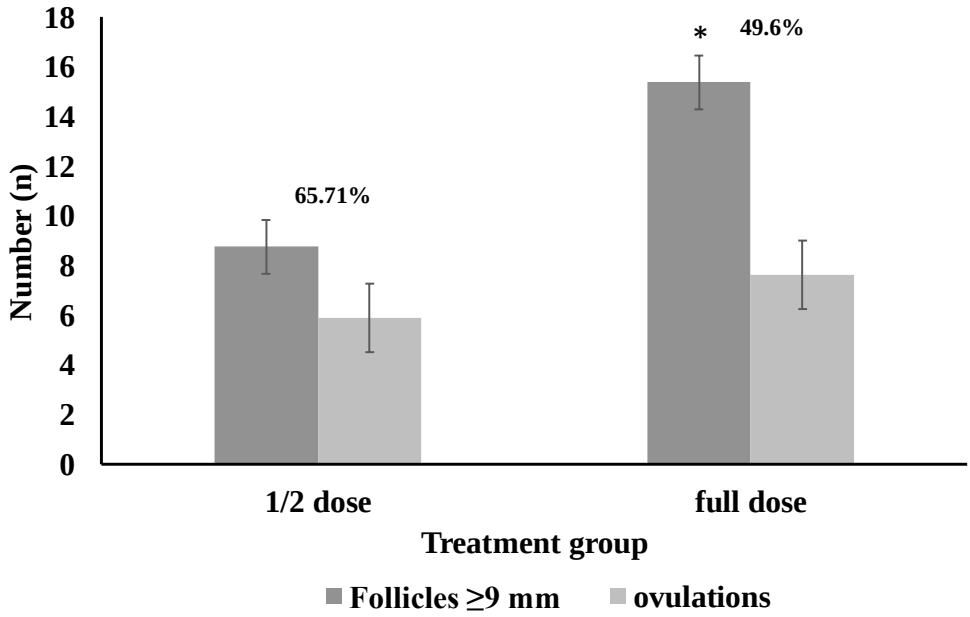

Figure 3.5: Effect of ½ or full doses of FSH on proportions of ovulations and follicle numbers ≥ 9mm on day before ovulation. (1/2 dose n=8, full dose n=8)
* Denotes significantly different follicle, P <0.05

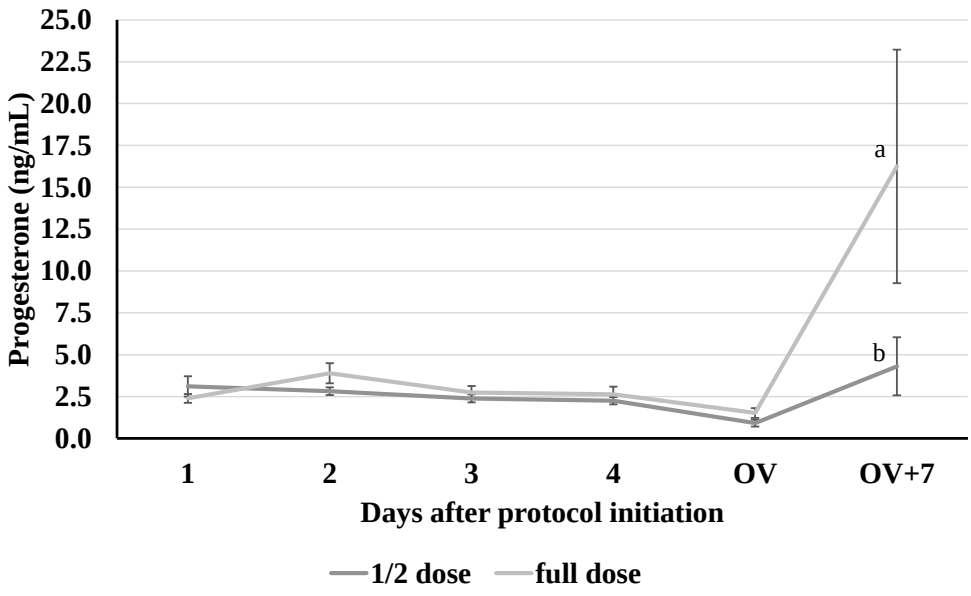

Figure 3.6: Effect of ½ or full doses of FSH during super ovulation of heifers on progesterone concentration by day of treatment. (1/2 dose n=8, full dose n=8)
OV is the day of ovulation and OV+7 is 7 days post-ovulation.
a,b Denotes statistical differences in concentrations of progesterone, P <0.05

DISCUSSION

Our studies aimed to investigate whether manipulation of progesterone concentration to induce a lower progesterone environment at key follicular development points during superstimulation would produce numbers of ovulatory follicles and ovulations and whether these techniques could be utilized with ½ doses of FSH and maintain satisfactory numbers of ovulations. The most significant findings of these studies were that moderate levels of progesterone, with removal of influence shortly after the final FSH dose, results in the highest proportion of ovulations. The data also confirmed that ½ doses of FSH resulted in equivalent numbers of ovulations with our modified protocols compared to full doses and industry standards.

It has been well documented that progesterone concentration plays a key role in the development of growing follicles (Adams et al, 1992). High circulating concentrations of progesterone can prolong growth of the DF and increase time to new wave emergence, while lower concentrations can result in premature ovulations (Adams et al, 1992; Burke et al., 1994). Theories for this phenomenon depend on the change in negative feedback on LH pulsatility. Higher magnitudes of progesterone concentration result in decreased LH pulsatitlity (Bergfeld et al., 1996; Fike et al., 2004). While removal of progesterone influence results in increased LH release correlating with the magnitude of the change in concentrations (Fike et al., 2004). Luteinizing hormone is responsible for the maturation of the ovulatory follicle and a final LH surge induces ovulation (Stumpf et al., 1991). Estradiol concentrations play a role in this feedback system as increased 17b-estradiol concentrations positively influence the magnitude of LH pulses (Stumpf et al., 1989; Cupp et al., 1995a). Manipulation of this tri-folded feedback mechanism results in changes in follicular development patterns and ovulatory response. Our experiments modified

progesterone concentrations during superstimulation protocols, likely affecting this trinity of steroid response.

Protocols in Experiment 3 resulted in three lengths of progesterone influence during the stimulation period, short (PG-E/C-N), moderate (PG-L/C-N, PG-L/C-E, and PG-E/C-E), and long (PG-L/C-L and PG-E/C-L). The short progesterone environment resulted in premature ovulation in all heifers (n=6) in Group 1 (PG-E/C-N). Premature being defined as ovulation prior to cessation of FSH administration. Early removal of the CL and lack of CIDR application likely allowed for a premature LH surge and ovulation of the present d 5 DF in the absence of sufficient progesterone to suppress LH activity. These findings were similar to those by Bednar and Pursley, 2000 where groups without supplemental progesterone ovulated the present DF before maturation of the FSH-stimulated follicles and subsequently did not experience multiple ovulations. Meanwhile, prolonged progesterone presence in both CIDR-late groups (PG-L/C-L and PG-E/C-L) likely had an influence on their low ovulation numbers. CIDRs were not removed until 24 h after the final FSH treatment in these groups. Circulating progesterone from the CIDRs were much greater than expected. The intent of utilizing used CIDRs was to provide a low P4 environment allowing sufficient LH pulses to keep follicles $\geq$ 9 mm in diameter growing but also control timing of the LH surge. This would ensure the greatest numbers of follicles possible to reach the ovulatory pool of follicles. Circulating P4 was likely too high in these studies to allow for sufficient LH pulses. Therefore, developing follicles may have turned over and become atretic, thus unable to ovulate. Although both CIDR-late groups (PG-L/C-L and PG-E/C-L) had large number of ovulatory sized follicles, less than 30% successfully ovulated. This is in stark contrast to the three moderate length progesterone groups that had progesterone influence removed by the day of final FSH administration (PG-L/C-N, PG-L/C-E, and PG-E/C-E). These groups all successfully ovulated

over 58% of their follicles ≥9 mm with the PG-E/C-E heifers ovulating close to 80% of their ovulatory sized follicles (76.4 ± 10.2% (SEM)). These data suggest that prolonged suppression of LH pulsatility in the CIDR-late groups resulted in follicular turnover and inability of follicles to respond to the resulting ovulatory LH surge after progesterone influence was removed. This correlates with the findings of Adams et al., 1992 who demonstrated that prolonged exposure to increased progesterone levels during the growing phase of follicular development resulted in premature turnover of new wave follicles.

Previous studies by our group (Ahearne et al., 2020) had answered the question of proper dose administration protocol (decreasing, twice daily dosing) and Experiment 3 determined the appropriate timing of $PGF_{2\alpha}$ treatment and CIDR administration (PG-early/CIDR-early). We utilized these protocols to re-test ½ dosing versus full dosing of FSH. There was no significant difference between the ½ doses or full doses on numbers of ovulations even though the full dose of FSH stimulated more follicles to reach ovulatory size. Although ovulation rate was not significantly different, 50% of ovulatory sized follicles did not ovulate in the full compared with the ½ dose group. The reason is unclear, but failure to ovulate is a common occurrence following most superovulation protocols. Several possible explanations for failure of ovulatory size follicles to ovulate are feasible. Firstly, elevated amounts of estradiol, that has been well documented in superovulated animals (Monniaux et al, 1983), from the increased number of large follicles in the full dose group may have a negative influence on follicular development at the level of LH receptors resulting in an inability of some ovulatory follicles to respond to the LH surge. Another postulation is that the higher amount of FSH is directly causing atresia of ovulatory follicles in the full dose group perhaps by decreasing LH receptor formation.

The second experiment revealed that the progesterone concentrations 7d post ovulation were different between the ½ dose and full dose groups. The full dose group had significantly higher levels of progesterone than the ½ dose group. It is unclear why the ½ dose group seems to have modulated CL function post ovulation despite similar numbers of ovulations.

In summary, these data reiterate the importance of appropriate steroid levels in the development of follicles during superovulation protocols. Future experiments would be useful in investigating the effects of these new protocols on LH activity and fertilization rates. The post-ovulation progesterone levels do raise the question as to how the low levels noted in the ½ dose group may influence early embryonic development. Further investigation into these modified protocols will continue to answer these important questions and aid in making these techniques more widely applicable on the modern dairy farm. We can conclude that the newly developed protocol can reliably stimulate a large number of follicles with acceptable numbers of ovulations via manipulation of progesterone concentrations throughout stimulation and using only ½ doses of FSH

CHAPTER 4

CONCLUSION

INTRODUCTION

The primary objective of this thesis was to increase ovulation rates in Holstein heifers following superovulation and to characterize the effect of varying pharmaceutical strategies on daily follicular development and numbers of ovulations. Once characterized, this information was used to develop a protocol to maximize ovulation rates with ½ doses of FSH while maintaining ovulation rates on par with industry standards (AETA, 2018 census).

CURRENT STATUS OF SUPEROVULATION

Superovulation and embryo transfer have been utilized in the dairy industry on a commercial scale since the early 1970's, as outlined in numerous reviews (Betteridge, 1981; Hasler, 2014; Mapletoft et al., 2002), following the first successful transfer in cattle performed by Willett et al. in 1951. Since that time, many efforts have been put forth to improve techniques and maximize profitability. These investigations answered questions about which gonadotropin formulations and dosages were superior for stimulation (Cole and Hart, 1930; Rowson, 1951; Gonzalez et al., 1990; Alkemede et al., 1993) as well as the appropriate timing for dose administration (Lindsell et al., 1986; Fricke et al, 1994). Over the years, protocols have become more streamlined as many embryo transfer programs have adopted a common method. Current protocols involve the administration of a full 20 mL vial containing 400 mg NIH-FSH-P1(Folltropin V, Vetoquinol, Quebec, Canada), in decreasing doses, over four to five consecutive days beginning on day 8 to 10 of the donor estrous cycle.

Despite close to 50 years of commercial use, embryo transfer techniques continue to produce low numbers of viable embryos. Currently, the AETA reports that an average of 5.3 viable embryos are produced with each embryo recovery procedure. These numbers have stayed consistent over the years despite development of more advanced reproductive management

techniques. While average numbers of oocytes have remained consistent, the response of individual animals continues to be highly unpredictable (Mapletoft, 2002). In order to continue to advance the applications of embryo transfer technologies we must develop protocols that reduce the variability between animals by qualifying and then maximizing follicular recruitment and ovulation rates.

POTENTIAL FOR ADVANCEMENT

Throughout this thesis we investigated some of the key physiologic components of superovulation. In Chapter 2 we characterized follicular development under the influence of various doses and application procedures of FSH. We successfully induced a new wave of stimulated follicles under the influence of a d5 DF, a hurdle that had been previously disputed (Adams et al., 1993; Guilbault et al., 1991; Ko et al., 1991). Our data indicated that not only were we able to induce a new wave of follicles, we were also able to induce comparable numbers of ovulation with ½ doses of FSH compared to full doses. The second investigation in Chapter 2 confirmed that the industry standard of twice daily, decreasing doses of FSH results in the greatest number of ovulations. These investigations then raised questions about the ovulatory response of superstimulated follicles to varying levels of progesterone during stimulation.

Chapter 3 experiments set out to answer questions about the physiologic effects of progesterone modulation at different stages of stimulation and explore the potential implications on protocol development. Data from Experiment 3 demonstrated that moderate levels of progesterone, with removal of influence shortly after the final FSH dose, resulted in the highest proportion of ovulations. Experiment 4 then confirmed that ½ doses of FSH are adequate to maintain ovulation numbers on par with full doses. This suggests that producers may be able to

utilize lower doses while still recovering a predictable number of embryos. The specific protocol is outlined in Figure 4.1, indicating timing of procedures and FSH dosing.

In conclusion, investigations throughout this thesis successfully developed protocols to increase ovulation rates in response to superovulation. Many questions were raised about the deeper physiologic implications of steroid manipulation during superovulation and thus, open new and exciting avenues for future investigation. It is our hope that this research can serve as a new step-off point for future experiments and that the developed protocol can aid in increasing reliability of active embryo transfer protocols.

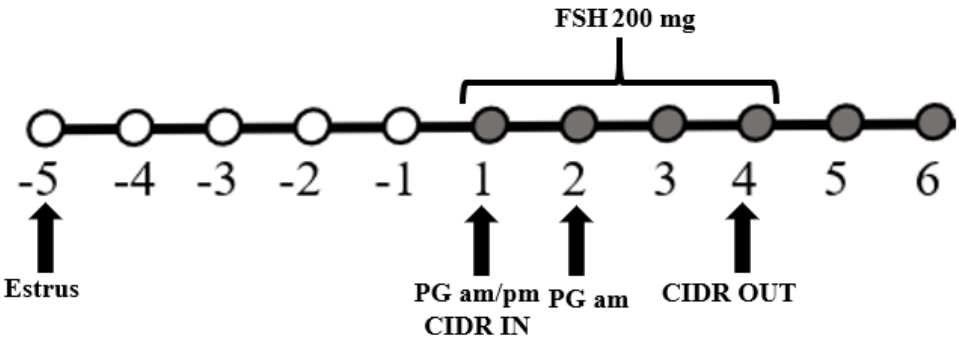

Figure 4.1: A diagram outlining the proposed CIDR SO protocol. FSH administration is as follows: 2.6 mL, 1.3 mL, 0.65 mL, 0.45 mL (administered twice daily on the indicated d).

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