COMPARISON OF PREGNANCY OUTCOMES IN DAIRY HEIFERS ARTIFICIALLY INSEMINATED WITH SEXED SEMEN DEPOSITED IN THE UTERINE HORNS VERSUS THE UTERINE BODY

By

SARA JOYCE KIRKS

(Under the Direction of Roberto A. Palomares)

ABSTRACT

Utilization of sex-sorted semen in dairy heifers has become a frequent topic of research due to its positive attributes to a herd, but the full adoption of this technology into practice has been overshadowed by low economic gain due to lower conception rates compared to conventional semen. In this study, deep intra-cornual insemination ipsilateral to the ovary with the preovulatory follicle was used to evaluate the effect of site of semen deposition with sexed semen on pregnancy per timed artificial insemination (P/TAI). The results show that deep intra-cornual insemination resulted in greater P/TAI compared to deposition in the uterine body (56.7%; 44.32%: P = 0.038). Different inseminators had an effect on P/TAI (P = 0.09), and there was a higher numeric tendency for pregnancy in the left versus the right horn (66.3%; 53.9%). This suggests that deep intra-cornual insemination may help overcome the lower P/TAI previously achieved with sex-sorted semen.

INDEX WORDS: Sex-sorted semen, intra-cornual insemination, artificial insemination

COMPARISON OF PREGNANCY OUTCOMES IN DAIRY HEIFERS ARTIFICIALLY INSEMINATED WITH SEXED SEMEN DEPOSITED IN THE UTERINE HORNS VERSUS THE UTERINE BODY

By

SARA JOYCE KIRKS

B.S., The University of Georgia, 2017

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

COMPARISON OF PREGNANCY OUTCOMES IN DAIRY HEIFERS ARTIFICIALLY INSEMINATED WITH SEXED SEMEN DEPOSITED IN THE UTERINE HORNS VERSUS THE UTERINE BODY

By

SARA JOYCE KIRKS

Major Professor:	Roberto A. Palomares
Committee:	Pedro Melendez Maria S. Ferrer

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

DEDICATION

This thesis is dedicated in loving

memory to my grandfather Larry Larson.

Thank you for everything Opa-z.

To my parents Amy and Rusty,

whose constant and unwavering

support carried me through this process.

To my fiancé Reed,

whose love and comfort never ceased,

and whose encouragement never failed

to get me back on track.

ACKNOWLEDGMENTS

I would like to express my greatest gratitude to my major professor Dr. Roberto Palomares for his encouragement, patience, support, and guidance throughout my years before and during this program.

I would also like to give my gratitude to Dr. Pedro Melendez and Dr. Maria S. Ferrer for their guidance and support as my committee members throughout my time at the University of Georgia.

I am grateful to everyone at the University of Georgia, College of Veterinary Medicine who helped shape my graduate career, with special thanks to the members of the Group for Reproduction in Animals, Vaccinology, and Infectious Diseases (GRAVID) and also to Lisa Norris for her help and advice in both graduate studies and life.

Thank you to Southern Comfort Dairy LLC for allowing us to perform this research at your farm.

A special thanks to my family and friends for their support and encouragement throughout my graduate career. Without you all I would be lost.

Many thanks to my Athens Church small groups throughout the years, with a special thanks to Marilou Braswell. I would not be who I am today without you.

Lastly, I thank God for being a constant companion and the ultimate encourager through the highs and lows. My strength alone did not get me here and I will continue following where you lead me.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Advanced reproductive technologies (ARTs) can be used to improve reproductive performance and the economic efficiency of cattle operations. One such technology is the utilization of sex-sorted semen instead of conventional, unsexed semen when artificially inseminating heifers. Although this technology has been around for nearly 30 years, more recent advancements in biochemical laboratory procedures and high-throughput machinery has made it more easily accessible to those looking to increase their herds genetic gain through genomic selection (Vishwanath and Moreno, 2018). The introduction of sexed semen into the herd's breeding program allows for a more bio-secured herd expansion as all heifers will most likely originate from that same farm and also decreases the number of low value bull calves born which also plays a role in cow welfare. With the advancements in technology today, there is a roughly 90% reliability in the predetermination of calf sex when utilizing sexed semen (Vishwanath and Moreno, 2018).

While sexed semen does have the potential of becoming a widely adopted technology throughout both beef and dairy operations, conception rates continue to fall below those of conventional semen. The majority of studies evaluating sexed semen performance have found that conception rates from sexed semen only achieve 70% to 80% of conception rates seen with conventional semen, which is a main reason why sexed semen is mostly used in heifers as a non-pregnancy does not affect the lactating herds' milk production (Butler et al., 2014). The actual sorting process and subsequent cryopreservation of the sperm are two likely reasons for the

reduced fertility, as it has been shown that fresh, non-sorted semen has a lower incidence of acrosome reacted sperm compared to cryopreserved, sex-sorted semen (Mocé et al., 2006). The sorting process also contains an upwards of 20 steps the sperm must endure before being cryopreserved compared to the three or four steps non-sorted sperm go through before being cryopreserved (Vishwanath, 2014). This makes using sexed semen not as acceptable as conventional semen, as a reduction in fertility reduces any financial gain that could benefit the farm (Holden and Butler, 2018). One technique that could possibly alleviate that negative impacts on fertility from sexed semen is deep intra-cornual insemination. This technique is routinely used in mares and bitches with adequate success. The logic behind this technique is that since sex-sorted, cryopreserved semen have a higher percentage of acrosome reacted sperm it does not require as much time in the female reproductive tract to achieve oocyte fertilization. Semen deposition in closer proximity to the site of fertilization and also closer to the time of ovulation may increase the pregnancy per AI with sex-sorted semen. Other studies have been conducted using this rationale, and most all of them have contradictory results and final conclusions. The current thesis comprises a thorough literature review about reproductive physiology, ovulation synchronization and timed artificial insemination of heifers (Chapter 2) and a field study to elucidate the effect of the site of sexed-semen deposition on pregnancy outcomes in dairy heifers (Chapter 3). The results of this study can contribute to the growing field of sexed semen technology and its application, and how site of semen deposition can affect pregnancy per TAI in heifers. A better understanding of breeding protocols and tools that maximize reproductive performance of heifers may directly lead to increase the farm profitability and open the door to state new hypotheses on the lactating herds.

CHAPTER 2

LITERATURE REVIEW

Puberty

Puberty is the stage of life when an animal reaches reproductive competence (Senger, 2012). This typically happens over a period of time rather than one specific point during the animal's life cycle. Puberty in females can be defined by the age of first estrus, the age of first ovulation and/or the age that they can successfully maintain cyclicity and carry a pregnancy, without risks. Although these criteria are helpful in determining puberty in the female, there is not one single chronological age that all pre-pubertal female cattle reach puberty, rather a physiological age. The onset of puberty can be affected by different environmental and social cues, along with certain genetic factors which lead to differing time periods a pre-pubertal female may reach physiological sexual maturity (Senger, 2012).

Nutrition, weight, and body composition affect age at Puberty

The time period at which puberty is reached depends on age and weight among other factors and is variable among different breeds of cattle with a range of 9 to 24 months across species and breeds of cattle (Perry, 2016).

Table 2.1: List of average ages that puberty is reached in female cattle of different breeds (Senger, 2012).

Average Age of Female at Puberty Among Different Cattle Species					
Breeds	Holstein	Brown Swiss	Angus	Hereford	Brahman
Age (Months)	8-10	12	11-12	13	19

Several studies have shown that in order for a pre-pubertal heifer to reach sexual maturity, she must have an adequate body weight (Perry, 2012). Across different breeds, heifers reached puberty when they were between 55% and 60% of their breeds' mature body weight (Patterson et al., 1991; Curtis et al., 2018). Heifers that developed slower and reached this weight range when they were older did not attain sexual competence as early as heifers who were younger when they reached this body weight range (Perry, 2016). Another study showed that there is no significant difference in pregnancy rate for pubertal heifers achieving between 55% to 65% of mature body weight. However, heifers at 55% body weight had less pregnancies compared to heifers at 65% of mature body weight. Moreover, lighter weight heifers also had a longer postpartum period, which lengthened their return to their first estrous cycle (Perry, 2012).

In order for heifers to be reproductively efficient throughout their lives, the optimal age for conception should be 13 to 15 months of age (Gasser et al., 2006). This timing relies heavily on when heifers reach puberty, as they must be at a certain weight and body composition so that the hypothalamic-pituitary (HP) axis can initiate a higher pulse frequency of gonadotropin-releasing Hormone (GnRH) which initiates puberty (Alves et al., 2017). One study stated that 4 to 8 months on age is the critical window of obtaining adequate nutrition and body weight increase for activation on of the HP axis in heifers to induce an earlier age of puberty (Alves et al., 2017). Body weight at weaning can also impact age of puberty according to some studies (Buskirk et al., 1995; Gasser et al., 2006). One study found that as body weight at weaning increased, so did the probability of beef heifers reaching puberty before their first breeding season and conceiving from their first insemination (Buskirk et al., 1995). Gasser et al. (2006) reported that early weaning and feeding a high concentrate diet to beef heifers can induce an early puberty, and this early puberty is preceded by an increasing frequency of LH pulses.

Another study found that weaning at 3 to 4 months of age and supplementing with a high concentrate diet until 7 months of age results in 50% of the heifers reaching puberty at an earlier age compared with heifers that were not fed the diet (Day and Anderson, 1998). Adequate nutrition and subsequently adequate body weight and composition are pivotal factors in reaching puberty. Any inadequacies such as poor nutrition in the critical window during the pre-pubertal stage and low body weight at weaning may result in a delayed puberty, delayed first breeding, and could lead to a delayed return to estrus post-calving. Neglecting to reach puberty at an appropriate time is the major reason that heifers do not conceive during their first ovulation synchronization treatment and breeding (Yelich et al., 1996).

Initiation of Puberty

Before a young heifer calf can reach puberty, multiple endocrinological and synaptic events must occur to drive estrus, ovulation, and subsequent normal luteal function (Day and Anderson, 1998). The onset of puberty can be delayed if the hypothalamus does not secrete an adequate amount of GnRH to cause the production of gonadotropins (Senger 2012). The hypothalamus contains two important structures that directly affect the initiation and onset of puberty in female cattle. One structure is called the tonic GnRH center and the other is the preovulatory GnRH center or surge center. The surge center must be fully developed before ovulation can occur. The Gonadostat hypothesis describes the process of the initiation of puberty as a decreased sensitivity of the hypothalamic-pituitary-gonadal (HPG) axis to the inhibitory feedback of estradiol which eventually stimulates a Luteinizing hormone (LH) surge and ovulation (Atkins et al., 2013; Kenny et al., 2018). In the pre-pubertal female, low frequency GnRH pulses from GnRH neurons in the tonic center result in low frequency pulses of follicle stimulating hormone (FSH) and LH from the anterior pituitary gland, secreted at a basal level.

That is, the low frequency pulses of GnRH do not stimulate a high frequency release of FSH or LH from the anterior pituitary gland, which is needed for the development of follicles on the ovaries. Follicles on the ovaries are responsible for gonadal estradiol secretion. Once the concentration of estradiol increases and surpasses a certain threshold concentration, the surge center is triggered to release high amplitude pulses of GnRH. The pre-pubertal ovary does not secrete an adequate amount of estradiol to stimulate the preovulatory GnRH surge by itself. The tonic center is highly sensitive to negative feedback, so that low concentrations of estradiol are able to inhibit the HP axis, resulting in low levels of GnRH, and consequently low frequency and amplitude FSH and LH secretions. During the transition to puberty, the tonic center's sensitivity to estradiol steadily decreases and as a result more GnRH is released, which increases the frequency that LH and FSH are released, along with increased estradiol concentrations and an increase in follicle growth on the ovaries (Senger, 2012.; Atkins et al., 2013). Once concentrations of estradiol reach a threshold level, the high amplitude preovulatory GnRH surge occurs, which then stimulates a surge of LH released from the anterior pituitary gland. This event leads to the first ovulation and the subsequent beginning of the estrus cycle in pubertal heifers.

HPG Axis Role Throughout the Estrous Cycle and Hormones Affecting Puberty

In female cattle, the hypothalamic-pituitary-gonadal axis or HPG axis controls the production of hormones throughout the stages of the estrous cycle using feedback mechanisms by mean of communication between the hypothalamus, the pituitary gland, and the ovaries. Positive and negative feedbacks produce specific responses of activation or deactivation of the hormones produced in the organs of the axis. As the hypothalamic tonic center's estradiol sensitivity decreases, the concentration of estradiol in the bloodstream causes an increase in GnRH concentration. This continual increase in concentration leads to the preovulatory GnRH

surge that then causes the LH surge, which is required for juvenile female cattle to ovulate and begin cyclicity. This feedback system and its maturation facilitates an earlier sexual development in cattle (Kenny et al., 2018). The maturation of this reproductive neuroendocrine axis is defined by the episodic release of LH that happens in response to the preovulatory GnRH surge (Macedo et al., 2019).

Estradiol Receptors, ARC and Kisspeptin

The main deciding factor of when juvenile female cattle reach puberty is when the tonic center becomes less sensitive to estradiol negative feedback. The estrogen receptors in the different areas of the hypothalamus trigger the cascade of events that ultimately leads to ovulation. One study observed that as heifers got closer to puberty, estrogen receptors in the hypothalamus and pituitary gland decreased (Day et al., 1987). Another study found a decrease in estrogen receptors in the anterior and the medial basal areas of the hypothalamus was associated with an increase in LH pulse frequency (Atkins et al., 2013). These findings are consistent with the Gonadostat hypothesis, as it states that the sensitivity to estrogen in the hypothalamus decreases as the animal reaches the pubertal transition. Estrogen receptors are pivotal in signaling the beginning of puberty, as one study using estrogen receptor alpha (αER)-KO mice showed that estrogen receptor -α, or ESR1 is the primary mediator of estradiol signaling that controls the episodic release of gonadotropin (Dorling et al., 2003). The hypothalamic neurons contain most of the estrogen receptors within the HPG axis, with most of these neurons being located in the medial preoptic area (MPOA), anterior hypothalamus (AH), ventrolateral septum, the bed nucleus of the *stria terminalis*, the ventromedial hypothalamus (VMN) and the arcuate nucleus (ARC) (Atkins et al., 2013).

The arcuate nucleus plays an important role in the regulation of metabolic and reproductive functions by mediating signals from peripheral tissues to different regions of the brain (Kenny et al., 2018). The ARC region also contains neurons dealing with metabolic sensing through neuronal and glial circuits that regulate GnRH release and GnRH pulse regulation (Alves et al., 2017; Kenny et al., 2018). Although estrogen receptors are key in signaling the preovulatory GnRH pulse, GnRH neurons that signal this pulse do not contain estrogen receptors (Atkins et al., 2013; Amstalden et al., 2014). A neuropeptide called kisspeptin is found in the hypothalamus and it is able to stimulate GnRH neuronal activity and gonadotropin secretion (Ahmed et al., 2009). Neuropeptides like kisspeptin are regulators of multiple physiological processes like reproduction, energy metabolism, circadian rhythm and can even help to regulate body temperature homeostasis. Neural and glial cells produce hypothalamic neuropeptides, which make up most of the cellular population, target different neurons at the hypothalamus and are released into the HP portal system. This is the circulation network by which hormones can be transported to and from the pituitary gland without being diluted (Senger, 2012) in order to stimulate or inhibit the generation and/or release of hormones in the pituitary gland (DeAtley et al., 2018). Kisspeptin neurons are classified as an important contributor to the cellular pathway by which estradiol regulates the secretion of GnRH (Amstalden et al., 2014). The ARC region of the hypothalamus contains a large number of kisspeptin neurons, with most of these neurons containing estrogen receptors as shown in Figure 2.1 (Atkins et al., 2013). Kisspeptin neurons within the ARC region are key elements to GnRH pulse generation, as they stimulate GnRH neurons in and around the neurosecretory zone (Kenny et al., 2018). Macedo et al. (2019) found that an injection of kisspeptin to heifers induce a quicker LH release as compared to heifers given a GnRH agonist injection. Therefore, kisspeptin

neurons may act as an intermediate effector of the feedback control of estradiol on GnRH secretion, which affects the beginning of puberty and could potentially also be a part of the mechanisms that changes the hypothalamus' sensitivity to estradiol as an animal reaches puberty; though more research needs to be done to validate this theory (Amstalden et al., 2014; Kenny et al., 2018).

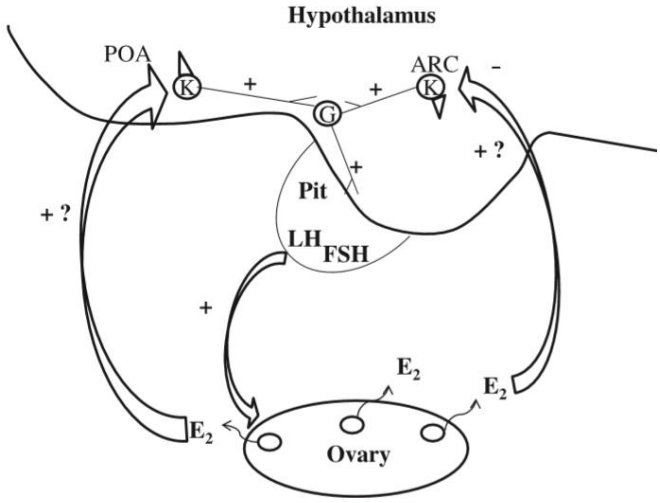


Figure 2.1: This figure depicts a hypothetical HPG axis and the roles of kisspeptin. Before puberty, estradiol (E_2) from the ovaries bind to their receptors (triangles) in the hypothalamus to create the negative feedback. As the animal progresses towards puberty, the number of estrogen receptors diminishes, which could increase kisspeptin (K) concentration within the hypothalamus that could then lead to kisspeptin stimulating the GnRH neurons (G). This cascade would then stimulate the release of LH and FSH from the pituitary gland (Pit) which stimulates follicle growth and more estradiol secretion that would create a positive feedback loop within the system. (Atkins et al., 2013).

The GnRH receptors at the anterior pituitary gland do not change over time or as the animal ages. These GnRH receptors are able to cause the release of gonadotropins in response to GnRH in heifers as young as one month old. The pituitary holds ample concentrations of LH and

FSH throughout the animals' pre-pubertal stage, and as the heifer grows and gets older and after the GnRH stimulus has occurred, higher concentrations of these gonadotropins are released. Theoretically, with the stored concentrations of LH and FSH, the pituitary gland itself could induce follicular growth and ovulation before puberty by secreting these hormones. While the pituitary gland may be able to function this way, estradiol negative feedback still remains the limiting factor for GnRH, FSH and LH secretions (Atkins et al., 2013).

Leptin Feedback

As stated in a previous section, nutrition plays a vital role in the age of onset of puberty in both male and female cattle. A study in beef heifers revealed that dietary energy restrictions lowered LH pulse frequencies and delayed puberty onset (Yelich et al., 1996). These authors also observed that increasing energy intake to heifers previously submitted to a nutrient restrictive diet increased frequency of LH pulses within two weeks of diet improvement, which led to a quicker onset of puberty (Yelich et al., 1996). The amount of adipose tissue an animal has may play a role in and affect the mechanisms that control the age of puberty. Adipose tissue contains adipokines (cytokines secreted by adipose tissue) that serve as paracrine and endocrine hormones that regulate functions such as appetite, satiety, fat distribution, and blood pressure, among others. They can also send signals to the brain and a plethora of metabolic and neuroendocrine tissues (Kenny et al., 2018).

Leptin, one such adipokine, has been demonstrated to have a very important function in beef cattle with regards to the control of the onset of puberty (D'Occhio et al., 2019). The more adipose tissue an animal has, the more concentration of leptin is released into the bloodstream. Kisspeptin is sensitive to nutritional changes and plays a pivotal role in the timing of the onset of puberty (Amstalden et al., 2014; Kenny et al., 2018). Leptin uses the receptor GPR54 present on

kisspeptin neurons in the hypothalamus to stimulate the kisspeptin release that leads to the maturation of the GnRH neurons. As these pre-pubertal heifers age and grow, they accumulate more adipose tissue which creates a positive feedback loop between adipose tissue, leptin and kisspeptin release (D'Occhio et al., 2019). While leptin may have a role in kisspeptin concentration and therefore GnRH neurons' maturation, leptin concentration is not a limiting factor for the onset of puberty. One study found that in ruminants, leptin will only signal an endocrine response if the animal had previously been fasted or had chronic low or negative energy balance. Researchers also observed that serum leptin concentration increased the closer heifers came to puberty, but a change in diet did not alter leptin concentrations when total carcass fat was similar between animals. They concluded that the determinant of an endocrinological response to leptin depends on the established metabolic state of the animal (Kenny et al., 2018).

Season affects pubertal transition in heifers

Cattle are polyestrous, which means they have the ability to be bred year-round. Although theoretically this is true, it has been shown that the age at which heifers go through the pubertal transition may be influenced by the season of the year that they are born in. Heifers born in the spring and fed a high plane of nutrition attained puberty earlier than heifers born in the fall (Moran et al., 1989). This could be due to the fact that supplemental lighting, like that found in longer photoperiods during the summer months has been shown to decrease the age at first ovulation in heifers (Moran et al., 1989; Rius et al., 2005). Schillo et al. (1983) performed a study raising heifers born in March or September in natural conditions until six months of age, and then the animals were moved to controlled environmental chambers until 12 months of age (with either spring-summer-autumn or autumn-winter-spring seasonal characteristics). They

observed that heifers born in September reached puberty earlier than those born in March. But between the two environmental chambers, those kept in the spring-summer-autumn conditions reached puberty quicker than both the March- and September-born heifers combined. This study showed that environmental factors such as season do play a role in age of puberty (Schillo et al., 1983).

Reproductive Tract Scoring

A useful tool to help determining whether or not puberty has been achieved in heifers is a reproductive tract scoring (RTS) system. An RTS can be performed via transrectal palpation or with the help of ultrasonography. The RTS system was designed to help estimate the stage of maturation in heifers to determine which heifers are good candidates for ovulation synchronization and breeding (Holm et al., 2015; Kasimanickam et al., 2016). When this system is implemented, it allows producers and veterinarians to screen and select heifers from a herd based on pubertal status in order make appropriate decisions about each heifer within the herd for the benefit of the herd reproductive performance and farm profitability. Reproductive tract scoring is replicable between people and is an accurate way to measure pubertal status within herds (Holm et al., 2009). The RTS system is based on ovarian follicular development and maturation, the presence of a *corpus luteum* (CL) and the estimated size of the reproductive tract. Once measurements are taken, heifers are given a score between 1 and 5, with a 1 meaning immature or prepuberal stage and a 5 meaning mature or pubertal stage (Kasimanickam et al., 2016). An RTS is typically performed four to six weeks before the start of breeding programs as this is an opportune time to give prebreeding vaccinations (Atkins et al., 2013). Table 2.2 gives an example of the measurement parameters used when evaluating heifers using the RTS system.

Table 2.2: Uterine and ovarian measurements and descriptions for reproductive tract scores (Atkins et al., 2013).

RTS	Classification	Uterine Horn Diameter(mm)	Ovarian Length(mm)	Ovarian Height(mm)	Ovarian Width(mm)	Structures
1	Prepubertal	<20, no tone	15	10	8	No palpable follicles
2	Prepubertal	20-25, no tone	18	12	10	8mm follicles
3	Peripubertal	20-25, slight tone	22	15	10	8-10mm follicles
4	Pubertal	30, good tone	30	16	12	>10mm follicles, CL may be present
5	Pubertal	>30	>32	20	15	CL present

Estrous Cycle Phases and Stages

In cattle, the average length of the estrous cycle is 21 days but can normally be between 17 and 24 days (Kojima, 2003; Boer, et al., 2010). The estrous cycle is divided into two distinct phases: a luteal phase and a follicular phase. These phases are each controlled by different dominant ovarian structures, and different dominant hormones are produced to exert positive and negative feedbacks on the HPG axis. The primary ovarian structure of the follicular phase is the preovulatory follicle on the ovary, with estradiol as the main hormone. The primary ovarian structure that controls the luteal phase is the *corpus luteum* (CL), with progesterone being the main hormone produced (Senger, 2012; Adams and Singh, 2014). The luteal and follicular phases can be broken further into four stages, with two stages per phase. The follicular phase is characterized by the proestrus and the estrus stages, and the luteal phase is characterized by the metestrus and diestrus stages (Adams and Singh, 2014). The luteal phase comprises 80% of the duration of the estrous cycle, while the follicular phase only accounts for 20% of the estrous cycle (Senger, 2012).

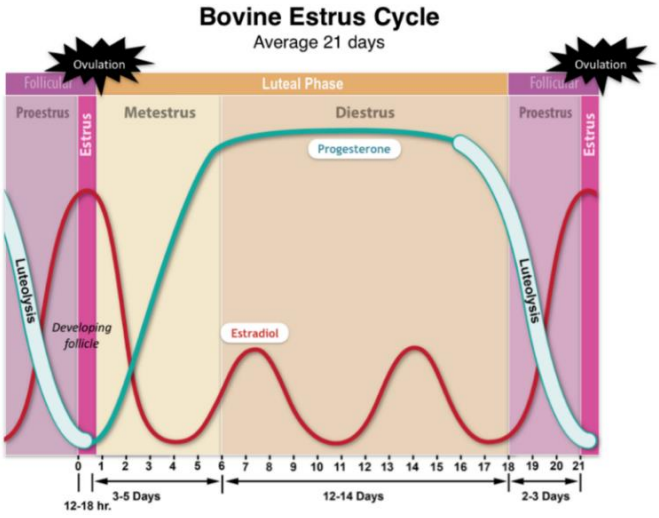


Figure 2.2: This figure depicts the estrous cycle in cattle. The average days per stage is listed along with the names of each stage. The dominant hormones progesterone and estradiol's concentrations in each stage are also shown. (Palomares, 2019).

Day 1 of the estrous cycle begins with the estrus stage. During this stage estradiol concentrations increase until a threshold level has been reached. Once this threshold has been surpassed, the high concentrations of estradiol signal GnRH neurons in the hypothalamus to secrete large amounts of GnRH, which then stimulate the anterior lobe of the pituitary gland to secrete a preovulatory surge of LH, inducing ovulation (on metestrus). These increased levels of estradiol cause female cattle to display estrus behaviors until full sexual receptivity, or 'standing to be mounted', occurs. Follicular maturation and ovulation along with the absence of a functional CL are also characteristics of the estrus stage (Senger, 2012; Adams and Singh, 2014). The estrus stage lasts an average of 12 to 18 hours and continues with ovulation during metestrus. Before ovulation, the basement membrane of the preovulatory follicle that separates the granulosa and theca interna cells begins to deteriorate and allows the intermingling of these two cell types to begin. Both of these cell types are responsible for steroid production within the follicle before ovulation (Downey, 1980). Ovulation concludes the follicular phase of the estrous

cycle and starts the luteal phase beginning with the metestrus stage. After the follicle's wall collapses and begins to fold, the granulosa and theca interna cells continually mix along with the remnants of the basement membrane. The cells are transformed into luteal tissue via the process of luteinization under the influence of LH, while the remaining basement membrane forms the connective tissue network of the CL (Peters, 1985; Senger, 2012).

Metestrus typically starts after estrus on day 2 and proceeds through day 5 of the estrous cycle. This stage is characterized by the formation of the transitional structure known as *corpus hemorrhagicum* (CH) and the beginning of an increase in progesterone secretion (Adams and Singh, 2014). This transitional structure received this named because of the rupture of small blood vessels around the site of ovulation, and it appears as a blood clot on the ovary (Senger, 2012). The CH luteinizes during metestrus and starts producing progesterone as it develops into a CL. The third stage of the estrous cycle that directly follows metestrus is diestrus. The diestrus stage accounts for the majority of the estrous cycle and can last from 10 to 14 days in cattle. This stage is characterized by a fully functional CL and high progesterone secretion and lasts until luteolysis of the CL, which stops progesterone secretion (Senger, 2012). During mid-diestrus, luteal cells undergo hypertrophy, an increase in cell size and volume density, which coincides with peak progesterone production (Adams and Singh, 2014). It has also been found in the mid-luteal phase that a release of progesterone follows LH pulses which indicates an association between LH pulses and progesterone secretion (Quintal-Franco et al., 1999). The high levels of progesterone signal the uterus to prepare an environment for early embryo development and subsequent embryo implantation to the endometrium. The amount of time the CL remains functional and produces progesterone is directly related to how long the female remains in the diestrus stage (Senger, 2012). When the theca interna and granulosa cells luteinize, they

transform into two new types of luteal cells. From the granulosa cells come the large luteal cells and from the theca interna cells come small luteal cells, but as the CL progresses through the cycle the small luteal cells differentiate into large luteal cells under the influence of LH (Kojima, 2003). There is a sharp decrease in estradiol around the time of ovulation as the ovulatory follicle collapses. The main role of the CL is to produce progesterone to prepare the uterine environment for embryo implantation, block successive ovulations of dominant follicles during diestrus and to maintain the gestation (Kojima, 2003). The formation and lysis of the CL are very dynamic processes within the estrous cycle (Adams and Singh, 2014). The amount of plasma progesterone concentration secreted by the CL during the early developmental stages is positively correlated with the weight, volume and histomorphology of the CL. The CL grows in size during its formation and subsequently regresses during proestrus and it has been shown that the progesterone concentrations follow the same pattern of increase and then decrease (Veronesi et al., 2001; Adams and Singh, 2014). Large luteal cells also secrete oxytocin which can affect the functionality of the CL. Oxytocin action is mediated by oxytocin receptors housed along the membrane of the CL and can stimulate prostaglandin ($PGF_2\alpha$) secretion. Oxytocin secretion supports luteolysis since it is a regulator of the amplitude of pulsatile $PGF_2\alpha$ secretion (Kotwica et al., 1998). Prostaglandin F2 α is produced by the endometrial cells after oxytocin stimulation and is transported from the uterine vein to the ovarian artery through a countercurrent mechanism (Senger, 2012).

Immediately following lysis of the CL due to $PGF_2\alpha$ secretion from the uterus, the fourth stage of the estrous cycle, proestrus, begins and the cycle is now considered to be back in the follicular phase (Adams and Singh, 2014). Proestrus typically begins 3 days before the onset of estrus. As progesterone secretion decreases due to lack of a functional CL, estradiol secretion

increases and becomes the dominant hormone as a dominant follicle grows. The pre-ovulatory follicles that have been growing begin to mature until it is ready for ovulation (Peters, 1985). Secretion of GnRH, and subsequently FSH and LH are responsible for this transition into proestrus. As estrogen levels continue to increase and an antral follicle achieves complete dominance, the female transitions from proestrus to estrus and the cycle begins again at day 1 (Hurnik et al., 1975).

Estrous Cycle and Behavioral Changes in Response to Puberty

As heifers mature and reach puberty, the changing levels of hormones within their system bring distinct behavioral changes that allow to determine whether or not a heifer is receptive to mating. This behavior is known as 'showing estrus or heat'. The term estrus is synonymous with the term heat in regard to the phase of the estrous cycle when female cattle display estrous behavior and will be used alternately throughout this literature review. The timing of this estrous behavior is tied to each animal's estrous cycle.

In order for a heifer to have successfully reached sexual maturity, it must have an estrus phase followed by a normal luteal phase (Burns et al., 2010). 'True estrus' is defined as when a cow or heifer stands immobile when mounted by a bull or another female within the herd. This standing behavior is the most dependable and most distinct visual sign of sexual receptivity and is the best indicator that a cow or heifer is in the pre-ovulatory stage of their cycle (Orihuela, 2000). The interval between the first sign of standing behavior and ovulation is approximately 27 hours, while the duration of estrus from first standing event and last standing event has been recorded 7-18 hours (Boer et al., 2010). Within those 7 hours of recorded estrous behavior, there are multiple other behaviors that can also be displayed by female cattle that show readiness to mate. Examples of these behaviors are showing a Flehmen response, sniffing the vulva of other

herd-mates, mounting but not standing, resting their chin on the backs of other herd-mates, attempting to mount another herd-mate, attempting to mount the head side of another herd-mate, and then standing heat (Boer et al., 2010). Other secondary characteristics that may indicate a female is in heat are the swelling and reddening of the vulva and mucus discharge from the vagina. The change in appearance of the vulva occurs before heat and for a short time after heat and is not the best indicator of estrus. The clear mucosal discharge from the vagina is an indirect result of an increased circulating amount of estrogen and its effects on the cervix. This mucus production happens before, during and after estrus. The discharge might also not be seen externally unless the female is palpated and the mucus along with other fluids excreted from the cervix are pushed out (O'Connor, 1993). Females that are in heat are also more restless than those not in heat and are more alert to their surroundings and will also follow other females around in order to attempt mounting them. Although these secondary characteristics are not a guaranteed sign of an animal in heat, animals displaying these signs should be carefully monitored until a standing heat or 'true estrus' has been exhibited (O'Connor, 1993). Not all female cattle display all of these behavioral signs, but as the frequency and level of sexual behavior increases, the closer to the time of mating receptivity the female is.

Multiple factors can affect when a female will come into heat, and these factors can also affect how, when, and the frequency at which females actually display estrous behavior. Their environment, health and nutrition, housing and herd-mate activity can positively or negatively affect a female's cyclicity and the onset of estrus (O'Connor, 1993). Herd interaction is very important for estrous behavior displays. Herd interaction allows female cattle to show mounting and chin resting behavior, which are both signs of the onset of estrus. The floor surface material where females are housed also play an important role in mounting behavior (Britt et al., 1986). A

slippery surface is more prone to females attempting to mount and falling due to lack of sturdy footing. A study conducted in North Carolina concluded that in cows housed on dirt flooring the number of mounting activity and standing behavior doubled compared to concrete flooring (Britt et al., 1986). Another study conducted with heifers at Perdue University found that standing and mounting behavior was higher in bedded areas compared to animals housed in the dry lot and free stall areas (O'Connor, 1993). Poor body condition, infection, parasitism, poor nutrition, anemia, and nutrient deficiencies may also result in a higher number of anestrous cattle and therefore no estrous behaviors would be displayed. Overfed or undernourished beef cows showed a delayed postpartum oestrus compared to healthy, good body conditioned beef cows (Orihuela, 2000). Environmental factors such as weather, temperature and day length also affect cyclicity and estrus timing within female cattle. Heavy amounts of rain, wind and high humidity have been shown to suppress estrus-related behaviors in female cattle. Extended periods of high temperatures that can lead to heat stress have also been reported to shorten the duration of and reduce the intensity of estrus behaviors (Orihuela, 2000). Late fall and early winter months also reduced the intensity and amount of mounting behaviors events (Wolfenson et al., 1988). According to one study done in Canada, the time of the day also affects the mounting activity among cows. They observed that throughout a 24-hour period, 70% of all mounting behavior occurred during the hours of 7 pm and 7 am (O'Connor, 1993). This suggests that during cooler periods of the day there is an increase in estrous behavior compared to hotter periods of the day.

Follicular Dynamics

Follicular growth and subsequent atresia occur continuously throughout the estrous cycle (Noseir, 2003.; Senger, 2012). Follicular development is dependent upon the levels of FSH and LH, and these follicles follow a specific pattern of growth and degeneration known as follicular

waves. Most estrous cycles consist of two or three follicular waves, and this will determine the cycle length (Adams, 1994). The process of folliculogenesis includes four stages: recruitment, selection, dominance, and atresia (Senger 2012). The follicular waves can happen during the majority of reproductive states of female cattle including the prepubertal period, the estrous cycle, gestation and postpartum anestrous (Smith et al., 2010). In cattle, the total number of primordial follicles are present in the ovaries at birth. These primordial follicles are housed in non-growing or static pools within the ovary. Before puberty is attained, follicles are recruited from the static pool into a growing pool of small follicles (Roche and Boland, 1991). Once the female transitions from pre-pubertal to pubertal status, folliculogenesis occurs and continues during each estrous cycle as the animal begins cyclicity. FSH is the main hormone that initiates the follicular waves (Kanitz, 2003). At the beginning of each wave, a cohort or group of antral follicles are recruited from a pool of small follicles (around 3 mm diameter) and continue to grow and begin to secrete estradiol in response to elevated FSH levels (Senger, 2012). One follicle (less frequently two) is selected and becomes dominant, while most of the recruited follicles undergo atresia or degeneration (Kanitz, 2003). During the time where selected follicles grow from 3 to 5-mm, they secrete larger amounts of estradiol and inhibin which suppress FSH secretion from the anterior pituitary, and also inhibit other follicles from growing (Kanitz, 2003). The suppression of smaller follicles is thought to be caused by not only the inhibitory effects of estradiol and inhibin from the larger follicles on FSH, but also a reduced blood supply to the smaller follicles. The more blood supply a follicle has, the better chance it has of receiving higher levels of gonadotropin, and the larger it will grow (Senger, 2012).

The last stage of folliculogenesis is when the dominant follicle undergoes atresia or ovulation based on the stage of the estrous cycle the animal is currently in. As these antral

follicles continue to grow, they become more dependent on LH and less dependent on FSH. While one follicle deviates from the rest of the now subordinate follicles and develops to 8-mm in diameter, the growth rate difference between the two largest follicles becomes more apparent and the transition from FSH to LH dependency also becomes evident. Granulosa cells from the dominant follicle have a greater LH binding ability compared to granulosa cells on the subordinate follicles. This greater LH responsiveness is associated with a greater mRNA expression and translation of LH receptors (Adams and Singh, 2014). Follicles can successfully ovulate once they reach around 10-mm in diameter, but ovulation majorly depends on LH secretion and these larger follicles require larger amounts of LH in order to induce ovulation (Kanitz, 2003). The main roles of LH are to stimulate the maturation and ovulation of the pre-ovulatory antral follicle, and also the formation and maintenance of the CL (Peters, 1985). Whether or not the dominant follicle ovulates or undergoes atresia depends on where in the estrous cycle the follicle is in and which hormone is mostly dominant. The CL plays a role in which path the dominant antral follicle takes. As previously stated, the CL maintains high levels of progesterone, which provides a negative feedback on pulsatile LH secretion from the anterior pituitary. Without high LH concentrations, necessary to induce the ovulation, the dominant antral follicle becomes atretic and the stage of follicular recruitment begins again (Kanitz, 2003; Kojima, 2003). The last follicular wave that leads to ovulation of the dominant follicle occurs after luteolysis, and the decline of progesterone which no longer inhibits GnRH and subsequently FSH-LH secretion from hypothalamus and pituitary, respectively (Senger, 2012). When levels of progesterone decrease during proestrus and estrus, estradiol and LH concentrations increase along with FSH, which creates an increase in GnRH concentrations in the hypothalamus that eventually cause the preovulatory LH surge (through a positive feed-back) that causes ovulation

of the dominant pre-ovulatory follicle. The new high frequency of LH pulses allows the dominant antral follicle to grow until it reaches ovulation. Once ovulation of the dominant follicle occurs, a new follicular wave may begin (Smith et al., 2010). This cycle is shown in Figure 2.3.

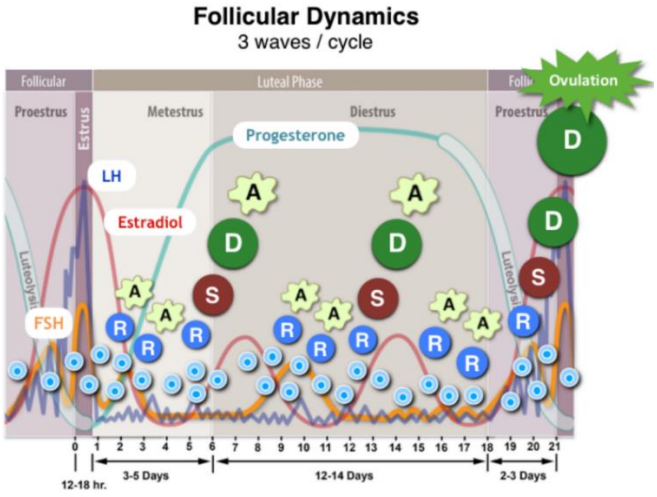


Figure 2.3: Graphical depiction of three follicular waves followed by ovulation based on stage of the estrous cycle. (Palomares, 2019).

Two-wave versus three-wave follicular dynamics

Multiple studies have found that over 95% of cattle typically have two or three follicular waves during their estrus cycle (Adams, 1994; Kanitz, 2003; Noseir, 2003; Smith et al., 2010; Adams and Singh, 2014) with some reporting up to four waves in some cattle. While there appears to be no age or breed specific predisposition for number of waves in *Bos taurus*, it has been shown that an increase in the proportion of females with a three-wave pattern have been associated with heat stress (Badinga et al., 1993; Wolfenson et al., 1995). Dairy heifers have more commonly three follicular waves, while cows have two (Adams and Singh, 2014). Cattle (beef and dairy) with a three-wave pattern generally have a longer estrus cycle compared to cattle displaying a two-wave pattern (20-24 days and 18-20 days, respectively) (Kojima, 2003;

Smith et al., 2010). On average, follicular waves are first detected as 4-5 mm follicles on days 0 and 10 of the estrous cycle in two-wave females and on days 0, 9 and 16 of the estrous cycle in three-wave animals (Noseir, 2003). The difference in length and number of the follicular waves affects the age and size of the ovulatory follicles produced, and this difference is thought to result in a lower fertility rate among the two-wave animals (e.g. dairy cows) compared to the three-wave animals (e.g. dairy and beef heifers). Since the ovulatory follicle in two-wave animals is older and larger compared to the ovulatory follicle in three-wave animals, the two-wave ovulatory follicle should produce more estradiol for a longer duration of time and in slightly larger amounts after luteal regression. One study revealed that a higher estradiol concentration during days 14 and 17 of the estrus cycle had a negative effect on pregnancy rate (Ahmad et al., 1997).

There is a correlation between the number of follicular waves and the duration of follicular dominance of wave 1. The onset of regression of the dominant follicle occurs later in two-wave animals compared to three-wave animals, and this dominance was also associated with a delay in time taken for the dominant follicle of wave 2 to reach maximum diameter along with being associated with an early onset of luteolysis (Adams and Singh, 2014). Therefore, heifers which have more often three follicular waves have shorter follicular waves and faster follicular turn over between waves. This physiological phenomenon has influenced the hormonal protocols to synchronize the ovulation of dairy heifers in an attempt to reach adequate pregnancy rates. In addition, factors that affect the growth and development of the dominant follicle in wave 1 regulate the wave pattern as a whole (Adams and Singh, 2014). The size and maturity of the largest preovulatory follicle at the time of $PGF_{2\alpha}$ administration to induce luteolysis can influence the time interval from $PGF_{2\alpha}$ injection to estrus and ovulation (Kastelic et al., 1990;

Kojima, 2003). Thus, cattle treated with $PGF_{2\alpha}$ during mid-diestrus had a longer interval from injection to estrus compared to cattle treated during early or late diestrus (Kastelic et al., 1990; Larson et al., 1992).

Estrus and Ovulation Synchronization in Dairy Heifers

Multiple hormonal protocols have been developed to manipulate the estrus cycle in an attempt to improve fertility and increase reproductive efficiency of cattle farm practices. Protocols used for estrus synchronization include exogenous hormonal application accompanied by either visual heat detection and artificial insemination (AI) or a timed artificial insemination (TAI). Although a cow standing to be mounted is the most accurate and reliable sign of estrus, 37 to 54% of detected ovulations are not preceded by standing estrus in Holstein cows and the rate of successful visual observation of a standing estrus is thought to be variable between 38% to 86% (Chanvallon et al., 2014). Synchronizing the estrus cycle in cattle helps to create a fixed, short, and predefined period of time to breed, which allows for scheduled calving periods, improving the reproductive efficiency and economic returns to producers (Islam, 2011). In most production levels and management systems, the optimal calving interval for a lactating dairy cow is around 13 months. A longer calving interval may result in economic losses due to reduced milk production per day, larger involuntary culling of lower producing cows and smaller replacement heifer herd size (Larson et al., 1992). Estrus-ovulation synchronization can shorten the time period of breeding from a 21-day to less than 5-day span depending on the protocol used, which facilitates the production of a uniform replacement calf crop and also produces a uniform calving interval (Larson et al.,1992; Islam, 2011). Estrus-ovulation synchronization also facilitates the use of AI and embryo transfer (ET), which allows cattle producers to incorporate

superior genetics into their herds without the costs of purchasing and maintaining the genetically superior animals themselves (Lamb et al., 2010).

These protocols are used to synchronize the maturation of the dominant follicle with the onset of regression of the corpus luteum (Smith et al., 2010). A successful synchronization has the ability to better control the precise time of ovulation without needing to detect estrus so that only TAI is necessary (Islam, 2011). The development of different synchronization protocols for sexually mature dairy females typically centers around inducing the regression of the corpus luteum by utilizing the hormone $PGF_{2\alpha}$ and produces adequate fertility, but the injection does not affect animals in the first 5 to 6 days of their estrus cycle which can undermine the overall synchronization rate (Smith et al., 2010; Yapura et al., 2018). Most protocols used for estrus synchronization use one or a combination of different forms of exogenously produced hormones with the most widely used ones including Gonadotropin Releasing Hormone (GnRH), $PGF_{2\alpha}$, and Progesterone.

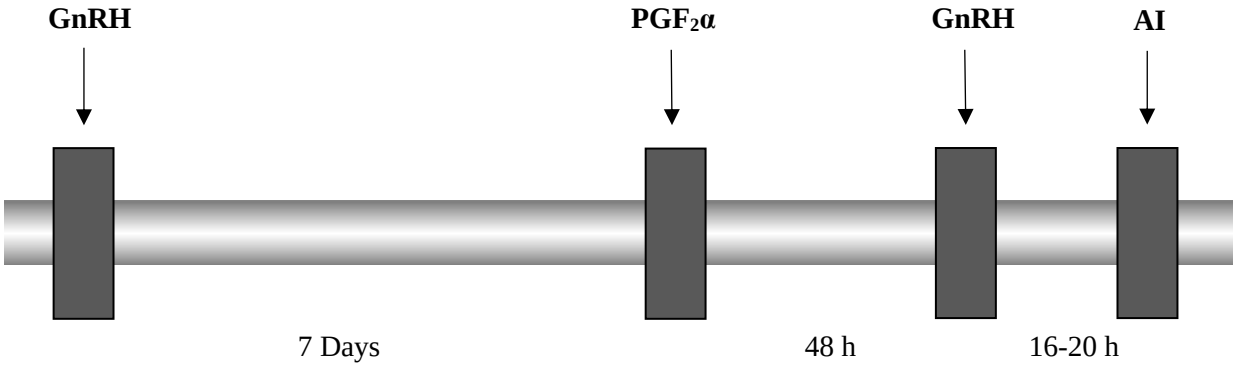
Each hormone plays a different role in estrus synchronization and each also targets specific reproductive organs to form a cascade of effects on the estrus cycle. The initial injection of GnRH acts on the anterior lobe of the pituitary gland to signal the surge release of LH from the anterior pituitary, and this LH surge induces ovulation (or luteinization) of the dominant follicle already present on the ovaries and formation of a CL. This initiates a new follicular wave, which would ensure the presence of a new young growing follicle containing a viable and competent oocyte for fertilization. The CL secretes progesterone which acts on the hypothalamus, uterine endometrium, and mammary glands to inhibit more GnRH secretion from the hypothalamus through a negative feed-back. The injection of $PGF_{2\alpha}$ according to most synchronization protocols lyses the CL and stops the secretion of progesterone. This removes the

negative feed-back on the HPO system, so that pulses of GnRH, FSH and LH start increasing in frequency and amplitude. Finally, an injection of GnRH is intended to help synchronizing the ovulation. This sequence of events allows dairy cattle to have a better synchronized insemination time which allows producers obtaining greater pregnancy per AI, as most of the animals included into an estrus synchronization protocol will ovulate around the same time. (Islam, 2011; Senger, 2012).

There are multiple different protocols used by producers that utilize at least one of the hormones listed above. The majority of dairy farmers tend to either subject their whole herd to TAI or combine the use of TAI and insemination at estrus detection together as a way to facilitate management at the time of increasing fertility. Applying estrus synchronization and TAI to a herd increases the number of cows eligible for AI and also reduces the number of days to an animals first insemination and days to first conception (Tibary et al., 2019). Two of the most common protocol groups are called Ovsynch and Cosynch. Both of these protocols include TAI, being Cosynch a modified version of Ovsynch (Carabă et al., 2013). Cosynch program is commonly combined with the use of exogenous progesterone (Cosynch + CIDR) and can be modified according to different protocol lengths, i.e. 4-day, 5-day, 7-day. In the Ovsynch protocol, a GnRH injection is given at the beginning of the protocol and seven days later an injection of $PGF_2\alpha$ is given to lyse the formed CL. Forty eight to 56 hours after the $PGF_2\alpha$ injection, another injection of GnRH is administered to cause ovulation of the dominant follicle that had formed. Finally, TAI is performed 16-20 hours after the last GnRH injection. The Cosynch protocol follows the same steps as the Ovsynch protocol, but the last GnRH injection is administered at the moment of TAI, which occurs 62 hours after $PGF_2\alpha$ injection (rather than 16 hours after GnRH administration) (Carabă et al., 2013). There are no differences in pregnancy

rates between the two programs (Carabă et al., 2013) and a 7-day Ovsynch protocol remains the most common program for synchronization of lactating dairy cows (Stevenson, 2016), but the reasoning for the modification was to decrease the amount of times producers would have to work their cattle (Stevenson, 2005; Alkar et al., 2011). Outlines of each protocol are illustrated below in Figure 2.4.

Ovsynch Protocol



Cosynch Protocol

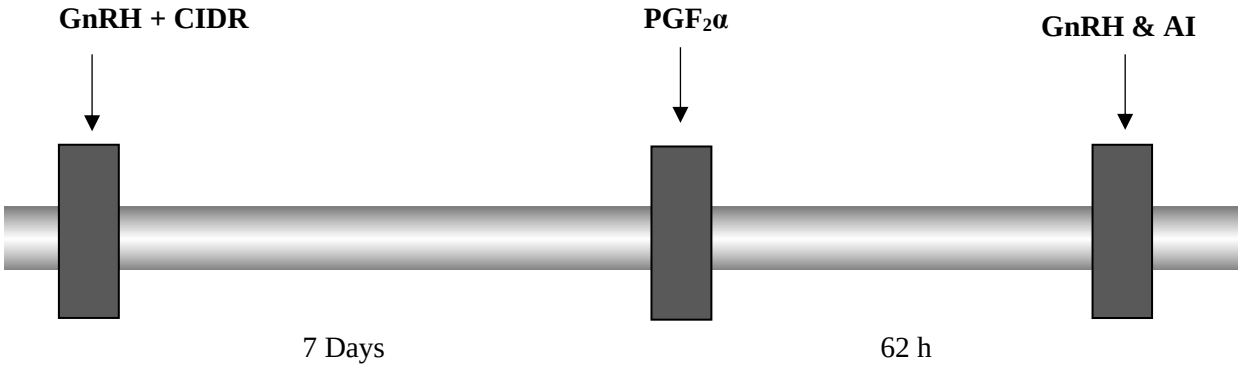


Figure 2.4: Depiction of a typical Ovsynch and Cosynch timeline (adaptation from Carabă et al., 2013).

Ovsynch and 7-day Cosynch protocols have multiple advantages and disadvantages. Advantages include allowing for the synchronization of an entire herd; it is applicable in all cows; it reduces the need for heat detection, and it shortens the voluntary waiting period and intercalving interval (Nowicki et al., 2017). In a three-experiment study done by Pursley et al.

(1995) researchers submitted lactating dairy cows and heifers to an Ovsynch protocol where animals received a GnRH injection on Day 0, a $PGF_{2\alpha}$ injection on day 7, a second GnRH injection 48 and 24 hours later for lactating cows and heifers, respectively; and TAI 24 hours after the second GnRH injection. They reported that 18 of 20 cows and 13 of 24 heifers had ovulation and formation of a new or accessory CL after the first GnRH injection, and this injection was also found to have initiated a new follicular wave in 100% of the cows and 75% of the heifers. Moreover, CL regression was found in 100% of the cows and 75% of the heifers after the $PGF_{2\alpha}$ injection. They also reported that all cows and 75% of the heifers ovulated a newly formed dominant follicle between 24 and 32 hours after the second GnRH injection. The study showed that 50% of the cows inseminated by TAI were pregnant (Pursley et al., 1995).

Disadvantages to the Ovsynch protocol include an increased embryonic mortality rate, increased cost of the hormones needed, possibility of hormonal treatment and AI in females with reproductive disorders, the difference in hormonal responses between individual cattle, low efficiency when the program is not initiated between days 5 and 9 of the estrus cycle, and very poor fertility in heifers. Dairy heifers submitted to an Ovsynch and TAI protocol have typically had conception rates of 20% to 40% lower than heifers bred via estrus detection (Schmitt et al., 1996; Pursley et al., 1997). This poor fertility could be due to the differences in the pattern of follicular development between dairy heifers and lactating cows, and given their shorter follicular waves compared to cows, heifers could display estrus close to the $PGF_{2\alpha}$ injection which can result in asynchrony at the time of AI (Sartori et al., 2004; Rivera et al., 2005). A study conducted by Pursley et al (1997) concluded that the pregnancy per AI between a group of lactating cows on a fixed-time AI with a synchronized ovulation were similar to pregnancy per AI for another group of lactating cows that followed the a.m.-p.m. rule after estrus had been

induced by a $PGF_{2\alpha}$ injection. However, when the fixed-time AI was done in heifers, the protocol was proven not effective given the absence of ovulation synchronization. Another experiment was conducted by Schmitt et al (1996) to compare fertility from AI at an estrus induced in the presence of a newly recruited follicle (induced with GnRH agonist), or with a 16-day old persistent first-wave dominant follicle in heifers. Pregnancy per AI for heifers in the GnRH-agonist group was 60.6%, while pregnancy per AI in the persistent dominant follicle group was 43.4%, concluding that the fertility is reduced when there is persistence of the first wave dominant follicle; while addition of a new dominant follicle resulted in a greater fertility (Schmitt et al., 1996).

Research on protocols including Cosynch and the addition of an intravaginal progesterone releasing device such as Controlled Internal Drug Release (CIDR®) have proven to be effective synchronizing the ovulation, including noncycling animals. This progesterone releasing device reduces the secretion of LH and prevents estrus and ovulation, and once it is removed, the reduced levels of progesterone eliminate the inhibitory negative feed-back, stimulating the release of GnRH and subsequently FSH and LH. These events lead to maturation of the dominant follicle and subsequent ovulation (de Graaff and Grimard, 2018). While the Cosynch + CIDR protocol is most commonly used in ovulation synchronization of beef cows, one study done in Holstein-Friesian cows found that between two groups of these cows submitted to either an Ovsynch protocol or a Cosynch + CIDR protocol, those submitted to the Cosynch + CIDR protocol were 2.1 times more likely to become pregnant after AI than those submitted to the Ovsynch protocol (Azevedo et al., 2014).

Detection of estrus and the timing of AI are crucial components to improving the reproductive efficiency of a successful dairy herd (Rankin et al., 1992). Given the low efficacy of

the 7-day CIDR synchronization protocols on dairy heifers due to the shorter follicular waves compared to dairy cows, researchers have studied the possibility of shortening the progesterone administration to five days instead of seven days in an attempt to achieve greater P/TAI in heifers. Bridges et al (2008) performed multiple experiments in beef cows to see how a difference in time of AI affects pregnancy per AI. In one experiment they concluded that pregnancy per TAI was 13.3% greater in cows on a 5-day Co-Synch + CIDR protocol with TAI and GnRH 72 hours after CIDR removal compared to cows on a 7-day Co-Synch + CIDR protocol with TAI and GnRH 60 hours after CIDR removal. They repeated this experiment and found a 9.1% greater pregnancy per TAI in the 5-day versus the 7-day protocol (Bridges et al., 2008). Lima et al (2011) performed a similar study in Holstein heifers to see how the 5-day timed AI + CIDR protocol combined with a GnRH injection at the initiation of the protocol affects ovarian dynamics and fertility. They also wanted to find how the effect of timing the final GnRH injection to induce ovulation relative to the timing of AI affected P/TAI. In their first experiment regarding the initial GnRH injection at the initiation of the AI protocol, Lima et al (2011) found that ovulation on day 0 of the study (35.4% vs. 10.6%), presence of a new CL at $PGF_{2\alpha}$ injection (43.1% vs. 20.8%), and progesterone levels on the day of AI (0.50 ± 0.07 vs. 0.28 ± 0.07 ng/mL) were greater in heifers that received an injection of GnRH at the beginning of the protocol compared to heifers who did not receive this injection, respectively. Despite this, the percentages of heifers in estrus at AI did not differ between the two treatments and averaged 66.8% and P/AI was not affected by the treatment at day 32 (GnRH = 52.5% vs. control = 54.1%) or at day 60 (GnRH = 49.8% vs. control = 50.0%) with a pregnancy loss average of 6.0% between both groups. They concluded that GnRH injection of the first day of a 5-day TAI protocol resulted in low ovulation rates and had no improvement in P/AI when heifers were

given one injection of PGF2α 5 days later. In their second experiment regarding timing of final GnRH injection on ovulation, Lima et al (2011) found that heifers receiving the GnRH injection concurrent with AI 72 hours after $PGF_{2\alpha}$ (COS72) had greater levels of estrus at AI (61.4% vs. 47.5%) compared to heifers receiving the GnRH injection 56 hours after $PGF_{2\alpha}$ and AI 16 hours (OVS56) later. Also, heifers in the COS72 group had a greater P/AI on day 32 (55.0% vs. 47.6%) and day 60 (53.0% vs. 44.7%) in heifers that did not display estrus compared to heifers in the OVS56 group. They also found that treatment did not affect P/AI on day 32 or day 60 in heifers that displayed estrus at the time of AI. They concluded that when following a 5-day TAI protocol, extending proestrus and delaying the final GnRH injection to be concurrent with the time of AI produced a greater P/AI in heifers who did not show signs of estrus at insemination.

Rabaglino et al (2010) conducted a study to determine the effects of a 5-day Co-Synch + CIDR + one or two injection of $PGF_{2\alpha}$ after CIDR removal on pregnancy at first and second insemination service in dairy heifers and to explore the possibility of this becoming an adequate method of synchronization in diary heifers. Results of this study showed no significant difference between one or two injections of $PGF_{2\alpha}$ (46.1% and 48.6%, respectively) and no significant difference between heifers bred after treatment with the 5-day TAI protocol and heifers bred via estrus detection (52.2% and 55%, respectively). However, researchers did observe that heifers that received a CIDR at the beginning of the 5-day TAI protocol had greater P/TAI (51.8%) compared to those that did not receive a CIDR at the beginning of the protocol (39.3%). They also performed a field study using this 5-day Co-Synch + CIDR with one $PGF_{2\alpha}$ injection protocol and obtained 58.2% pregnancy per TAI at 60 days for first service and a 47.5% pregnancy per TAI at 60 days for second service (Rabaglino et al., 2010).

Utilization of Sex-Sorted Semen in AI

The use of semen sorted by the sexual chromosome (X versus Y) allows producers to control the ratio of female to male calves their farms produce (Chang et al., 2017). The most reliable and repeatable method for sorting sperm and producing live, healthy, and functional offspring is by using flow cytometry (Seidel and Schenk, 2008; Zobel et al., 2011; Karakaya et al., 2014). During this process, the sperm are stained using a fluorescent substance, submitted to flow cytometry, and then sorted by their DNA content (bovine X chromosome has 3.9% more DNA), with about 90% accuracy. For the dairy industry, the sorting process is set up for X-chromosome bearing sperm (Karakaya et al., 2014). Using sex-sorted semen can be very beneficial to producers as it allows them to increase the number of replacement animals and lower their chances of producing bull calves, which provide little economic value. Cows giving birth to a greater number of heifers can reduce the likelihood of dystocia, as they are typically of lower birth weight than their male counterparts. They can also contribute to the expansion of the herd size and improve the herd biosecurity through the replacement heifers born in the same farm rather than purchasing heifers from another herd with unknown health/infectious status (Holden and Butler, 2018). Sexed semen also allows for a more efficient genetic selection within the herd, which reduces the generational interval and presents the opportunity to sell extra replacement heifers (Hohenboken, 1999). One study found that first lactation cows produced 1.3-1.6% more milk over a 305-day lactation along with an advantageous productivity into the second lactation if they gave birth to a heifer calf as opposed to a bull calf (Ingenhoff et al., 2017).

Another group of experiments performed by Sales et al (2011) showed the differences in timing of insemination in dairy and beef cattle when utilizing sex-sorted sperm. The first

experiment used Jersey heifers to see how type of semen and different times of insemination affected pregnancy per AI. They saw a greater pregnancy per AI in heifers inseminated with sexed semen at 60 hours (31.4%) than heifers inseminated with sexed semen at 54 hours (16.2%) (P = 0.06). They also saw that changing the time of AI of non-sexed semen did not have an effect of pregnancy per AI (60 hours at 51.8% and 54 hours at 50.5%, P = 0.95). In their second experiment, they used *Bos indicus* beef cows and submitted them to the same protocol as the Jersey heifers. They observed a lower P/AI when using sexed semen (41.8%) compared to non-sexed semen (51.8%) (P = 0.05), and evidenced a tendency for a greater P/AI (P = 0.11) when TAI was performed at 60 hours (50.8%) as opposed to 54 hours (42.8%). In their third experiment the same researchers enrolled *Bos indicus* cows into three separate TAI groups using sexed semen at 36 hours, 48 hours or 60 hours after a progesterone device was removed. They observed a greater P/AI in cows inseminated closer to ovulation, with 37.9% in those inseminated 0 to 12 hours before ovulation, 19.4% in those inseminated between 12.1 and 24 hours, and 5.8% in those inseminated > 24 hours before ovulation. Sales et al (2001) were able to conclude that although sex sorted semen has lower P/AI than non-sexed semen, it was possible to improve pregnancy per AI by delaying the time of insemination from 54 hours to 60 hours post exogenous progesterone removal.

Despite advantages of using sex-sorted semen, the laboratory process can be harmful to the sperm resulting in lower fertility of animals bred with sexed semen due to DNA damage. The sorting process can also affect the longevity of the sperm cells in the reproductive tract (Gosálvez et al., 2001). The pregnancy per AI (P/AI) in cows inseminated with sexed semen has generally been reported to be 70-80% of the P/AI obtained with non-sexed semen, with some variations (Karakaya et al., 2014; Chang et al., 2017; Ingenhoff et al., 2017) (Table 2.3). During

the flow cytometric sorting process sperm go through multiple different steps to achieve the 90% sorting accuracy, but these steps can often damage the structure of the sperm and decrease its viability (Chang et al., 2017). The flow cytometry process is expensive, and is done very slowly at a rate of 10-18 million/hour with a low number of sperm, about 2.0 million packaged in each sex-sorted semen straw (0.25 mL) equaling roughly 10% of amount of semen packaged in a conventional or un-sexed semen straw (Zobel et al., 2011; Chang et al., 2017). In a study assessing sexed sperm efficiency on *in vitro* fertilization, Blondin et al. (2009) found that the freezing process rather than the sexing process had greater negative effects on semen quality. In addition, because of the lower P/AI of sex-sorted semen, this semen is more typically utilized in heifers rather than lactating cows as heifers normally show higher fertility compared to cows. Although the lower P/AI obtained by using sex-sorted semen in heifers will not lengthen or affect the average days in milk of the lactating herd, the negative effects of using sex-sorted semen results in higher reproductive costs as the average age at calving for heifers will increase, delaying the incorporation of the heifers into the lactating herd (Ingenhoff et al., 2017). Although there are higher reproductive and financial costs of using sexed semen, the 90% heifer calf rate produced by using sex-sorted semen has caused a tendency to shift from conventional to sexed semen in the dairy industry within the last few years (Chang et al., 2017).

Table 2.3: Effect of sex-sorted sperm on pregnancy per AI and pregnancy loss of dairy cows on an Ovsynch Protocol (Karakaya et al., 2014). Abbreviations: ARR, Average Relative Risk.

	Treatment				
	Sex-Sorted	Conventional	ARR	(95% CI)	p
Pregnant, % (n/n)					
Day 31	31.8 (47/148)	40.9 (63/154)	0.80	(0.60-1.06)	0.09
Day 62	25.7 (38/148)	39.0 (60/154)	0.68	(0.49-0.94)	0.01
Pregnancy loss, % (n/n)	19.1 (9/47)	4.8 (3/63)	4.07	(1.20-13.81)	0.02

In the study done by Blondin et al (2009), their objective was to see how different variations of bovine semen affected the number of blastocysts produced and to find which variation produced the highest percentages of blastocysts compared to the others. The four variations of semen used were fresh and frozen unsexed, and fresh and frozen sexed and each variation was collected from three different bulls. Oocytes from slaughterhouse ovaries were fertilized *in vitro* with each variation, and differing levels of heparin were added to the culture medium (2, 5, or 10 micrograms/mL for sexed semen and 10 micrograms/mL for non-sexed semen). They found that variations of sexed semen produced less blastocysts than non-sexed semen variations ($P < 0.05$), and sexed semen that appeared to have a greater level of capacitation did not require a large amount of heparin in the IVF media compared to sexed semen that did not have a large amount of capacitation to produce the maximum number of blastocysts. Through further analysis, researchers also reported that compared to frozen-thawed sexed and non-sexed semen, fresh sexed and non-sexed semen had significantly lower percentages of spermatozoa with acrosome reactions, lower percentages of spermatozoa that were damaged, and also lower mitochondrial activity. They also observed that frozen-thawed non-sexed semen had a greater percentage of damaged spermatozoal DNA compared to frozen-thawed sexed semen. Bull effect on blastocyst percentage was also taken into account and it was reported that the different levels of heparin did not significantly affect the cleavage or blastocyst rates when the three bulls were analyzed together, but when differing levels of heparin concentrations for oocytes fertilized with sexed semen were combined each bull performed differently with blastocyst percentages ranging from 8.8 to 12.7%. These findings led them to conclude that the cryopreservation procedure has a greater negative effect on semen quality than the sexing process; reduced fertility in *in vitro* situations is not due to an increased percentage of

damaged DNA due to the sexing process; and different concentrations of heparin added to the culture media may optimize the IVF conditions and maximize blastocyst percentages.

Intra-cornual Insemination as a Novel Approach for Sex-Sorted Semen

In cattle, it is widely accepted that the target site of semen deposition when utilizing AI is the uterine body. The site of fertilization within the female's reproductive tract is known to be the ampullary-isthmic junction of the oviduct (Hunter, 2001). Regardless of the site of semen deposition during natural mating, the distal portion of the caudal isthmus has been accepted as the functional sperm reservoir. This reservoir is where viable spermatozoa are released from as ovulation in the female approaches (Hunter, 2001; Hunter 2003). The path to the epithelium of the caudal isthmus where sperm "dock" to promote viability within the reproductive tract is hostile and prone to spermatozoal loss through retrograde flow of uterine mucus or by phagocytosis (Senger, 2012; Diskin, 2018). During estrus, the female reproductive tract is mainly controlled by estradiol. Under this hormonal influence neutrophils are recruited from circulation within the uterine and vaginal mucosa (through the process of diapedesis) and attack any foreign agents presented into the tract to prevent infections. While the neutrophils protect the reproductive tract from microorganisms introduced via natural copulation they do not discriminate against any foreign objects and will attack and phagocytize spermatozoa as well (Senger, 2012). The reproductive tract itself can also be a hindrance to sperm transport, as each portion of the tract contains multiple folds, ridges, and grooves that spermatozoa can swim to and become lost and eventually die.

Given these obstacles, semen deposition in the uterine horns has become a topic of study among researchers. Intra-cornual insemination using sex-sorted semen can provide many benefits that uterine body insemination may not be able to provide including: increase the

fertility of genetically superior bulls with sub-optimal non-return rates and increase the efficiency when using low number of spermatozoa per insemination dose. Moreover, this technique would permit to use a small number of sex-sorted sperm produced by flow cytometry (Hunter, 2003). Given that sex-sorted semen has been reported to have a higher percentage of membrane- and DNA-damaged sperm and a lower sperm concentration per insemination straw compared to conventional semen, implementing intracornual deposition could potentially help overcome this problem by shortening the distance the sperm must travel to meet the oocyte (Hunter, 2001). The membrane damage sustained by the sex-sorted sperm resembles that of when non-sexed sperm undergo capacitation. The biochemical changes during capacitation are necessary in order for sperm to become able to fertilize an oocyte (Moće et al., 2006; Blondin et al., 2009). This early capacitation can leave sperm cells vulnerable to the harsh uterine environment as they do not have a protective outer membrane. Therefore, intracornual insemination may allow sex-sorted sperm cells a greater likelihood of fertilizing an oocyte as they do not have to traverse the entire female reproductive tract in order to become capacitated, spending a shorter time within the uterine environment (Hunter, 2003). While overall beneficial, intra-cornual insemination also has drawbacks. In order to determine the horn ipsilateral to the ovary containing the preovulatory follicle, a technician must perform transrectal ultrasonography on each ovary, which require certain level of expertise to avoid disruption of the preovulatory follicle. Damage to the uterine wall by the insemination rod is speculated that may occur if the deep horn AI technique is not performed adequately. This could cause potential fertility and animal welfare issues. The risk of polyspermic fertilization has been reported to be higher, as the spermatozoa have by-passed many of the reproductive tract's defense systems (Hunter, 2003). Multiple studies have found contradictory results in conception rate when comparing uterine

body versus intracornual insemination, with most sources finding little to no difference, some studies finding a negative results with intracornual insemination and few studies showing positive results (McKenna et al., 1990; Graves et al., 1991; Seidel and Schenk, 2008; Zobel et al., 2011; Carvalho et al., 2013; Chang et al., 2017; Ingenhoff et al., 2017;). A small pilot trial using 26 Holstein heifers inseminated with sexed semen in Taiwan (Chang et al., 2017) found that pregnancy rate was greater in heifers inseminated in the uterine horn (71%, n=14) compared to those inseminated in the uterine body (42%; n=12). That study did lack accuracy, statistical significance, and power due to the very small sample size. Ingenhoff et al (2017) found no significant difference between inseminations sites in Holstein heifers inseminated with sexed ($P = 0.528$) and non-sexed semen ($P = 0.886$). Contrarily, using conventional semen when inseminating in either the uterine body or the uterine horn had opposite effects, with a 62.9% pregnancy per AI in Jersey cows and heifers inseminated in the uterine body and only 54.2% pregnancy per AI in a bilateral horn insemination (Graves et al., 1991).

Although results on uterine body and intra-cornual insemination continue to be polarized, intra-cornual insemination may still be useful when utilizing low quality semen or when the microenvironment of the uterus is weakened or damaged (López-Gatius, 1999). Compared with semen deposition in the uterine body, intra-cornual AI with sexed semen would minimize sperm exposure to the uterine environment which could result in higher number of sperm housed within the functional sperm reservoir as well as higher likelihood of viable sperm suspended in the epithelium of the caudal isthmus waiting to be released for fertilization (Hunter, 2001; Senger, 2012).

CHAPTER 3

COMPARISON OF PREGNANCY OUTCOMES IN DAIRY HEIFERS ARTIFICIALLY INSEMINATED WITH SEXED SEMEN DEPOSITED IN THE UTERINE HORNS VERSUS THE UTERINE BODY

Kirks, Sara. To be submitted to Theriogenology.

Abstract

Utilization of sex-sorted semen in dairy heifers has become a frequent topic of research due to its positive attributes to a herd, but the full adoption of this technology into practice has been overshadowed by low economic gain due to lower conception rates compared to conventional semen. In this study, deep intra-cornual insemination ipsilateral to the ovary with the preovulatory follicle was used to evaluate the effect of site of semen deposition with sexed semen on pregnancy per timed artificial insemination (P/TAI). The results show that deep intra-cornual insemination resulted in greater P/TAI compared to deposition in the uterine body (56.7%; 44.32%: P = 0.038). Different inseminators had an effect on P/TAI (P = 0.09), and there was a higher numeric tendency for pregnancy in the left versus the right horn (66.3%; 53.9%). This suggests that deep intra-cornual insemination may help overcome the lower P/TAI previously achieved with sex-sorted semen.

Introduction

A key requirement to ensure a sustainable livestock production with sufficient economic returns for dairy producers is to achieve a high herd's reproductive efficiency (Baruselli et al., 2018). Artificial insemination (AI) is the cornerstone of the breeding program in most dairy operations worldwide and continues to provide multiple benefits on reproductive performance and genetic improvement for dairy herds. A combination of several factors can affect the efficacy of this technology, including heat detection, ovulation synchronization protocols, type of semen (sex-sorted versus conventional), AI technique and technician expertise, environmental conditions, milk yield, health status, hygienic conditions and biosecurity, among others (García-Ispierto et al., 2007).

The preselection of the sex of future offspring by using AI with sex-sorted semen (also known as sexed semen, SS) has tremendous benefits for dairy producers by increasing the

overall farm efficiency, profitability, and environmental sustainability (López-Gatius, 2012; Holden et al., 2018). The use of SS has the capability of increasing the number of replacement heifers, reducing the chances of producing bull calves (which have lower economic return) and therefore the risks of dystocia. The utilization of SS can also contribute to expand the herd size (increasing the number of heifers) and improve the herd biosecurity by reducing new additions from farms with unknown infectious status (Holden et al., 2018). The increment of replacement heifers population allows for a more efficient genetic selection within the herd, reducing the generational interval and genetic lag (difference between genetic level of the bull and the cows), which increases the farm's profit margins (Vishwanath et al., 2018; Oikawa et al., 2019). Further, significant genetic gain may be achieved with SS through genomic selection and progeny tests of sires based on the greater number of daughters produced within a shorter period of time (Kumar et al., 2016; Patel et al., 2019).

Sex-sorted semen technology has improved over the years, but its prominent use throughout the cattle industry has been limited due to high costs and lower conception rates when used for AI (McCullock et al., 2013). Fertility of female cattle inseminated with SS is typically lower compared to cattle inseminated with conventional semen, reaching approximately 70-80% of the conception rate obtained with non-sexed semen (Murphy et al., 2016; Oikawa et al., 2019; Patel et al., 2019). This reduction in pregnancy outcomes has been mainly attributed to the lower sperm concentration and greater level of sperm damage due to the sex-sorting process (DeJarnette et al., 2008; Frijters et al., 2009). The commercially available SS straws contain a sperm concentration of 2×10^6 sperms/0.25 mL straw compared to a sperm concentration of 15×10^6 sperms/0.54 mL straw for conventional semen (López-Gatius, 2012; Diskin, 2018; Patel et al., 2019). This is a considerable factor that affects cows' fertility when using SS, being a barrier

for the implementation of this technology within routine AI programs (Hutchinson et al., 2013; Holden et al., 2018).

Flow cytometry remains the most successful, reliable, and only commercially available method to sex-sort sperm cells with a 90% accuracy. This method is based on discrimination of the 3.9% difference in DNA content between X and Y chromosome-bearing sperm (Rai, 2018; Vishwanath et al., 2018). Unfortunately, cell sex-sorting based on flow cytometry is a slow process and appears to have negative effects on sperm viability and quality leading to a reduced pregnancy per AI (Carvalho, et al., 2010; López-Gatius, 2012; Kurykin, 2017; Holden et al., 2018; Vishwanath et al., 2018; Oikawa et al., 2019; Patel et al., 2019). Some of the alterations observed after the sex sorting process include chromatin stability damage, and acceleration of capacitation and acrosome reaction, which reduces the sperm's lifespan. Moreover, oxidative stress during the cryopreservation process further damages the membranes and decreases the motility of frozen-thawed sex-sorted sperm (Kurykin, 2017).

Spermatozoa must go through a series of biochemical changes known as 'capacitation' in order to be able to reach fertilization ability. This process requires sperm stay in the female reproductive tract during 6 to 12 hours. Seminal plasma and seminal plasma proteins provide nourishment, helps with transportation, and contributes to the physiological events that sperm undergo during capacitation (Westfalewicz et al., 2017). The target site of non-sexed semen deposition during AI is the uterine body to provide sperm the environment, distance, and time necessary for interaction with the female reproductive tract to be capacitated before fertilization (López-Gatius, 2012). Since the sex-sorting process results in structural changes in acrosome and plasma membrane resembling capacitation, deposition of sexed semen nearer the site of fertilization and close to the time of ovulation might enhance the efficiency of the pre-

capacitated sex-sorted sperm. Horn insemination has been extensively used in equine and canine when using frozen-thawed semen with low sperm doses. Thus, horn insemination might represent an alternative to overcome the low sperm concentration, and the increased cell damage and structural/biochemical changes associated with sperm capacitation observed in bull SS.

Different approaches have been used in an attempt to increase the efficiency of using SS for AI in dairy cattle, including increasing sperm concentration (4 x 10^6 sperms/straw), improving cell sorting platform, protocols and media, enhancing cryopreservation methods, adapting the ovulations synchronization protocols and the timing of AI close to ovulation, using different AI techniques, among others (Sharpe et al., 2009; Butler et al., 2014; Seidel, 2014; Holden et al., 2018; Vishwanath et al., 2018). Intracornual semen deposition has been recently proposed by other researchers and our team as a promising technique to improve conception rates in dairy heifers inseminated with SS (López-Gatius et al., 2000; Chang et al., 2017; Kirks et al., 2020).

In this study we hypothesize that dairy heifers inseminated with SS deposited in the uterine horn (UH) ipsilateral to the ovary containing the preovulatory follicle (POF) have greater pregnancy per TAI (P/TAI) and lower pregnancy loss (PL) compared to heifers receiving TAI in the uterine body (UB). The objective of this study was to compare P/TAI and pregnancy loss (PL) in dairy heifers inseminated with sexed semen deposited in the uterine horn (UH) ipsilateral to the ovary containing the preovulatory follicle (POF) versus TAI in the uterine body (UB).

Materials and methods

Heifers husbandry and housing

The experimental protocols applied in this study were previously approved by the Clinical Research Committee of the College of Veterinary Medicine at the University of

Georgia. A total of 459 virgin Holstein and Holstein x Jersey heifers (12 months of age) with a body condition score between 2.5 and 3.5 (scale 1-5, where 1 is too thin and 5 is too fat, Edmondson et al., 1989) from two commercial dairy farms located in South Georgia, USA, were initially considered as experimental population for this study. Heifers grazed on 8- and 20-acre pastures of Coastal (hybrid of *Cynodon dactylon*), Tifton 85 (hybrid of *Cynodon dactylon*) and Jiggs grass (hybrid of *Cynodon dactylon*) with access to natural shade (e.g. trees), free choice hay of Bermuda grass (*Cynodon dactylon*), and Bailage supplementation during the winter, minerals and water *at libitum*. Additionally, heifers received supplementation once daily of a total mixed ration that met or exceeded the nutritional requirements for Holstein breeding heifers according to NRC (*Nutrient*, 2001). Heifers were dewormed using Doramectin (10 mg/mL) (Dectomax® Zoetis Animal Health) subcutaneously (SC). The heifers were vaccinated with a modified-live virus vaccine containing Bovine viral diarrhea virus 1 and 2, Bovine herpes virus 1, Bovine respiratory syncytial virus, Bovine parainfluenza virus 3 and 5 serovars of Leptospira (Bovi-shield ™ Gold FP 5 L5; Zoetis Animal Health; 2ml intramuscularly, IM). In addition, animals received a bacterin-toxoid vaccine containing Clostridium killed isolates [Ultrabac® 7 (5mL SC)] 30 days before breeding. Heifers were given free choice minerals (All season Altosid® HIGH MAG FLY CONTROL MINERAL) each day. The study was performed over the course of three years (2017, 2019 and 2020) between January and April. For the implementation of synchronization protocols, insemination, blood collections and ultrasound evaluations, all heifers were handled in chutes and head-locking stations.

Experimental design, ovulation synchronization and timed artificial insemination

This study represents a completely randomized controlled study. A total of 358 Holstein and Holstein x Jersey cross heifers (out of the 459 animals synchronized and originally

considered as initial experimental population) were enrolled in this study to be submitted to timed artificial insemination (TAI) after receiving an ovulation synchronization hormonal treatment. In order to find a difference of 15% in conception rate between a control group (CR=45%) and a treated group (CR=60%), with a 95% confidence and 80% of power, a sample size of 184 heifers per group was required (SAS, 2017).

All heifers were submitted to a modified 5-day Co-Synch + CIDR protocol. Each heifer received an intravaginal insert (Controlled Internal Drug Release, CIDR; Eazi-Breed CIDR®; Zoetis Animal Health, Florham Park, NJ, USA) containing 1.38 g of progesterone for 5 days. On the day of CIDR removal, 25 mg of dinoprost tromethamine, a $PGF_2\alpha$ analog (Lutalyse; Zoetis Animal Health) was injected intramuscularly (IM). Seventy-two hours after CIDR removal, heifers were injected with 100 micrograms of gonadorelin, a GnRH analog (Factrel; Zoetis Animal Health) IM and at the same time received TAI with sex-sorted semen.

Two hours before TAI, all heifers were reproductively evaluated using transrectal ultrasonography (TRUS) with a 5-MHz linear transducer to determine which ovary contained the pre-ovulatory follicle (POF), and animals were randomly assigned to one of the two experimental groups based on the site of semen deposition during the TAI, as follows:

1. …Uterine Horn (UH, n=173): Heifers received TAI with SS deposition in the uterine horn ipsilateral to the ovary containing the POF.
2. …Uterine Body (UB, n= 185): Heifers received TAI with SS deposition in the uterine body, regardless the location of the POF.

Ultrasonography

Trans-rectal ultrasonography (TRUS) was performed by four experienced veterinarians working simultaneously using ultrasound units equipped with 5-MHz probes (Evo Lite E.I.

Medical Imaging, CO, USA). Examination of the reproductive tract was performed using transrectal palpation and TRUS on all heifers before CIDR insertion in order to determine their reproductive status based on the characteristics of the uterus (including uterine tone) and the main ovarian structures [follicles, *corpus hemorrhagicum* (CH), and *corpora lutea* (CL)]. The diameter of the uterine horns and the ovarian follicles was estimated. Only heifers with a normal reproductive tract were included in this study. Heifers with congenital (e.g. Freemartinism) or acquired reproductive pathologies (e.g. ovarian cysts or mucometra) were eliminated from the study. This evaluation also allowed to exclude immature pre-pubertal heifers with infantile reproductive tracts (uterine horns and ovaries with less than 1cm diameter).

Heifers that had not shown signs of estrus (standing to be mounted) during the first 48 hours after CIDR removal were assigned to receive TAI 72 hours after CIDR removal. These heifers were evaluated via TRUS two hours before TAI to identify the ovary (left or right) that contained the POF, and its diameter was estimated. In addition, TRUS was used for pregnancy diagnosis 35 days following TAI and for pregnancy reconfirmation 30-60 days later.

Estrus detection

The heifer breeding program of the commercial operations described in this study included heat detection during the first 48 hours after CIDR removal plus AI of those estrous heifers 12 hours later. Estrus detection was done by visual observation twice a day during one hour during the first two days after CIDR removal. The sexual behavior 'standing to be mounted' was considered the main sign of estrus. Of the total 459 heifers initially considered as the experimental population, 81 showed signs of estrus (standing to be mounted) during the first 48 hours after CIDR removal and received AI approximately 8-12 hours later, based on the am-pm rule. These animals inseminated based on heat detection were not considered for the purpose of

the present study. Moreover, heifers that showed estrus between 48 and 72 hours after CIDR removal and those that were not detected with signs of estrus behavior after CIDR removal were considered for the present study and submitted to TAI using sex-sorted semen at 72 hours post CIDR withdrawal.

Artificial Insemination

Heifers included in the study were fixed time artificially inseminated by five experienced inseminators with known insemination efficiency. Artificial insemination was performed using commercial frozen-thawed sex-sorted semen from eight different sires from two different certified semen centers (Select Sires® and ABS®). Sex-sorted semen from one, five and two sires were used on years 2017, 2019 and 2020, respectively. Sex-sorted semen straws contained 2×10^6 sperms per 0.25 mL straw. Semen was thawed at 37 degrees Celsius for 30 seconds and then deposited transcervically into either the uterine body or deep into the middle-cranial portion of the uterine horn ipsilateral to the ovary containing the POF, according to group assignment. The whole artificial insemination procedure lasted between 15 and 60 seconds/animal.

Pregnancy diagnosis and pregnancy outcomes

Pregnancy diagnosis was performed at 35 days after TAI via ultrasonography per rectum. The pregnancy was confirmed if an embryonic heartbeat was detected. Heifers diagnosed pregnant were reexamined via palpation and TRUS of uterine contents at 30-60 days after initial pregnancy diagnosis. Pregnancy rate at 35 days after TAI was calculated by dividing the number of heifers diagnosed pregnant at 35 days after TAI by the total number of heifers inseminated. Pregnancy loss was determined by dividing the number of heifers that lost the pregnancy between days 35 and 60-90 of gestation by the number of heifers diagnosed pregnant at 35 days after TAI.

Statistical Analysis

All statistical analyses were performed using a commercial statistical software (Statistical Analysis System, SAS® version 9.3; SAS Institute, Cary, NC, USA). A logistic regression model using the Proc Logistic, Chi square test and Proc Frequency were run to compare the variables pregnancy per TAI (P/TAI) and pregnancy loss (PL) between groups. Adjusted odds ratios (OR) were calculated for each comparison, including 95% confidence intervals. The outcome variable was adjusted by and also included the variables inseminator (1 to 5), year (2017, 2019, 2020), body condition score (scale 1 to 5, 0.25 units increment) and diameter of the POF (mm, classifying the population in two groups using the median value of the distribution; ≥ 15 mm or < 15 mm) as covariates. Descriptive statistics were used to analyze data of the side of occurrence of the POF (right or left), as well as the effect of inseminator, year, BCS, and diameter of the POF on P/TAI. For all analyses, values of $P < 0.05$ were considered significant and $0.05 < P < 0.1$ was considered a tendency.

Results

Reproductive evaluation at the initiation of the protocols indicated that a high proportion of heifers had moderate to high uterine tone and a uterine diameter equal or larger than 2 cm. Moreover, 47.5% (177/373) of the heifers had a CL in one of the ovaries at the time of CIDR insertion (Table 3.1). There was no difference in the proportion of cycling heifers between years. Among all of the heifers 34.0% had a follicle of 8 mm or greater diameter as their main ovarian structure, and 11.8% had a follicle less than 8 mm, while 6.7% of the animals had no significant structures in their ovaries.

Heifers inseminated with SS in the UH ipsilateral to the ovary containing the POF had a greater P/TAI (56.7%, 98/173) than heifers inseminated with SS in the UB regardless of the

location of the POF (44.32%, 82/185; $P = 0.038$; Figure 3.1). Heifers receiving intra-cornual insemination with SS were 1.6 times (OR =1.6 95% CI: 1.02-2.43, $P = 0.05$) more likely to become pregnant than those inseminated in the UB. Pregnancy loss (PL) was not statistically different between groups (UB: 9.9%, 7/71; UH: 7.2%, 6/83) and was within the normal ranges for dairy heifers at 8.0% (13/154). There were no significant effects of years, inseminators, BCS and POF diameter on PL (Figure 3.8).

In general inseminators 2, 3, 4, and 5 had adequate overall P/TAI using SS. There was no significant difference in P/TAI among these inseminators (55.7%, 52.78%, 57.5%, and 45.59% for inseminators 2, 3, 4, and 5, respectively). However, inseminator 1 tended to have lower ($P = 0.09$) P/TAI compared to the other inseminators (36.9%) (Figure 3.2). All inseminators (except for inseminator 1) had a greater P/TAI when semen was deposited within the UH ipsilateral to the ovary containing the POF, compared to that when TAI was done in the UB (Figure 3.4). There was no significant difference in P/TAI among years regardless of inseminator ($P = 0.389$) (Figure 3.3). On all years, heifers inseminated in the UH tended to have greater P/TAI compared to heifers inseminated in the UB (Figure 3.5).

The ultrasound evaluation before TAI revealed that the POF was more frequently observed in the right ovary compared to the left ovary (49.9 and 37.6% for the right and left ovary, respectively). In addition, 12.5% of the heifers had either large follicles of similar size in both ovaries, or did not have a follicle of significant diameter before TAI. In general, (during 2019 and 2020 breeding programs and 4 out of 5 inseminators), there was numerical tendency of greater P/TAI when UH insemination with SS was performed in the left horn (62.3%) versus the right horn (52.5%) (Figure 3.7). However, for only one inseminator (2017 breeding program and inseminator 5) the tendency was opposite, having numerically greater P/TAI (without statistical

difference, P = 0.47) when intracornual TAI with SS was done in the right UH (57.1%) compared to the left horn (44.4%) (Figure 3.6).

Heifers having a POF of 15 mm or greater diameter did have a higher likelihood (53.9%) of conceiving compared to heifers with a follicle smaller than 15 mm (47.1%), without statistical difference (P = 0.20). There was no difference in P/TAI between heifers with BCS of 3.0 or above versus heifers with a BCS lower than 3.0 (50% and 50.63%, respectively).

Discussion

Deep intracornual AI with sex-sorted semen in the uterine horn ipsilateral to the ovary containing the POF resulted in greater P/TAI than insemination with sexed semen in the uterine body regardless of the location of the POF. These findings support our hypothesis and agree with the results of previous pilot trials showing that AI with sexed semen in the uterine horn ipsilateral to the ovary of the impending ovulation is associated with higher fertility (Chang et al., 2017; Kirks et al., 2020). This protocol may prove to be beneficial for dairy operations attempting to increase the number of replacement heifers by using TAI with sex-sorted semen in a more efficient manner, overcoming the reported limitations associated with lower pregnancy rate.

Multiple studies have been done to compare the pregnancy outcomes in cattle inseminated in the uterine horns versus the uterine body. In most of those trials intracornual insemination has resulted in similar or lower pregnancy outcomes compared to AI in the uterine body (Williams et al. 1988; Kurykin et al., 2007; Seidel and Schenk, 2008; Sá Filho et al., 2012). However, consistent with the present study, various field studies inseminating a large number of cows demonstrated that conception rate was improved when semen was deposited into the uterine horn versus the uterine body (López-Gatius et al., 1988; Senger et al., 1988; Graves et al.,

1991; Meirelles et al., 2012), especially in farms and inseminators with low insemination efficiency (Diskin et al., 2018).

In a study assessing the competition between marked and unmarked sperm, deep uterine horn AI improved access of sperm to the oocyte based on numbers of accessory sperms (Dalton et al., 1999). The authors concluded that deposition of unsexed semen (containing sperm with a semi-flattened head) into the mid-cranial portion of the uterine horn may improve fertilization and subsequent pregnancy success compared with deposition of semen into the uterine body. However, other studies showed no effects of the site of semen deposition on the fertilization rates (Hawk et al., 1986; Dalton et al 1999). Further, experiments in superovulated cows receiving AI with non-sexed semen into the uterine body or both uterine horns (half semen dose in each horn) concluded that semen deposition into the uterine horns did not improve the fertilization rate or embryo quality (Hawk et al., 1988; Carlvalho et al., 2013).

A more recent trial performed in Holstein heifers using sexed and non-sexed semen inseminated with a fixed-time AI protocol showed that there was little or no differences in P/TAI between the sites of semen deposition (Ingenhoff et al., 2017). In that study, heifers inseminated with non-sexed semen in the uterine horn ipsilateral to the POF had 44.4% P/TAI compared with 43.5% for those inseminated in the uterine body; and heifers inseminated with sexed semen in the uterine horn ipsilateral to the POF had 25.6% P/TAI compared to 22.3% for uterine body inseminations. Another study comparing the P/TAI in Angus cows inseminated with different sexed semen doses and deposition sites revealed that there was no significant difference between sites of semen deposition (Seidel and Schenk, 2008). Likewise, additional studies using frozen conventional (Williams et al. 1988) and sex-sorted semen (Kurykin et al., 2007; Seidel and

Schenk, 2008; Sá Filho et al., 2012) did not observe significant differences in pregnancy outcomes between semen deposition into the uterine body and uterine horn.

The evidence shows that the application and benefits of horn insemination over AI in the uterine body have been controversial topics for more than 30 years. The high variation and contrasting results with the current trial and among different studies have been influenced by numerous factors including differences in synchronization protocols, timing of insemination, inseminator's experience, type of semen, semen quality and management practices, among others.

Despite flow cytometry-based sperm sorting technology rapidly developed and spread worldwide, the lower pregnancy rates achieved when using sex-sorted semen has been a limitation for its use in AI programs (Seidel, 2007; Frijters et al., 2009; González-Marín et al., 2018). The reduction in conception rates obtained with sexed semen accounts for 70-80% of the conception rates achieved with conventional semen (DeJarnette et al., 2008; Boustan et al., 2014). The cell sorting process is slow, only providing a suboptimal dose at 2-4 x 10^6 sperm compared to that of conventional semen (between 15-20 x 10^6 sperm; Frijters et al., 2009). The production rate of this technology is very limited and only 30% of the sperm containing the desired sex are able to be sorted out of one ejaculate (Kurykin, 2017). In addition, sex-sorting affects total sperm motility (Blondin et al., 2009) and induces changes in the sperm membrane which can accelerate the acrosome reaction sequence and capacitation post-cryopreservation (Vishwanath and Moreno, 2018). In a recent study, sex-sorted cryopreserved semen exhibited a higher percentage (23%) of live-acrosome-reacted sperm compared to fresh non-sorted sperm (9%) (Mocé et al., 2006). Therefore, investigation of strategies to overcome these limitations are warranted. Research efforts have been focused on improving laboratory procedures and media to

increase cell sorting efficiency that may result in greater sperm concentration and lower cell damage, with promising results (Vishwanath and Moreno, 2018). In addition, hormonal TAI protocols that provide appropriate temporal-spatial interaction of sperm with the female reproductive tract and the oocyte may help to improve fertility and maximize the efficiency of the use of SS for AI.

In this study, heifers bred by UH insemination with SS achieved optimal P/TAI (57%), while AI in the UB reached acceptable values, which are similar to those previously described for sexed semen (Vishwanath and Moreno 2018). Furthermore, pregnancy loss in the current study was 7.5%, which is within the normal range of 0.4% to 10.6% reported in dairy heifers (Forar et al., 1995). Successful conception relies heavily on the timing of insemination relative to ovulation to achieve fertilization, as well as adequate uterine (and endocrine) environment to support embryo development and implantation. In order to attain an adequate conception rate, it has been suggested to perform AI with non-sexed semen 7-18 h before the synchronized ovulation (Schels et al., 1978; Lim et al., 2018). In contrast, AI with conventional semen performed 25-40 hours before or 8 -12 hours after ovulation have been associated with low fertilization and conception rates (Schels et al., 1978; Lim et al., 2018). When using sex-sorted semen, it is highly recommended to perform AI close to the time of ovulation, based on the shorter sperm survival time in the female reproductive tract and the pre-capacitation status of sexed sorted semen. Ovulation has been reported to occur 84-90 hours after CIDR removal in dairy heifers treated with the 5-day CoSynch CIDR protocol (Colazo et al., 2011; Fishman et al., 2019). In the present study AI with sexed semen was performed 72-74 hours after CIDR removal. This appropriate timing of AI along with adequate semen quality may have contributed with the observed optimal conception rates.

In the current study, the efficiency of the inseminator affected P/TAI with sexed semen. One of the inseminators had a significantly lower overall P/TAI compared with the other four inseminators. While those four inseminators attained 8.5-32% greater P/TAI when performing TAI in the UH compared to the UB, the inseminator 1 obtained similar P/TAI with both semen deposition sites. Artificial insemination in the mid-cranial portion of a particular uterine horn requires certain level of technical expertise. However, in most cases learning the 'horn AI technique' takes only few attempts (in cows) for an experienced AI technician. García-Ispierto et al (2007) demonstrated that the likelihood of pregnancy decreased by a factor of 0.25 when comparing the worst and best inseminators. A study assessing inseminators' capability to adequately performing intra-cornual or body insemination revealed that after appropriate training almost all inseminators correctly deposited semen into uterine horn or body with >95% accuracy. However, the authors concluded that the accuracy of AI in the uterine body (but not in the uterine horns) can decrease to 75% with the time (Senger et al.,1988). Therefore, the improvements in P/TAI when using AI in the uterine horn versus the uterine body observed here and in other studies may be associated to the benefit of horn insemination preventing the occurrence of cervical AI, which has been proven to reduce the conception risk (Moller et al., 1972). Consequently, the success of intracornual insemination as a strategy to increase conception rates with sex-sorted semen will highly depend on application of an appropriate AI technique. There was no significant effect of the years and sires on P/TAI. In contrast, one study done in Holstein cows using conventional and sexed semen have shown significant effects of individual sires on conception rates (Abdalla et al., 2019).

Heifers with a larger POF (≥ 15 mm) had greater P/TAI compared to heifers with a POF smaller than 15 mm. In prior studies in beef cattle, larger POF showed greater ovulation rate and

resulted in greater P/AI (Perry et al. 2007; Sa´ Filho et al. 2010). Moreover, ovulation of larger follicles could be associated with greater estradiol production, oocyte competence, subsequent formation of a larger CL and greater progesterone concentration which may positively affect the fertility after TAI (Baruselli et al., 2012). A study performed by Lopes et al (2007) revealed that the diameter of the POF on the day of AI was larger in animals that became pregnant compared to animals that resulted non-pregnant. The authors also observed that progesterone concentration after day 5 post-AI rose in animals that became pregnant compared to animals that did not (Lopes et al., 2007). The results of that study also concluded that pregnancy is positively correlated with the diameter of the POF, which determines the diameter of the CL and the subsequent production of progesterone (Lopes et al., 2007).

In the present study the POF was more frequently observed in the right ovary at 72 h after CIDR removal, being consistent with the results of previous studies showing that the number of ovulations occurring in the right ovary was greater than that in the left ovary (Reece & Turner 1938; Ginther et al. 2014). It has been observed that the right ovary is larger and more active than the left ovary (reviewed in López-Gatius 2000). However, Ginther et al. (2014) observed that this was not the case for dairy heifer showing three follicular waves, in which there was no difference in the proportion of ovulations between both ovaries, contrasting with the results of presented here.

Pregnancy per TAI was numerically greater in heifers receiving horn TAI when the POF was present in the left ovary. Therefore, the POF development in the left ovary and subsequent TAI with sexed semen in the left UH appears to be associated with increased fertility. Similarly, Miura and Izumi (2017) evaluated the effects of the location (left or right ovary) of the POF detected via trans-rectal palpation, on conception rate of dairy heifers. In that study conception

rate after AI into the uterine body with both sexed and non-sexed semen was significantly higher when the POF was in the left ovary (60.1%) versus the right ovary (46.2%). Specifically, conception rates using sexed semen were significantly higher in the left POF than in the right POF (57.3% vs. 44.4%). Similar findings in lactating dairy cows indicated that conception rate tended to increase when the POF developed in the left ovary (Townson et al. 2002). The physiological mechanisms underlying these differences still remain unclear but may be associated to the reported size asymmetry of the ovaries and uterine horns (López-Gatius 2000), or the locational relationships among the POF and the regressed CL and the number of the occurring follicular wave (first, second or third) (Miura and Izumi, 2017). One of these authors working with dairy heifers observed that the first-wave dominant follicle located ipsilateral to the CL in the ovary is associated with reduced conception rates (Miura et al. 2015).

In summary, intracornual AI of dairy heifers with sex-sorted semen in the uterine horn ipsilateral to the ovary of the impending ovulation resulted in greater P/TAI (72 hours after CIDR removal) compared with that of heifers inseminated in the uterine body. Since nowadays ultrasonography is a common tool used in dairy farms (Van Schyndel et al., 2018), the utilization of this technology to identify the POF could contribute to the establishment of horn insemination with sexed semen within the routine breeding program for dairy heifers as a strategy to increase P/TAI with sexed semen in dairy operations. This could become a powerful tool to improve the economical and genetic gain within dairy herds. This study had the limitation of relatively small sample size and differences in the inseminator experience. Further prospective studies should be performed in large dairy operations to determine the effects of intracornual insemination with sexed semen in lactating dairy cows to determine how this approach might improve reproductive performance and genetic progress within the milk production herd.

References

Abdalla, H., Elghafghuf, A., Elsohaby, I. 2019. Evaluating sire effects on cow fertility: Timed AI and repeat-breeder dairy cows. Animal Reproduction Science. 209: 106147.

Adams, G. P., Singh, J. Ovarian Follicular and Luteal Dynamics in Cattle. 2014. Bovine Reproduction. Pg. 219-244.

Adams, G.P. 1994. Control of Ovarian Follicular Wave Dynamics in Cattle: Implications for Synchronization & Superstimulation. Theriogenology. 41:19-24.

Ahmad, N., Townsend, E.C., Dailey, R.A., Inskeep E.K. 1997. Relationship of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Animal Reproduction Science. 49:13-28.

Alkar, A., Tibary, A., Wenz, J.R., Nebel, R.L., Kasimanickam, R. 2011. Presynchronization with GnRH 7 days prior to resynchronization with CO-Synch did ont improve pregnancy rate in lactating dairy cows. Theriogenology. 76: 1036-1041.

Alves, B. R.C., Cardoso, R. C., Doan, R., Zhang, Y., Dindot, S. V., Williams, G. L., Amstalden, M. 2017. Nutritional programming of accelerated puberty in heifers: alterations in DNA Methylation in the arcuate nucleus. Biology of Reproduction. 96(1): 174-184.

Amstalden, M., Cardoso, R.C., Alves, B.R.C., Williams, G.L. 2014. Reproduction Symposium: Hypothalamic neuropeptides and the nutritional programming of puberty in heifers. Journal of Animal Science. 92:3211-3222.

Atkins, J. A., Pohler, K. G., Smith, M. F. 2013. Physiology and Endocrinology of Puberty in Heifers. Vet Clinical Food Animal. 29:479-492.

Azevedo, C., Maia, I., Canada, N., Simões, J. 2014. Comparison of fertility, regular returns-to-estrus, and calving interval between Ovsynch and CO-synch + CIDR protocol in dairy cows. Theriogenology. 82: 910-914.

Badinga, L., Thatcher, W.W., Diaz, T., Drost, M., Wolfenson, D. 1993. Effect of Environmental Heat Stress on Follicular Development and Steroidogenesis in Lactating Holstein Cows. Theriogenology. 39: 797-810.

Baruselli, P.S., Sá Filho, M.F., Ferreira, R.M., Sales, J.N.S., Gimenes, L.U., Vieira, L.M., Mendanha, M.F., Bó, G.A. 2012. Manipulation of Follicle Development to Ensure Optimal

Oocyte Quality and Conception Rates in Cattle. Reproduction in Domestic Animals. 47 Supplemental: 134-141.

Baruselli, P.S., Ferreira, R.M., Sá Filho., M.F., Bó, G.A. 2018. Review: Using artificial insemination v. natural service in beef herds. The Animal Consortium. 12: S1; s45-s52.

Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P., King, W.A. 2009. Analysis of bovine sexed sperm for IVF from sorting to the embryo. Theriogenology. 71: 30-38.

Boer, H.T.M., Veerkamp, R.F., Beerda, B., Woelders, H. 2010. Estrous behavior in dairy cows: identification of underlying mechanisms and gene functions. Animal. 4:3, 446-453.

Boustan, A., Javaremi, A.N., Shahrbabak, M.M. 2014. Economic and genetic aspects of using sexed semen in traditional and genomic evaluations of Iranian Holstein dairy cattle: a simulation study. Journal of Agriculture Science Technology. 16: 801-810.

Bridges, G.A., Helser, L.A., Grum, D.E., Mussard, M.L. Gasser, C.L., Day, M.L. 2008. Decreasing the interval between GnRH and $PGF_{2\alpha}$ from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology. 69: 843-851.

Britt, J.H., Scott, R.G., Armstrong, J.D., Whitacre, M.D. 1986. Determinants of Estrous Behavior in Lactating Holstein Cows. Journal of Dairy Science. 69:2195-2202.

Burns, B.M., Fordyce, G., Holroyd, R.G. 2010. A review of factors that impact on the capacity of beef cattle females to conceive, maintain a pregnancy and wean a calf - Implications for reproductive efficiency in northern Australia. Animal Reproduction Science. 122:1-22.

Buskirk, D.D., Faulkner, D.B., Ireland, F.A. 1995. Increased postweaning gain of beef heifers enhances fertility and milk production. Journal of Animal Science. 73: 937-946.

Butler, S.T., Hutchinson, I.A., Cromie, A.R., Shalloo, L. 2014. Applications and cost benefits of sexed semen in pasture-based dairy production systems. Animal. 8:s1, 165-172.

Carabă, I. Velicevici, S. 2013. Using Ovsynch Protocol versus Cosynch Protocol in Dairy Cows. Animal Science and Biotechnologies. 46: 63-65.

Carvalho, J.O., Sartori, R., Machado, G.M., Mourão, G.B., Dode, M.A.N. 2010. Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in *in vitro* embryo production. Theriogenology. 74: 1521-1530.

Carvalho, P.D., Souza, A.H., Sartori, R., Hackbart, K.S., Dresch, A.R., Vieira, L.M., Baruselli, P.S., Guenther, J.N., Fricke, P.M., Shaver, R.D., Wiltbank, M.C. 2013. Effects of deep-horn AI on fertilization and embryo production in superovulated cows and heifers. Theriogenology. 80: 1074-1081.

Chang, L., Chou, C., Shiu, J., Tu, P., Gao, S., Peng, S., Wu, S. 2017. Artificial insemination of Holstein heifers with sex-sorted semen during the hot season in a subtropical region. Tropical Animal Health Production. 49: 1157-1162.

Chanvallon, A., Coyral-Castel, S., Gatien, J., Lamy, J.M., Ribaud D., Allain, C., Clément, P., Salvetti, P. 2014. Comparison of three devises for the automated detection of estrus in dairy cows. Theriogenology. 82:734-741.

Colazo, M.G., Ambrose, D.J. 2011. Neither duration of progesterone insert nor initial GnRH treatment affected pregnancy per timed-insemination in dairy heifers subjected to a Co-synch protocol. Theriogenology. 76: 578-588.

Curtis, G., Argo, C.M., Jones, D., Grove-White, D. 2018. The impact of early life nutrition and housing on growth and reproduction in dairy cattle. PLOS One. 13: 1-20.

D'Occhio, M. J., Baruselli, P. S., Campanile, G. 2019. Influence of nutrition, body condition and metabolic status on reproduction in female beef cattle: A review. Theriogenology. 125:277-284.

Dalton, J.C., Nadir, S., Bame, J.H., Saacke, R.G. 1999. Effect of a deep uterine insemination on spermatozoal accessibility to the ovum in cattle: a competitive insemination study. Theriogenology. 51:883–90.

Day, M. L., Anderson, L.H. 1998. Current concepts on the control of puberty in cattle. Journal of Animal Science. 76:1–15

Day, M. L., Imakawa, K., Wolfe, P. L., Kittok, R. J., Kinder, J. E. 1987. Endocrine Mechanisms of Puberty in Heifers. Role of Hypothalamo-Pituitary Estradiol Receptors in the Negative Feedback of Estradiol in Luteinizing Hormone Secretion. Biology of Reproduction. 37:1054-1065.

De Graaff, W., Grimard, B. 2018. Progesterone-releasing devices for cattle estrus induction and synchronization: Device optimization to anticipate shorter treatment durations and new device developments. Theriogenology. 112: 34-43.

DeAtley, K. L., Colgrave, M. L., Cánovas, A., Wijffels, G., Ashley, R. L., Silver, G. A., Rincon, G., Medrano, J. F., Islas-Trejo, A., Fortes, M. R.S., Reverter, A., Porto-Neto, L., Lehnert, S. A., Thomas, M. G. 2018. Neuropeptidome of the Hypothalamus and Pituitary Gland of Indicine X Taurine Heifers: Evidence of Differential Neuropeptide Processing in the Pituitary Gland before and after Puberty. Journal of Proteome Research. 17:1852-1865.

DeJarnette, J.M., Nebel, R.L., Marshall, C.E., Moreno, J.F., McCleary, C.R., Lenz, R.W. 2008. Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows. Journal of Dairy Science. 91: 1778-1785.

Diskin, M.G. 2018. Review: Semen handling, time of insemination and insemination technique in cattle. The Animal Consortium. 12: S1; s75-s84.

Dorling, A.A., Todman, M.G., Korach, K.S., Herbison, A.E. 2003. Critical Role for Estrogen Receptor alpha in Negative Feedback Regulation of Gonadotropin-Releasing Hormone mRNA Expression in the Female Mouse. Neuroendocrinology. 73: 204-209.

Downey, B. R. 1980. Regulation of the Estrous Cycle in Domestic Animals-A Review. The Canadian Veterinary Journal. 21:301-306.

Edmondson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G. 1989. A Body Condition Scoring Chart for Holstein Dairy Cows. Journal of Dairy Science. 72: 68-78.

Fishman-Holland, H., Stoskute, A., Ferrer, M.S., Veal, D., Bittar, J.H.J., Rollin, E., Lourenço, J., Palomares, R.A. 2019. Comparison of Follicular Development, Timing of Ovulation and Serum Progesterone, Estradiol, and Luteinizing Hormone Concentrations in Dairy Heifers Treated with 4- or 5-Day CoSynch + CIDR Protocols. Veterinary Medicine Science. 5: 379-389.

Forar, A.L., Gay, J.M., Hancock, D.D. 1995. The frequency of endemic fetal loss in dairy cattle: A review. Theriogenology. 43: 989-1000.

Frijters, A.C.J., Mullaart, E., Roelofs, R.M.G., van Hoorne, R.P., Moreno, J.F., Moreno, O., Merton, J.S. 2009. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology. 71: 64-67.

García-Ispierto, I., López-Gatius, F., Santolaria P., Yániz, J.L., Nogareda, C., López-Béjar, M. 2007. Factors affecting fertility of high producing dairy herds in northeastern Spain. Theriogenology. 67: 632-638.

Gasser, C.L., Grum, D.E., Mussard, M.L., Fluharty, F.L., Kinder, J.E., Day, M.L. 2006. Induction of precocious puberty in heifers I: Enhanced secretion of luteinizing hormone. Journal of Animal Science. 84:2035-2041.

González-Marín, C., Góngora, C.E. Gilligan, T.B., Evans, K.M., Moreno, J.F., Vishwanath, R. 2018. *In vitro* sperm quality and DNA integrity of SexedULTRA™ sex-sorted sperm compared to non-sorted bovine sperm. Theriogenology. 114: 40-45.

Gosálvez, J., Ramirez, M.A., López-Fernández, C., Crespo, F., Evans, K.M., Kjelland, M.E., Moreno, J.F. 2011. Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. Theriogenology. 75: 206-211.

Graves, W.M., Dowlen, H.H., Kiess, G.A., Riley, T.L. 1991. Evaluation of Uterine Body and Bilateral Uterine Horn Insemination Techniques. Journal of Dairy Science. 74: 3454-3456.

Hawk, H.W., Conley, H.H., Wall, R.J., Whitaker, R.O. 1988. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. Theriogenology. 29:1131–42.

Hohenboken, W.D. 1999. Applications of Sexed Semen in Cattle Production. Theriogenology. 52: 1421-1433.

Holden, S.A., Butler, S.T. 2018. Review: Applications and benefits of sexed semen in dairy and beef herds. The Animal Consortium. 12: S1, s97-s103.

Holm, D.E., Nielen, M., Jorritsma R., Irons, P.C., Thompson, P.N. 2015. Evaluation of pre-breeding reproductive tract scoring as a predictor of long term reproductive performance in beef heifers. Preventative Veterinary Medicine. 118:56-63.

Holm, D.E., Thompson, P.N., Irons, P.C. 2009. The value of reproductive tract scoring as a predictor of fertility and production outcomes in beef heifers. Journal of Animal Science. 87:1934-1940.

Hunter, R.H.F. 2001. New Breeding Opportunities with Deep Cornual Insemination: Exploiting Modern Sperm Technologies in Cattle. Reproduction in Domestic Animals. 36: 217-222.

Hunter, R.H.F. 2003. Advances in deep uterine insemination: a fruitful way forward to exploit new sperm technologies in cattle. Animal Reproduction Science. 79: 157-170.

Hurnik, J.F., King, G.J., Robertson, H.A. 1975. Estrous and Related Behavior in Postpartum Holstein Cows. Applied Animal Ethology. 2:55-68.

Hutchinson, I.A., Shalloo, L., Butler, S.T. 2013. Expanding the dairy herd in pasture-based systems: The role of sexed semen use in virgin heifers and lactating cows. Journal of Dairy Science. 96: 6742-6752.

Ingenhoff, L., Hall, E., Ranjbar NI, S., House, J.K. 2017. Effect of insemination site and diameter of the pre-ovulatory follicle on the odds of pregnancy in heifers using sexed or non-sexed semen. Australian Veterinary Journal. 95: 317-324.

Islam, R. 2011. Synchronization of Estrus in Cattle: A Review. Veterinary World. 4(3):136-141

Kanitz, Wilhelm. 2003. Follicular dynamic and ovulation in cattle-a review. Arch. Tierz., Dummerstorf. 46:187-198.

Karakaya, E., Yilmazbas-Mecitoglu, G., Keskin, A., Alkan, A., Tasdemir, U., Santos, J.E.P., Gumen, A. 2014. Fertility in Dairy Cows After Artificial Insemination Using Sex-Sorted Sperm or Conventional Semen. Reproduction in Domestic Animals. 49: 333-337.

Kasimanickam, R.K., Whittier, W.D., Hall, J.B., Kastelic, J.P. 2016. Estrous synchronization strategies to optimize beef heifer reproductive performance after reproductive tract scoring. Theriogenology. 86:831-838.

Kenny, D.A., Heslin, J., Byrne, C.J. 2018. Early onset of puberty in cattle: implications for gamete quality and embryo survival. Reproduction, Fertility and Development. 30:101-117.

Kojima, F. N. 2003. Symposium Paper: The estrous cycle in cattle: Physiology, Endocrinology, and Follicular Waves. The Professional Animal Scientist. 19:83-95.

Kotwica, J., Skarzynski, D., Miszkiel, G. 1998.Oxytocin modulates the pulsatile secretion of prostaglandin $F_{2\alpha}$ in initiated luteolysis in cattle. Research in Veterinary Science. 66: 1-5.

Kumar, A., Vineeth, M.R., Sinha, R., Singh, R.K., Thakur, A., Gupta, S.K. 2016. Current status, scope and constraints of sexed semen – An Indian perspective. Agricultural Reviews. 37: 240-244.

Kurykin, J. 2017. Sex-Sorted Semen: Efficiency of Insemination and Opportunities to Increase Outcome of Pregnancies in Dairy and Beef Cattle. A Review. Veterinary Medicine and Zootechnics. 75: 22-29.

Kurykin, J., Jaakma, Ü., Jalakas, M., Aidnik, M., Waldmann, A., Majas, L. 2007. Pregnancy percentage following deposition of sex-sorted sperm at different sites within the uterus in estrus-synchronized heifers. Theriogenology. 67:754–9.

Lamb, G. C., Dahlen, C. R., Larson, J. E., Marquezini, G., Stevenson, J. S. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: A review. Journal of Animal Science. 88: E181-E192.

Lim, H.J., Yoon, H.B. 2018. Investigation of Relation between the Ovulation Confirmation and Conception Rate in Dairy Cattle. Journal of Embryo Transfer. 33: 55-59.

Lopes, A.S., Butler, S.T., Gilbert, R.O., Butler, W.R. 2007. Relationship of pre-ovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. Animal Reproduction Science. 99: 34-43.

López-Gatius, F. 1999. Site of Semen Deposition in Cattle: A Review. Theriogenology. 53: 1407-1414.

López-Gatius, F. 2012. Factors of a noninfectious nature affecting fertility after artificial insemination in lactating dairy cows. A review. Theriogenology. 77; 1029-1041.

López-Gatius, F., Camón-Urgel, J. 1988. Increase of pregnancy rate in dairy cattle after preovulatory follicle palpation and deep cornual insemination. Theriogenology. 29: 1099-1103.

Macedo, G.G., Mingoti, R.D., Batista, E.O.S., Monteiro, B.M., Vieira, L.M., Barletta, R.V., Wiltbank, M.C., Nogueira, G.P., Rennó, F.P., Maio, J.R., Baruselli, P.S. 2019. Profile of LH release in response to intramuscular treatment with kisspeptin in Bos indicus and Bos taurus prepubertal heifers. Theriogenology. 125:64-70.

McCullock, K., Hoag, D.L., Parsons, J., Lacy, M., Seidel Jr., G.E., Wailes, W. 2013. Factors affecting economics of using sexed semen in dairy cattle. Journal of Dairy Science. 96: 6366-6377.

McKenna, T., Lenz, R., Fenton, S., Ax, R. 1990. Nonreturn Rates of Dairy Cattle Following Uterine Body or Cornual Insemination. Journal of Diary Science. 73: 1779-1783.

Meirelles, C., Kozicki, L.E., Weiss, R.R., Segui, M.S., Souza, A., dos Santos, I.W., dos Santos Breda, J.C. 2012. Comparison between Deep Intracornual Artificial Insemination (DIAI) and Conventional Artificial Insemination (AI) Using Low Concentration of Spermatozoa in Beef Cattle. Brazilian Archives of Biology and Technology. 55: 371-374

Miura, R., Haneda, S., Kayano, M., Matsui, M. 2015. Short communication: development of the first follicular wave dominant follicle on the ovary ipsilateral to the corpus luteum is associated with decreased conception rate in dairy cattle. Journal of Dairy Science. 98; 318-321.

Miura, R., Izumi, T. 2018. Relationship of the conception rate and the side (left or right) of preovulatory follicle location at artificial insemination in dairy heifers. Animal Science Journal. 89: 328-331.

Moće, E., Graham, J.K., Schenk, J.L. 2006. Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. Theriogenology. 66: 929-936.

Moller, K., Macmillan, K.L., Shannon, P. 1972. Site of insemination and subsequent non-return rates in cows. New Zealand Journal of Agricultural Research. 15:252-254.

Moran, C., Quirke J. F., Roche, J. F. 1989. Puberty in Heifers: A Review. Animal Reproduction Science. 18:167-182.

Murphy, C., Shalloo, L., Hutchinson, I.A., Butler, S.T. 2016. Expanding the dairy herd in pasture-based systems: The role of sexed semen within alternative breeding strategies. Journal of Dairy Science. 99: 6680-6692.

Noseir, Wael MB. 2003. Ovarian follicular activity and hormonal profile during estrous cycle in cows: the development of 2 versus 3 waves. Reproductive Biology and Endocrinology. 1:50.

Nowicki, A., Barański, W., Baryczka, A., Janowski, T. 2017. OvSynch Protocol and its Modifications in the Reproduction Management of Dairy Cattle Herds – an Update. Journal of Veterinary Research. 61: 329-336.

Nutrient Requirements of Dairy Cattle. Seventh revised edition. Washington, DC: National Academy of Science; 2001.

O'Connor, Michael. Heat Detection and Timing of Insemination for Cattle. Pennsylvania State University. 1993.

Oikawa, K., Yamazaki, T., Yamaguchi, S., Abe, H., Bai, H., Takahashi, M., Kawahara, M. 2019. Effects of use of conventional and sexed semen on the conception rate in heifers: A comparison study. Theriogenology. 135; 33-37.

Orihuela, A. 2000. Some factors affecting the behavioral manifestation of oestrus in cattle: a review. Applied Animal Behavior Science. 70:1-16.

Palomares, R.A. 2019. The Bovine Estrus Cycle. Educational Resources. College of Veterinary Medicine. The University of Georgia.

Patel, S.B., Jethva, P.C. 2019. Use of Sexed Semen in Indian Dairy Cattle: A Case study. The Indian Journal of Veterinary Sciences & Biotechnology. 14: 54-57.

Patterson, D.J., Corah, L.R., Brethour, J.R., Spire, M.F., Higgins, J.J., Kiracofe, G.H., Stevenson, J.S., Simms, D.D. 1991. Evaluation of reproductive traits in *Bos taurus* and *Bos indicus* crossbred heifers: Effects of postweaning energy manipulation. Journal of Animal Science. 69: 2349-2361.

Perry, G.A., Smith, M.F., Roberts, A.J., MacNeil, M.D., Geary, T.W. 2007. Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. Journal of Animal Science. 85: 684-689.

Perry, G.A. 2012. Physiology and Endocrinology Symposium: Harnessing basic knowledge of factors controlling puberty to improve synchronization of estrus and fertility in heifers. Journal of Animal Science. 90, 4; 1172.

Perry, G.A. 2016. Factors affecting puberty in replacement beef heifers. Theriogenology. 86:373-378.

Peters, A.R. 1985. Hormonal Control of the Bovine Oestrus Cycle. I. The Natural Cycle. British Veterinary Journal. 141: 564-575.

Pursley, J.R., Mee, M.O., Wiltbank, M.C. 1995. Synchronization of Ovulation in Dairy Cows using $PGF_{2\alpha}$ and GnRH. Theriogenology. 44: 915-923.

Pursley, J.R., Wiltbank, M.C., Stevenson, J.S., Ottobre, J.S., Garverick, H.A., Anderson, L.L. 1997. Pregnancy Rate Per Artificial Insemination for Cows and Heifers Inseminated at a Synchronized Ovulation or Synchronized Estrus. Journal of Dairy Science. 80: 295-300.

Quintal-Franco, J.A., Kojima, F.N., Melvin, E.J., Lindsey, B.R., Zanella, E., Fike, K.E., Wehrman, M.E., Clopton, D.T., Kinder, J.E. 1999. Corpus Luteum Development and Function in Cattle with Episodic Release of Luteinizing Hormone Pulses Inhibited in the Follicular and Early Luteal Phases of the Estrous Cycle. Biology of Reproduction. 61:921-926.

Rabaglino, M.B., Risco, C.A., Thatcher, M.J., Kim, I.H., Santos, J.E.P., Thatcher, W.W. 2010. Application of one injection of prostaglandin $F_{2\alpha}$ in the five-day Co-Synch + CIDR protocol for estrous synchronization and resynchronization of diary heifers. Journal of Dairy Science. 93: 1050-1058.

Rai, J. 2018. Sperm sexing of dairy cattle: economics, animal welfare and technological challenges. Current Science. 114; 1438-1442.

Rius, A.G., Connor, E.E., Capuco, A.V., Kendall, P.E., Auchtung-Montgomery, T.L., Dahl, G.E. 2005. Long-Day Photoperiod that Enhances Puberty Does Not Limit Body Growth in Holstein Heifers. Journal of Dairy Science. 88:4356-4365.

Rivera, H., Lopez, H., Fricke, P.M. 2005. Use of intravaginal progesterone-releasing inserts in a synchronization protocol before timed AI and for synchronizing return to estrus in Holstein heifers. Journal of Dairy Science. 88: 957-968.

Roche, J.F., Boland, M.P. 1991. Turnover of Dominant Follicles in Cattle of Different Reproductive States. Theriogenology. 35:1.

Sá Filho, M.F., Crespilho, A.M., Santos, J.E., Perry, G.A., Baruselli, P.S. 2010. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled Bos indicus cows. Animal Reproduction Science. 120: 23-30.

Sá Filho, M.F., Girotto, R., Abe, E.K., Penteado, L., Campos Filho, E.P., Moreno, J.F., Sala, R.V., Nichi, M., Baruselli, P.S. 2012. Optimizing the use of sex-sorted sperm in timed artificial insemination programs for suckled beef cows. Journal of Animal Science. 90:1816–23.

Sales, J.N.S., Neves, K.A.L., Souza, A.H., Crepaldi, G.A., Sala, R.V., Fosado, M., Campos Filho, E.P., de Faria, M., Sá Filho, M.F., Baruselli, P.S. 2011. Timing of insemination and fertility in dairy and beef cattle receiving timed artificial insemination using sex-sorted sperm. Theriogenology. 76: 427-435.

Sartori, R., Haughian, J.M., Shaver, R.D., Rosa, G.J.M., Wiltbank, M.C. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. Journal of Dairy Science. 87: 905-920.

Schels, H.F., Mostafawi, D. 1978. The effect of GnRH on the pregnancy rate of artificially inseminated cows. Veterinary Record. 103:31-32

Schillo, K.K., Hansen, P.J., Kamwanja, L.A., Dierschke, D.J., Hauser, E.R. 1983. Influence of Season on Sexual Development in Heifers: Age at Puberty as Related to Growth and Serum Concentrations of Gonadotropins, Prolactin, Thyroxine and Progesterone. Biology of Reproduction. 28:329-341.

Schmitt, E.J.P., Drost, M., Diaz, T., Roomes, C., Thatcher, W.W. 1996. Effect of a Gonadotropin-Releasing Hormone Agonist on Follicle Recruitment and Pregnancy Rate in Cattle. Journal of Animal Science. 74: 154-161.

Seidel, G.E. 2007. Overview of sexing sperm. Theriogenology. 68: 443-446.

Seidel, G.E. 2014. Update on sexed semen technology in cattle. Animal. 8:s1, 160-164.

Seidel, G.E., Allen, C.H., Johnson, L.A., Holland, M.D., Brink, Z., Welch, G.R., Graham, J.K., Cattell, M.B. 1997. Uterine Horn Insemination of Heifers with Very Low Numbers of Nonfrozen and Sexed Spermatozoa. Theriogenology. 48: 1255-1264.

Seidel, G.E., Schenk, J.L. 2008. Pregnancy rates in cattle with cryopreserved sexed sperm: Effects of sperm numbers per inseminate and site of sperm deposition. Animal Reproduction Science. 105: 129-138.

Senger, P.L., Becker, W.C., Davidge, S.T., Hillers, J.K., Reeves, J.J. 1988. Influence of cornual insemination on conception in dairy cattle. Journal of Animal Science. 66; 3010-3016.

Senger, PL. Pathways to Pregnancy & Parturition. 3rd ed., Current Conceptions, 2012.

Sharpe, J.C., Evans, K.M. 2009. Advances in flow cytometry for sperm sexing. Theriogenology. 71: 4-10.

Smith, M.F., Perry, G.A., Atkins, J.A., Jinks, E.M., Pohler, K.G., Patterson, D.J. 2010. Physiological Principles Underlying Synchronization of Estrus. Applied Reproductive Strategies in Beef Cattle. 29-52.

Stevenson, J.S. 2005. Breeding Strategies to Optimize reproductive Efficiency in Dairy Herds. Veterinary Clinics of North America Food Animal Practice. 21: 349-365.

Tenhagen, B.A., Kuchenbuch, S., Heuwieser, W. 2005. Timing of Ovulation and Fertility of Heifers After Synchronization of Oestrus with GnRH and Prostaglandin $F_{2\alpha}$. Reproduction in Domestic Animals. 40: 62-67.

Tibary, A., Patino, C., Ciccarelli, M. 2019. Synchronization of Estrus and Ovulation in Dairy Cattle. Spermova. 9(1): 1-13.

Townson, D.H., Tsang, P.C., Butler, W.R., Frajblat, M., Griel Jr, L.C., Johnson, C.J., Milvae, R.A., Niksic, G.M., Pate, J.L. 2002. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. Journal of Animal Science. 80: 1053–1058.

Van Schyndel, S.J., Bauman, C.A., Pascottini, O.B., Renaud, D.L., Dubuc, J., Kelton, D.F. 2018. Reproductive management practices on dairy farms: The Canadian National Dairy Study 2015. Journal of Dairy Science. 102: 1822-1831.

Veronesi, M.C., Gabai, G., Battocchio, M., Mollo, A., Soldano, F., Bono, G., Cairoli F. 2001. Ultrasonographic appearance of tissue is a better indicator of CL function than CL diameter measurement in dairy cows. Theriogenology. 58:61-68.

Vishwanath, R. 2014. SexedULTRA – raising the fertility bar of sexed sorted semen. In Proceedings of the 25th Technical Conference on Artificial Insemination and Reproduction, National Association of Artificial Breeders, September 2014, Wisconsin, USA, pp. 57–61.

Vishwanath, R., Moreno, J.F. 2018. Review: Semen sexing – current state of the art with emphasis on bovine species. The Animal Consortium. 12: S1; s85-s96.

Westfalewicz, B., Dietrich, M.A., Mostek, A., Partyka, A., Bielas, W., Niżański, W., Ciereszko, A. 2017. Analysis of bull (Bos taurus) seminal vesicle fluid proteome in relation to seminal plasma proteome. Journal of Dairy Science. 100: 2282-2298.

Williams, B.L., Gwazdauskas, F.C., Whittier, W.D., Pearson, R.E., Nebel, R.L. 1988. Impact of site of inseminate deposition and environmental factors that influence reproduction of dairy cattle. Journal of Dairy Science. 71: 2278-2283.

Wolfenson, D., Flamenbaum, I., Berman, A. 1988. Hyperthermia and Body Energy Store Effects on Estrous Behavior, Conception Rate and Corpus Luteum Function in Dairy Cows. Journal of Dairy Science. 71:3497-3504.

Wolfenson, D., Thatcher, W.W., Badinga, L., Savio, J.D., Meidan, R., Lew, B.J., Braw-Tal, R., Berman, A. 1995. Effect of Heat Stress on Follicular Development During the Estrous Cycle in Lactating Dairy Cattle. Biology of Reproduction. 52: 1106-1113.

Yapura, M. J., Zwiefelhofer, E. M., Pierson, R. A., Adams, G. P. 2018. Aromatase inhibitors: A new approach for controlling ovarian function in cattle. Theriogenology. 112: 18-25.

Yelich, J. V., Wetteman, R.P., Marston, T.T., Spicer, L.J. 1996. Luteinizing hormone, growth hormone, insulin-like growth factor-I, insulin and metabolites before puberty in heifers fed to gain at two rates. Domestic Animal Endocrinology. 13:325–338.

Zobel, R., Gereš, D., Pipal, I., Buić, V., Gračner, D., Tkalcic, S. 2011. Influence of the Semen Deposition Site on the Calves' Sex Ratio in Simmental Dairy Cattle. Reproduction in Domestic Animals. 46: 595-601.

Table 3.1: Reproductive cyclicity status on Day 0 of the protocol of all heifers initiated into the study

	Major Ovarian Structure on Day 0			
	CL	Follicle ≥8 mm (Large/Medium)	Follicle >8 mm (Small)	No Significant Structures
Total	177/373 = 47.5%	127/373 = 34.0%	44/373 = 11.8%	25/373 = 6.7%

One heifer had a luteal cyst as their main ovarian structure on Day 0.
Abbreviations: CL, Corpus luteum.

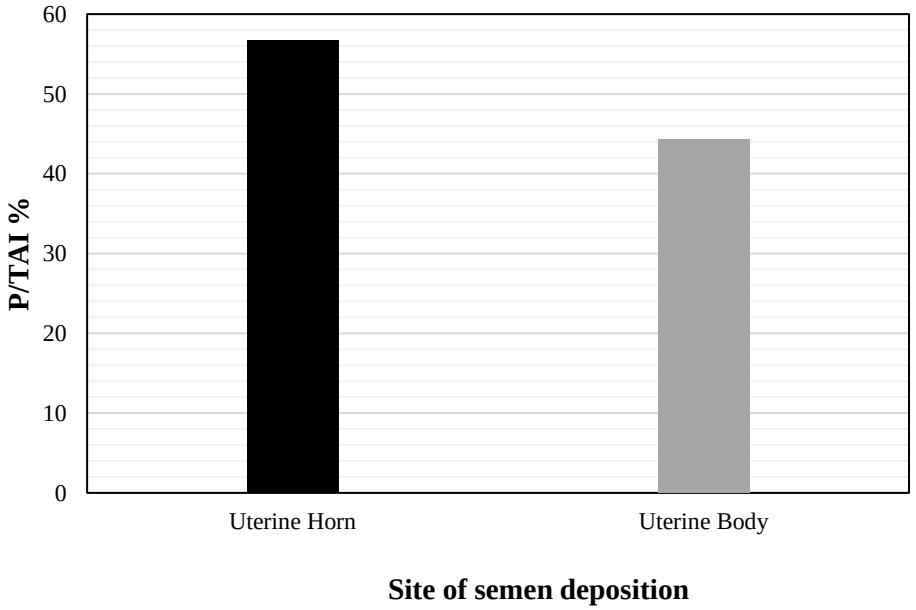


Figure 3.1: Overall pregnancy per TAI among heifers inseminated in the uterine horn ipsilateral to the ovary containing the preovulatory follicle versus heifers inseminated in the uterine body regardless of the location of the preovulatory follicle. This shows total P/TAI regardless of year or inseminator. There was a significant difference between groups (P = 0.038).

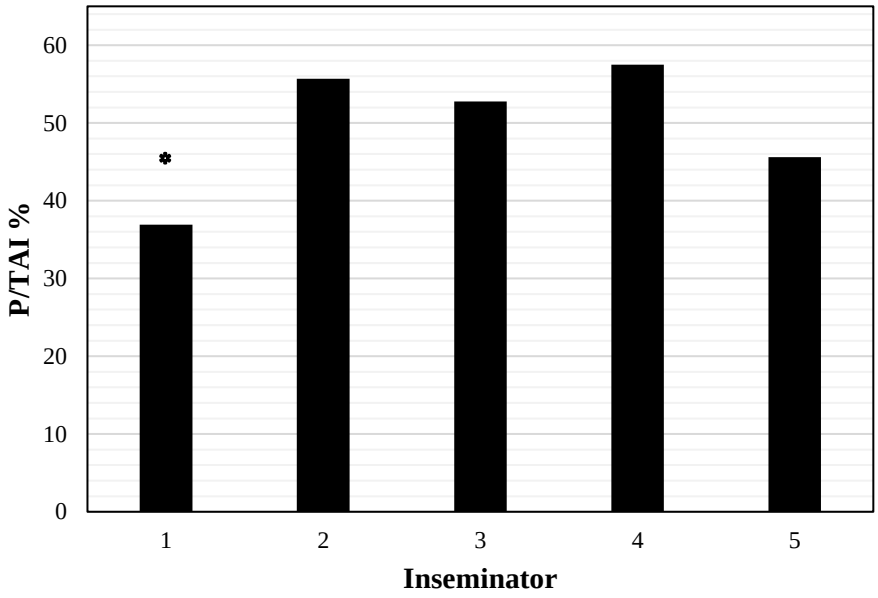


Figure 3.2: Overall pregnancy per TAI for each inseminator. It indicates the percentage of total pregnancies each inseminator had divided by the total number of heifers inseminated by each inseminator. *There was a tendency of lower overall P/TAI for inseminator 1 (P = 0.09).

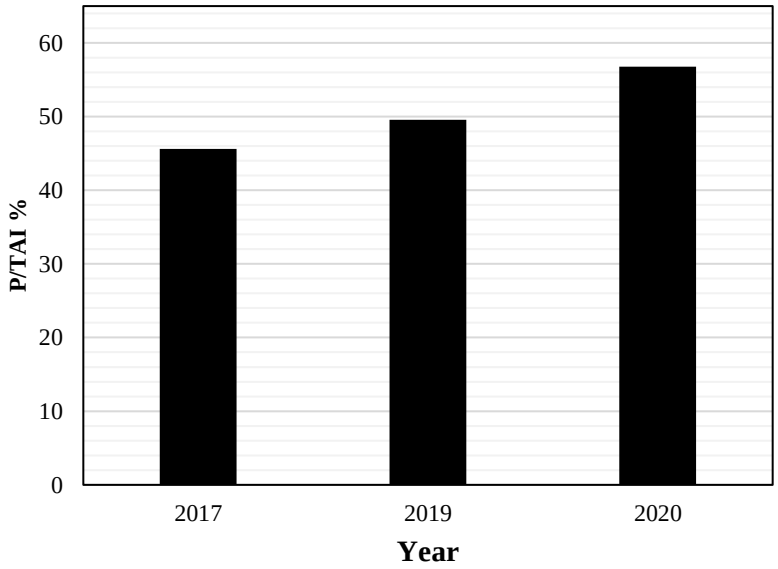


Figure 3.3: Overall P/TAI per year. It indicates the percentage of total pregnancies per year regardless of inseminator or group. There was no significant difference between years (P = 0.389).

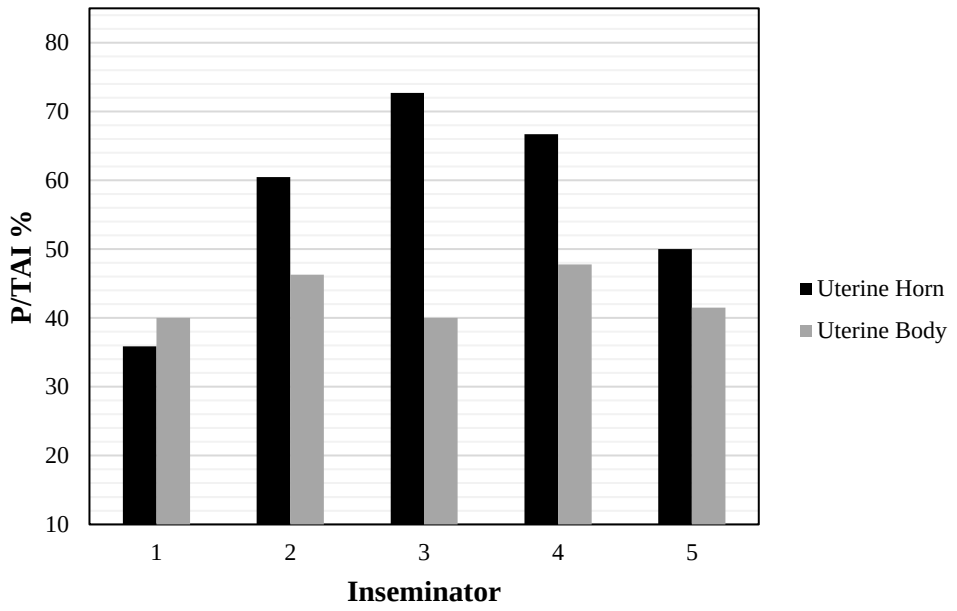


Figure 3.4: Comparison of the P/TAI based on the site of semen deposition (uterine horn versus uterine body) for each inseminator. There was a greater P/TAI for TAI with SS in the UH when compared with UB, for all inseminators, except for inseminator 1, which had a lower P/TAI (P=0.09).

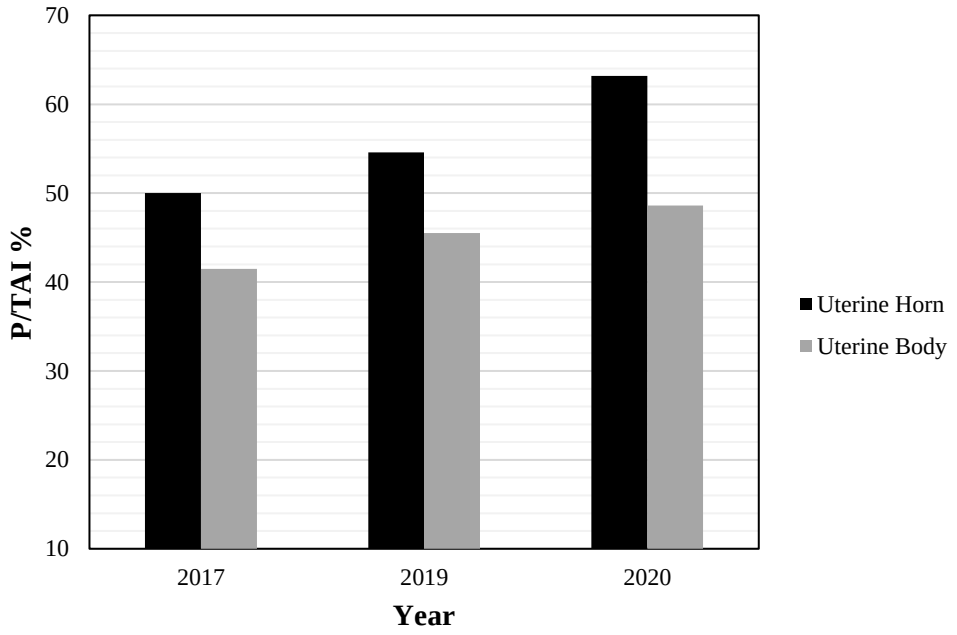


Figure 3.5. Comparison of the P/TAI according to the site of semen deposition (uterine horn versus uterine body) for each year, regardless of inseminator. There was not a significant difference among years (P>0.05).

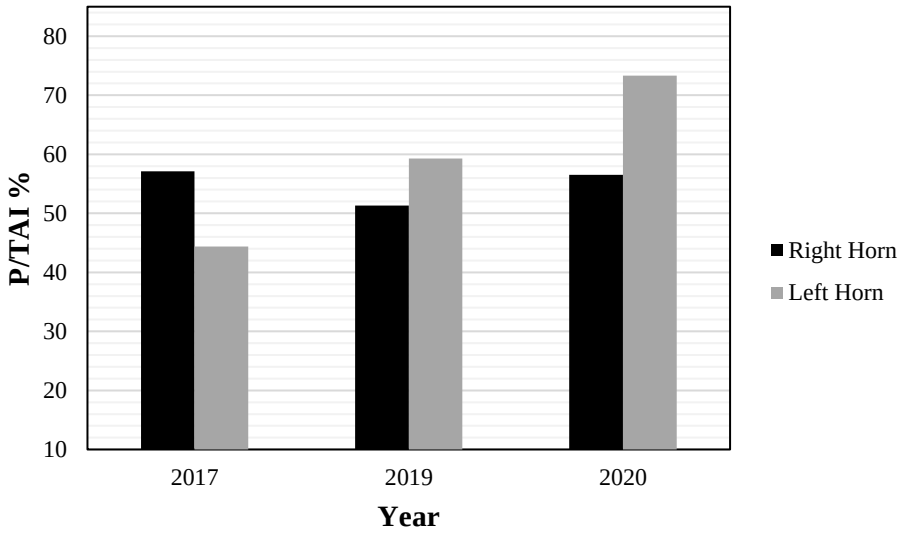


Figure 3.6. Comparison of the pregnancy per TAI in heifers artificially inseminated in the uterine horn (right versus left) ipsilateral to the ovary containing the preovulatory follicle per year.

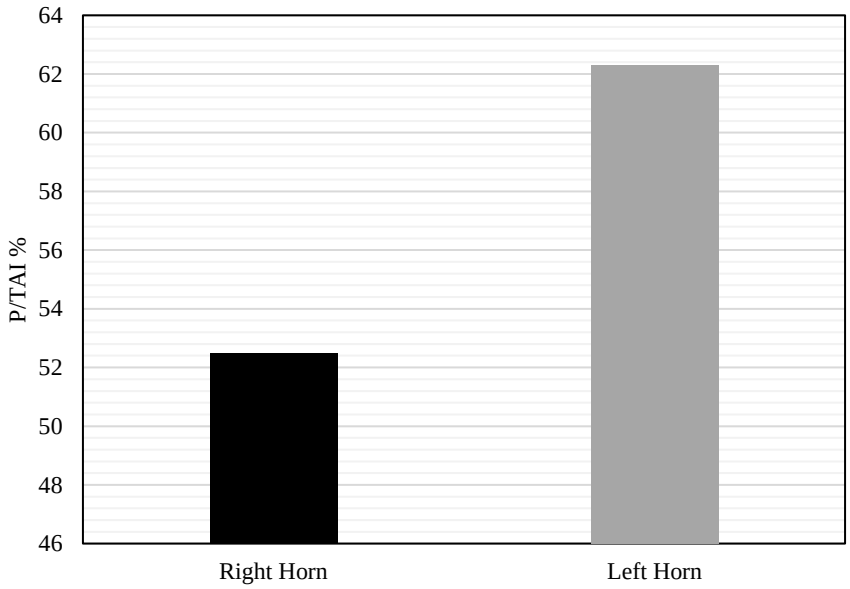


Figure 3.7. Total pregnancy per TAI in heifers artificially inseminated in the uterine horn ipsilateral to the preovulatory follicle in the 2019 and 2020 breeding season.

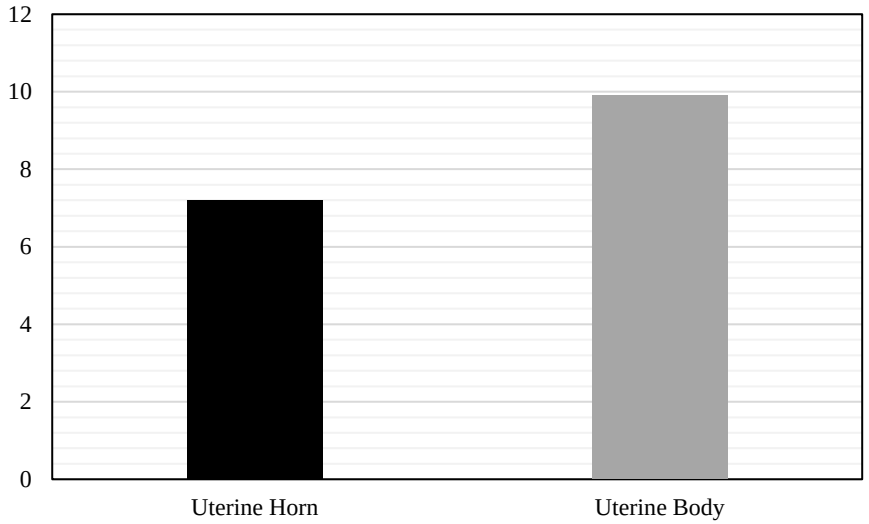


Figure 3.8. Comparison of percentage of pregnancy loss (PL) between heifers inseminated in the uterine horn ipsilateral to the ovary containing the preovulatory follicle versus heifers inseminated in the uterine body regardless of the location of the preovulatory follicle. Pregnancy diagnosis was performed by TRUS 35 days after TAI. Pregnancy reconfirmation was done 60-90 days after TAI. There was no significant difference between groups.