Strategies to improve fertility of *Bos taurus* beef females enrolled in estrous synchronization protocols

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ABSTRACT

Estrous synchronization (ES) protocols enahnces profitability of beef cow-calf operations by improving percentage of cows pregnant early in the breeding season and increasing kg of calf weaned per cow exposed. Many factors, however, influence the fertility of cattle enrolled to ES protocols. The overall goal of this work was to determine the influences of estrus expression and timming of artificial insemination (AI), as well as temperament on fertility of *Bos taurus* beef females exposed to ES protocols. Multiparous cows (n = 1,676) were enrolled to the 7-d CO-Synch+ Controlled internal drug releasing device (CIDR) protocol and cows expressing estrus were inseminated. Cows not expressing estrus received a gonadotropin-releasing hormone injection and were either inseminated immediately or delayed by 8 hours. Pregnancy rates were greater for cows expressing estrus to the protocol. Delaying AI for 8 hours in cows that failed to express estrus did not improve pregnancy rates. The effects of temperament on fertility of beef heifers enrolled to the 7-d CO-Synch+CIDR protocol were investigated. Heifers (n = 297) had temperament assessed by chute score and exit velocity, and classified as adequate or excitable. Hair and blood were collected for cortisol evaluation. Pregnancy rates were greater for adequate temperament heifers. Circulating cortisol concentrations were greater for excitable heifers, and overall plasma and hair cortisol concentrations were reduced from the start of the protocol to the end. Heifers with adequate temperament have improved fertility and ES protocols acclimated heifers and reduced plasma and hair cortisol concentrations due to handling.

General audience abstract

Infertility is a major issue that affects profitability of beef cow-calf producers. Estrous synchronization (ES) protocols are a tool that allow artificial insemination (AI) without estrus detection and increases the proportion of females pregnant at the start of the breeding season, providing a strategy to improve profitability. However, females enrolled in the ES protocol and that fail to express estrus have reduced pregnancy rates when compared to females expressing estrus. Furthermore, beef females with excitable temperament have reduced fertility when compared to females with adequate temperament. The effects of delaying insemination to 8 hours post injection of gonadotropin-releasing hormone for animals failing to express estrus during ES were determined in beef multiparous cows. Cows expressing estrus had greater pregnancy rates compared to cows not expressing estrus, and delayed insemination did not improve pregnancy rates. To determine the effects of temperament on fertility heifers were enrolled in an ES protocol and had temperament determined as adequate or excitable based on chute score and exit velocity. Hair and blood samples were collected for cortisol evaluation. Excitable heifers had reduced pregnancy rates and greater circulating cortisol concentrations, but hair cortisol did not differ between temperaments. Overall cortisol profiles indicate that heifers became acclimated to handling during ES protocol. Development of ES that maximizes estrus expression prior to AI and selection of cattle with adequated temperament can enhance profitability of cow-calf operations.

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List of Abbreviations

ACTH: Adrenocorticotropic hormone

AI: Artificial insemination

AVP: Vasopressin

BCS: Body condition score

CIDR: Controlled internal drug releasing device

CL: Corpus luteum

CRH: Corticotropin-releasing hormone

E2: Estradiol

eCG: equine chorionic gonadotropin

eCP: Estradiol cypionate

EPD: Expected progeny differences

ES: Estrus synchronization

ET-1: Endothelin-1

FSH: Follicle stimulating hormone

GnRH: Gonadotropin releasing hormone

GPR54: G protein coupled receptor 54

HPA: Hypothalamic-pituitary-adrenal

IFNT: Interferon-tau

ISG: Interferon stimulated genes

LH: Luteinizing hormone

M: Mastectomized

NS: Non-suckled

P/AI: Pregnancy to artificial insemination

P4: Progesterone

PGE2: Prostaglandin-E-2

PGF2α: Prostaglandin-F2α

RFRP: RF-amide-related peptide

S: Suckled

STAI: Split time artificial insemination

TAI: Fixed-time artificial insemination

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Chapter 1

Introduction

The world population is expected to reach 9.73 billion people by the year of 2050 and 11.2 billion people by 2100. This represents a growth of 31% and 51% of the present population, respectively. In order to meet the demand of this growing population, agriculture has the challenge of producing 50% more food than present production with limited expansion of land (F.A.O., 2018). In addition, there is a worldwide increase in the middle-class population, which is expected to reach nearly 5 billion people by the year of 2030 (Kharas and Gertz, 2010). Consumers of the middle-class tend to have a more diversified diet, and their desire for animal protein results in increasing global protein consumption (F.A.O., 2018). The challenge for the beef industry is to improve production so an adequate supply of beef is available to meet the increasing protein demands. In order to meet the increasing demand, whit limited expansion of land, the efficiency of the beef industry productivity should rely on the adoption of technology.

The main goal for cow-calf producers is to wean one healthy calf from each cow every year, however most operations fail to reach a weaning rate of 100% and the main trait responsible for that loss is infertility (Lamb et al., 2008). Infertility is of particular interest in Virginia, a traditional cow-calf state with a beef industry composed of over 650,000 cows distributed on more than 26,000 beef cattle operations across all regions of the state (Virginia Cooperative Extension). The economic impact of infertility may be severe.

The impact of infertility can be determined by simply calculating the revenue resulted by exposed cows in the herd. The average price for a 600lb. feeder calf in Virginia is $1.50/lb (Roanoke – Hollins weekly auction, 02/20/2018). Assuming 100%

of weaned calf per cow exposed, there is a profit of $900 per cow, whereas if 85% of weaned calf per cow exposed is assumed, the profit is reduced to $765 per cow resulting in a total loss of $9.00 per cow exposed for every 1% decrease in pregnancy rates.

In order to increase pregnancy rates, producers need to overcome several factors that influence fertility of beef cows and heifers during a breeding season. After parturition, cows will undergo a period of postpartum anestrus where follicular activity is present but animals fail to ovulate. Many factors will influence the length of postpartum anestrus, such as nutritional status, parity, suckling stimulus (Montiel and Ahuja, 2005) and temperament (Cooke et al. 2012b).

The understanding of the dynamic events of the estrous cycle in cattle has allowed the development of protocols that control the events of the cycle pharmacologicaly, leading to ovulation, facilitating adoption of AI and potentially inducing cyclicity in animals during postpartum anestrus (Meneghetti et al., 2009). The potential advantages of implementing such protocols in a herd are numerous and will be explored in the upcoming literature review.

Despite of the advantages of adopting ES, various factors will influence the fertility of animals enrolled to the protocol. These factors include temperament (Cooke et al., 2012b), estrus expression to the protocol (Bishop et al., 2016) ovulation response to the protocol (Vasconcelos et al., 1999) and follicle diameter at AI (Sa Filho et al., 2011). The comprehension of the effects of these factors on the fertility to ES protocols would provide valuable information for more efficient strategies to be created, allowing improvement of the efficiency of cow-calf operations.

The present work will demonstrate how the understanding of the dynamic factors that regulate the estrous cycle has allowed the development of ES protocols.

Further, analysis of different factors that influence the fertility of animals enrolled in ES protocols will be explored and strategies to overcome these effects will be discussed.

Chapter 2

Literature Review

Bovine Estrous Cycle

The estrous cycle is composed by dynamic events leading up to estrus expression and ovulation. In the bovine, these events are controlled in an endocrine manner, where hormones produced and secreted by the hypothalamus, the pituitary gland, the ovary and the uterus are responsible for regulating the estrous cycle through hormone feedback. The bovine estrous cycle is in average 21 days, but may range from 17 to 24 days according to individual variance (Lamb et al., 2010). The cycle is categorized, according to blood hormone profile, into 4 stages: 1) Estrus: period that ranges from 25 to 30 hours after acceptance of mount, is characterized by great blood concentrations of estradiol (E2) originated from the dominant follicle, which, in the absence of a mature and developed corpus luteum (CL), will trigger the surge of luteinizing hormone (LH) and ovulation. 2) Metestrus: period that ranges from 1 to 5 days after estrus, is a period characterized by blood hormone dominance profile transition with decreasing concentrations of E2 and increasing concentrations of progesterone (P4) originated by the newly formed CL. 3) Diestrus: period that ranges from 5 to 15 days after estrus, is characterized by great production of P4 by the mature CL, providing a blood P4 dominance profile. 4) Proestrus: period that ranges from 2 to 5 days prior to estrus, when transition of hormone dominance profile in the blood happens. Initiated by uterine production and secretion of prostaglandin-F2α (PGF2α), resulting in luteolysis and decreasing concentrations of P4, concomitantly with selection of the dominant follicle and increasing concentrations of E2 (Amstalden and Williams, 2014).

The Hypothalamic–Pituitary–Gonadal–Uterine Axis

The numerous events leading to ovulation and subsequent pregnancy maintenance in cattle are dynamic and the understanding of these events is key to comprehend how fertility may be impaired. The estrous cycle is controlled by several hormones produced by different organs and is regulated in a dynamic manner through hormone feedback. In the hypothalamus, there are two key neurons (Kiss-1 and Gonadrotopin Releasing Hormone (GnRH)) that are responsible for regulating the estrous cycle. Kiss-1 is responsible for producing and secreting a neuropeptide called kisspeptin. Kisspeptin bind and activate the G protein – coupled receptor 54 (GPR54) expressed on GnRH neurons, resulting on production and secretion of GnRH (Atkins et al., 2008). Gonadotropin releasing hormone produced by its neurosecretory neuron in the hypothalamus will be secreted from the median eminence into a primary capillary plexus and will travel the hypothalamo-hypophyseal portal system until it reaches a second capillary plexus present in the anterior pituitary gland. When GnRH reaches the pituitary, it stimulates the synthesis and secretion of the two gonadotropins, follicle stimulating hormone (FSH) and LH. Both gonadotropins will be released into the systemic blood stream and reach the ovaries, resulting in follicular growth and luteal activity (Clarke and Pompolo, 2005).

As follicles develop, one follicle acquires dominance over the others and is the main responsible for producing E2 and inhibin, which will be secreted into the bloodstream and exert a negative feedback with the pulsatile center of GnRH in the hypothalamus, resulting in decreased secretion of FSH and atresia of the remaining follicles.

In the absence of P4, produced and secreted by a developed and functional CL, great E2 concentrations will exert a positive feedback with the GnRH surge center in the hypothalamus, resulting in increased amplitude and frequency of LH production and

secretion. The increased amplitude and frequency of LH production shortens the interval between pulses and results in an LH surge and ovulation of the dominant follicle (Karsch, 1987).

After ovulation, the CL is formed and is responsible for producing P4 that acts developing the uterus for embryonic development and exerts a negative feedback with LH, blocking further ovulations. Progesterone negative feedback affects the surge center in the hypothalamus, preventing the E2 induced surge of LH and thus, ovulation (Karsch, 1987). Further detail of CL formation and luteolysis will be explored later in this review.

Follicle Dynamics

Follicles are tissues located in the cortex of the ovary, responsible for producing key hormones in controlling the estrous cycle and for developing the oocyte. The understanding of the follicular dynamics is key to develop strategies that control timing of ovulation of a competent follicle and oocyte when manipulating the cycle with ES protocols (Perry et al., 2005).

Follicular growth happens in a wave pattern, where several follicles will be stimulated to grow, and only one will acquire dominance. In average, the bovine estrous cycle is composed by 3 follicular waves but may range from 2 to 4 waves and only the dominant follicle of the last wave will ovulate (Perry, 2004).

In the ovary, primordial follicles formed during embryogenesis are recruited during the estrous cycle. As follicles develop, they grow in size and create a cavity, filled with follicular fluid, defined as antrum. In every follicular wave, several antral follicles will develop under the stimulus of FSH and LH (Perry, 2004).

Follicle Stimulating Hormone will provide proliferation of the antral follicles and prevent atresia and degeneration of the early antral follicles (Mihm et al., 2002).

The antral follicles selected will develop under FSH stimulus until they reach a size of 5 mm. At this point, follicles are able to produce E2 and inhibin, which in turn exert a negative feedback with FSH. When a follicle reaches a size of 8.5mm, it will further develop and may acquire dominance over the others (Ginther et al., 2001). The suppression of FSH surge is responsible for the deviation of follicular growth. The dominant follicle acquires LH receptors and is capable of growing under low FSH environment, and develops under the stimulus of LH pulses, whereas the remaining follicles will undergo atresia for failing in resume growth under a FSH deprived environment (Ginther et al., 1996). Atresia is defined as the apoptotic cell death a follicle will undergo. The dominant follicle can go under atresia or result in ovulation, depending on what follicular wave it is (Aerts and Bols, 2010).

The follicle has an important steroidogenic capacity, as it is the main source for circulating E2. Follicles produce E2 under the stimulus of both FSH and LH. It is proposed that LH bind and activates its receptors on the theca cells, resulting in the production of androstenedione from cholesterol. Androstenedione then is picked up by the granulosa cells, which under the stimulus of FSH, will upregulate the expression of aromatase. Aromatase is the enzyme responsible for producing E2 from androstenedione (Gervasio et al., 2014), resulting in increased production and secretion of E2. Increased concentrations of E2, provided by the dominant follicle from the last follicular wave, will be responsible for changing GnRH, and ultimately, LH pulsatility resulting in a surge and, in the absence of a functional CL and therefore, under low concentrations of P4, ovulation (Ginther et al., 1996).

When ovulation occurs, the oocyte along with the cumulus cells that surround the oocyte are released from the follicle into the oviduct and the remaining cells will form the CL (Aerts and Bols, 2010).

Corpus Luteum Formation

The development of a CL is essential for embryonic survival, once this tissue is the main source of blood P4, and P4 is essential for developing the uterus for embryonic development. Luteinization is defined by the morphological and functional changes the granulosa and thecal cells will undergo after ovulation (Stocco et al., 2007).

Luteinizing hormone is the main luteotropic hormone and is responsible for induction of luteinization, which is characterized by rapid cellular proliferation, differentiation and extensive angiogenesis (Silva et al., 2000). Luteal development starts with formation of small and large luteal cells. The large luteal cells derive from the granulosa cells, whereas small luteal cells derive from the theca interna cells (Alila and Hansel, 1984). The small and large luteal cells differ in function and characteristics. The large luteal cells lack LH receptors and therefore are not responsive to LH challenge however, they are greatly steroidogenic, contain PGF2α receptors and are capable of producing oxyoticn. The small luteal cells in turn, have little steroidogenic and oxytocin production ability, but have very few receptors for PGF2α and many receptors for LH and are responsive to LH stimulus (Fitz et al., 1984). The acquisition of steroidogenesis capacity to produce hormones such as P4, works by the LH-driven steroidogenic pathway alteration (Niswender et al., 2000).

The luteal tissue develops fast in its early formation and its growth rate can be compared to the growth of a tumor, and at an early stage, is capable to partially respond but fails to undergo complete luteolysis when challenged by PGF2α (Silva et al., 2000).

Luteolysis

Luteolysis is characterized as the functional and morphological changes the CL will undergo, leading to the regression of this tissue (Pate and Landis Keyes, 2001). Luteolysis is the key event to allow further ovulation as the CL is the main source of P4.

In addition, preventing luteolysis is an important event in pregnancy maintenance, as P4 is a key hormone to induce uterine changes for embryonic development.

Prostaglandin-F2α is a hormone produced by the endometrium of the non-pregnant uterus and is responsible for inducing luteolysis in the mid-cycle of the bovine CL. Progesterone acts in the endometrium maintaining oxytocin receptors down-regulated (Wathes and Lamming, 1995). The non-pregnant uterus under the stimulus of great E2 concentrations will up-regulate the expression of oxytocin receptors on the endometrium wall. The Oxytocin that induces CL regression is produced mainly by the CL itself, although we cannot discount other sources of oxytocin as key mediator in luteolysis. This oxytocin binds to its receptors in the uterus endometrium resulting in PGF2α production and secretion (Hansen et al., 2017).

Prostaglandin-F2α travels the uterine-ovarian counter current system and acts in the CL after it acquires luteolytic capacity and the effects of PGF2α are various. There is a direct effect of PGF2α on luteal cells, resulting in decreased P4 production, by inhibiting key enzymes for steroidogenesis. There is also an indirect effect of PGF2α on the endothelial cells within the CL. The effects of PGF2α on endothelial cells result in production and secretion of endothelin 1 (ET-1) which may act on luteal cells auxiliating steroidogenesis inhibition, or act with PGF2α on the endothelium, resulting in potent vasoconstriction and in result, a decrease in blood supply. This decrease in blood supply trigger further apoptotic mechanisms that facilitate further regression of the CL (Pate and Keyes, 2001).

With the regressing and non-functional CL, P4 concentrations in the blood will decrease, resulting in lack of negative feedback in the hypothalamus, enabling the E2 originated by the dominant follicle to alter pattern of GnRH secretion, and resilt in an LH surge and ovulation.

The CL needs to acquire luteolytic capacity, as injections of PGF2α analogs fail to induce luteolysis of the early cycle CL. While luteolysis is not the result observed, the early CL is not refractory to PGF2α as similar responses were seen in both early and mid cycle CLs when challenged with PGF2α. These changes include luteal ascorbic acid depletion, and decrease of PGF2α receptor expression, effects known to be part of PGF2α responses in the CL. The large luteal cells of the midcycle CL have a positive feedback loop response to PGF2α, resulting in more PGF2α being produce, which likely plays a role in luteolysis. This positive feedback loop mechanism is however, not seen in the early cycle CL. It seems like the early CL is capable of partially respond to the stimulus of PGF2α, but fails to continue the luteolytic process (Tsai and Wiltbank, 1998).

Luteal Rescue

When a viable embryo is present in the uterus, luteolysis fails to occur and the CL lifespan is prolonged with maintained production of P4 and progression of pregnancy. The protein responsible for the luteal rescue in ruminants was first identified in sheep, being produced by the conceptus trophectoderm between days 13 and 21 of the estrous cycle, contemporarily with the maternal recognition of pregnancy (Godkin et al., 1982). Intrauterine treatment of this protein in non-pregnant sheep successfully prolonged luteal maintenance, resulting in a pseudo-pregnancy (Godkin et al., 1984). The protein was later identified as Interferon-tau (IFNT) and is responsible for interacting with the endometrium, preventing the up-regulation of the expression of oxytocin receptors and therefore inhibiting PGF2α production (Hansen et al., 2017).

In addition to the anti-luteolytic role of IFNT, it also has a luteotropic role which can be determined by promoting the production of prostaglandin-E-2 (PGE2) from the endometrium without impacting PGF2α production and thus resulting in a greater

PGE2:PGF2α ratio. Prostaglandin-E-2 is a luteotropic factor produced by the endometrium and conceptus and is proposed to be a secondary mechanism participating on luteal rescue (Ealy and Yang, 2009).

The role of IFNT is not limited to the uterus. Pregnancy-dependent expression of interferon stimulated genes (ISGs) can be found in circulating leukocytes, and therefore, is possible that IFNT-mediated alterations in immune function facilitates maternal tolerance of the developing pregnancy. In fact, previous studies have reported that neutrophils with an increased expression of ISGs had decreased phagocytic activity (Sheikh et al., 2018), which may illustrate how the embryo is modulating the maternal immune system and allowing embryonic establishment.

The expression of ISGs may also be used as pregnancy diagnosis in heifers. The systemic circulating concentrations of IFNT is extremely low, however the response of ISGs on leukocytes can be measured and pregnant heifers have increased expression of ISGs when compared to non-pregnant heifers (Green et al., 2010). The quantification of the expression of ISGs can also suggest conceptus quality as the maternal recognition of pregnancy is a key event for pregnancy establishment and increased expression of ISGs suggests improved production of IFNT.

Artificial Insemination

Artificial insemination is not a recent technique and the first successful artificial insemination in mammals dates back to 1784, when a dog was fertilized and whelped 3 pups. Many years of research on semen collection, evaluation and processing were required to make this technology the main responsible for genetic improvement of cattle nowadays (Foote, 2002).

The adoption of AI technology not only enables the use of improved genetics, but also allows improved progeny performance by facilitating genetic enhancement and

reduces the risk of spreading sexually transmitted diseases (Ax et al., 2000). Artificial insemination however, is adopted by only 13.3% of cow-calf operations in the United States (Whittier, 2010).

The main reason producres fail to adopt the AI technique is the requirement of estrus detection. Many of the beef cows in the country are grazed on extensive systems, and the labor of observing estrus expression, and sorting animals based on estrus expression to be inseminated may not be cost-effective (Foote, 2002).

The adoption of ES protocols has facilitated the wide adoption of AI technology, since implementation of an ES protocol, followed by TAI allows 2 essential events: 1) limited frequency of handling the animals to handling facilities and 2) elimination of detection of estrus behavior by implementing TAI. In the past decades, several TAI protocols have been developed allowing AI while eliminating detection of estrus behavior and successfully provide satisfactory pregnancy rates (Lamb et al., 2010). The majority of these TAI protocols rely on the administration of exogenous P4, GnRH-induced ovulation and induced luteolysis by administration of PGF2α analog (Lamb et al., 2010).

Pharmacological Control of the Estrous Cycle

Advances in biotechnology and the comprehension of the dynamic events of the estrous cycle have allowed the development of hormonal protocols that manipulate the estrous cycle and control ovulation by administration of hormones, such as FSH, LH, progestins, GnRH and PGF2α, from either natural or synthetic origins. This section will discuss pharmacological agents that are widely used in the United States.

One of the first attempts to synchronize estrus of cows was to induce luteolysis by a singular injection of a PGF2α analog, followed by a period of estrus detection (Lauderdale et al., 1974). In this study, animals were assigned to receive either no

PGF2α injection, where animals were observed twice daily for estrus expression for a period of 18 to 25 days and artificially inseminated approximately 12 hours later, or to receive an injection of PGF2α. Animals receiving an injection of PGF2α were then assigned to either have their estrus expression observed twice daily and were artificially inseminated approximately 12 hours later, or to be inseminated twice at approximately 72 and 90 hours after PGF2α injection. There was no difference, however, for conception rates between the different treatments. This study was one of the first ES protocols developed, however the success of this protocol relies on the phase of the cycle the animal is when PGF2α is administered, since in order to the injection induce luteolysis, the CL has to have previously acquired luteolytic capacity, where the luteolytic response to a PGF2α administration will only happen when the animal is in the diestrus phase of the cycle. The injection of PGF2α to the animals in the metestrus phase failed to induce luteolysis and result in estrus expression, due to the presence of a young CL, unable to respond to the luteolytic stimulus of PGF2α, during this period of the cycle (Lauderdale et al., 1974). In addition, animals in anestrus and pre-pubertal females would also fail to respond, since no CL is present in those animals (Macmillan and Henderson, 1984).

Later, the introduction of GnRH as an ovulatory stimulus allowed a better control of follicular waves. The GnRH injection at a random period of the estrous cycle results in a surge of LH and allows the ovulation of cows with a follicle size ≥ 10mm. In consequence, a new follicular wave will resume at similar times among the treated animals. Combination of a GnRH injection to induce ovulation and initiate a new follicular wave, followed by an injection of PGF2α 7 days later to induce luteolysis, and an injection of GnRH along with AI 48 hours after PGF2α injection allowed producers to inseminate cows without checking for estrus behavior (Lamb et al., 2010). However,

5 to 15% of suckled beef cows enrolled in this protocol exhibit estrus behavior prior to and immediately after PGF2α injection (Kojima et al., 2000) and the ovulation response to the first injection of GnRH will depend on many factors. The ovulation response to GnRH in anestrus cows ranges from 52% to 54% (Lamb et al., 2001), it also varies regarding which phase of the cycle the animal is (Vasconcelos et al., 1999) and the stage of follicular development (Pursley et al., 1995).

The inclusion of an exogenous source of P4, in order to prevent the LH surge that results in ovulation, between the first GnRH injection and the PGF2α injection was investigated as a strategy to synchronize ovulation period of the animals. The most common source of exogenous P4 used nowadays is the CIDR and the patters of slow P4 release allows to maintain a basal serum concentration of P4 which in turn delays onset of ovulation in animals having natural luteolysis prior to the PGF2α injection. This protocol is known as the 7-day CO-Synch + CIDR protocol and allows a more synchronized ovulation of animals enrolled (Lamb et al., 2010).

The advantages of including a source of P4 in the protocol are not limited to the enhanced synchrony of ovulation. Progesterone based protocols also allow ovulation of non-cycling animals, providing similar pregnancy rates to cycling animals (Meneghetti et al., 2009). Also, animals in anestrus prior to protocol initiation and that failed to become pregnant to the protocol, will likely resume cyclicity due to exposure of the hormonones of the protocol (Larson et al., 2006).

Finaly, ES protocols have facilitated adoption of AI and its advantages are not only by allowing insemination without estrus detection, but by also allowing insemination of many cows in the same day, early in the breeding season. This results in a greater concentration of animals calving early in the calving season, which results in

calf uniformity and a greater period for calf performance, resulting in greater weight of calf weaned per cow exposed to the protocol (Rodgers et al., 2012).

Importance of estrus on fertility of timed AI protocols

Various Estrous synchronization protocols followed by TAI have made possible for producers to extensively implement AI without the necessity of detecting estrus behavior prior to AI. Despite current protocols yield acceptable pregnancy rates (Lamb et al., 2001), animals that fail to express estrus to the protocol prior to AI, yield lesser pregnancy rates when compared to animals that successfully express estrus (Bishop et al., 2016).

Cows detected in estrus have greater concentration of E2 in the blood when compared with cows not expressing estrus (Perry and Perry, 2008), and the physiological mechanisms underlying the positive effects of estrus expression on pregnancy rates can be supported by genomics. A study analyzing 58 targeted genes between cows that failed or succeeded to express estrus, enrolled to an E2 and P4 based ES protocol, demonstrated improved gene expression for critical genes involved in the maternal immune suppression and endometrium receptivity, as well as partial inhibition of PGF2α mRNA machinery and enhanced conceptus quality for the animals that succeeded to express estrus behavior (Davoodi et al., 2016). These results indicate that E2 concentrations prior to AI play an important role in signaling important factors for pregnancy establishment.

Preovulatory concentrations of E2 are important in modulating the uterine environment for pregnancy, capacitating and transporting the sperm through the female reproductive tract and in preparing follicular cells for luteinization (Perry and Perry, 2008). In addition, cows expressing estrus behavior had larger follicle diameter at both CIDR removal and TAI when compared with cows that failed to express estrus

behavior, as well as larger CL diameters, producing greater amounts of P4 seven days after TAI (Sa Filho et al., 2011). In addition, not only estrus expression but also the intensity of estrus expression has an influence on fertility parameters. Cows with an intense physical activity during the proestrus and estrus period, enrolled to a P4 and E2 based ES protocol had increased dominant follicle diameter at AI, as well as increased CL volume 7 days later, producing greater concentrations of P4 when compared to cows expressing less physical activity during the same period. Conceptus development was also improved in cows exhibiting intense estrus behavior as indicated by increased expression of *myxovirus resistance 2* mRNA 20 days after AI (Rodrigues et al., 2018), which is an indicator of conceptus quality and IFNT production.

Split time AI

The development of protocols that yieald greater proportion of animals expressing estrus behavior to the protocol have been investigated. The split time-AI (STAI) protocol consists of an ES protocol where animals that successfully express estrus behavior prior to AI are inseminated, whereas animals that failed to express estrus behavior have their insemination delayed, in contrast with TAI protocols where animals are inseminated at a determined time despite of estrus expression. The delayed insemination is proposed in order to increase the number of animals expressing estrus to the protocol and thus, enhance fertility to the protocol. Several studies were completed and two key studies can be explored as an example. These studies have shown the advantages of implementating STAI. Implementation of STAI has allowed increased overall estrus response when administration of second GnRH and AI were delayed, with 61% and 45% of cows exhibiting estrus for delayed GnRH at 90 h after PGF2α and GnRH at 60h after PGF2α, respectively (Bishop et al., 2016; 2017), and pregnancy rates to AI tended to increase ($P = 0.06$) when compared to TAI protocol (56% and

49%, respectively; Bishop et al., 2017). These results indicate that basing the time of insemination on the expression of estrus is a promising strategy to increase pregnancy rates to an ES protocol.

Factors Affecting Fertility of TAI

The estrous cycle is dictated through dynamic events but the controlling mechanisms result in changes in the hypothalamic pattern of GnRH production and secretion. The hypothalamus receives various metabolic signals that may, directly or indirectly, influence the reproductive axis and ultimately determine the reproductive capacity of the individual. This section purpose is to explore factors that change the metabolic status and ultimately influence fertility of beef females.

The metabolic status of an animal may unfavor reproduction and affect fertility. The challenge to overcome infertility is great, since many factors influence the homeostatic status in cattle, such as nutrition (Larson et al., 2006), the suckling stimulus (Marquezini et al., 2013) and temperament (Cooke et al., 2017) and the comprehension of these factors is crucial to develop better strategies to improve fertility in cattle.

Nutrition

In the bovine, nutrients are partitioned by priority to first maintain the dam's homeostasis and then to reproduction and species propagation, the priority of nutrient partitioning, therefore is as follows: 1) Basal metabolism; 2) Activity; 3) Growth; 4) Basic energy reserves; 5) Pregnancy; 6) Lactation; 7) Additional energy reserves; 8) Estrous cycles and initiation of pregnancy and 9) Excess reserves (Short et al., 1990). After parturition, a cow will face a period of increased energy demand, since the energy required for basal metabolism will be enhanced, due to the inflammatory process required for uterine involution. Energy demand will also be increased as the lactation demand for energy will also be enhanced. This results in a period of postpartum

anestrus, where follicular development of cows occurs but ovulation fails to happen (Montiel and Ahuja, 2005).

The influence of the nutrition status on reproduction is evident and many studies have been reported. The most common way to access nutrient reserve is through the use of body condition scores (BCS). To illustrate the influence of BCS on fertility of beef females, some studies can be explored. An experiment was conducted analyzing fertility of 3734 beef females, from 8 different herds. Results indicate that pregnancy success throughout a breeding season is reduced for lesser BCS when compared to greater BCS (BCS ≤ 4 yielded a 59% of pregnancy rate whereas BCS ≥ 5 yielded a 90% of pregnancy rate; $P < 0.05$; Rae et al., 1993).

The influence of nutritional status on reproduction can also be evidenced by other fertility related parameters. Cows with moderate BCS had greater follicle diameter at AI when compared to reduced BCS cows and an increase of one unit of BCS yielded 11.5% (Larson et al., 2006) and 22.9% (Lamb et al., 2010) improvement of pregnancy rate to a TAI protocol (in a scale from 1 to 9). Estrus expression and intensity are also improved as BCS increases. Cows expressing high estrus intensity had greater mean BCS (4.8) when compared to cows expressing low intensity estrus (4.7), which in turn were greater than cows not expressing estrus (4.5; $P < 0.01$; Rodrigues et al., 2018).

The physiological mechanisms responsible for the effect of nutrition in fertility are not fully elucidated however, there are strong evidence indicating that nutrition plays an important role in fertility of cows.

Studies indicate the role of leptin in reproduction homeostasis. Leptin is a hormone secreted by adipocytes and among other functions, seems to be the hormone responsible to convey information about the body's energy reserves with the brain. Leptin knockout mice are sterile, and exogenous administration of leptin can fully

restore fertility (Ahima and Flier, 2000). In addition, neurons present in the hypothalamus, responsible for GnRH secretion were found to have leptin receptors and its pulsatility secretion can be stimulated by leptin (Blüher et al., 2007). In the bovine, administration of leptin resulted in increased GnRH secretion and LH peak, also, great concentrations of leptin were found in the blood of heifers at the onset of puberty (Zieba et al., 2005) and feed restriction in prepubertal heifers results in reduced circulating concentrations of leptin and insulin, as well as decreased concentrations of leptin mRNA in adipose tissue (Amstalden et al., 1999). These data support the necessity of energy reserves for cyclicity.

To explore the effects of sub-optimal levels of nutrition on the reproductive physiology and cyclicity, a study evaluating the effects of weight loss on cyclicity can be detailed. This study monitored luteal activity, LH concentrations and pulsatility between cows fed to maintain their body weight, or cows with a restricted diet, formulated for 1% of body weight loss weekly. Results indicate that cows belonging to the restricted group had their luteal activity ceased after 26 weeks of feed restriction, as well as lesser LH concentration and pulse frequency, resulting in anestrus when the cows reach a BCS of 3.5 (in a scale from 1 to 9) (Richards et al., 1989). In the same study, increasing dietary energy density until the cows reach a BCS of 4.6 resulted in cyclicity resumption.

Suckling Stimulus

The presence of the calf can impair the dam's reproduction in both nutrition related and unrelated manners. The factors independent of nutrition will be explored in this session, whereas the interaction of the suckling stimulus with the increased energy demand for lactation will be explored in the Parity session.

The presence of the calf, suckling the dam will influence the length of postpartum anestrus. This period have been reported to range from 46 to 104 days in suckled cows, and calf removal usually results in cyclicity resumption within 5 days (Walters et al., 1982). To illustrate the downregulation of the suckling stimulus on cyclicity despite of the nutrient competition for lactation requirement, a study compared postpartum intervals between suckled (S), non-suckled (NS) and non-suckled mastectomized (M) cows, adjusted to maintain body weight, demonstrated that the postpartum period was shortened in the NS cows but were further shortened in the M group (65 days, 25 days and 12 days for S, NS and M, respectively)(Short et al., 1972). These results indicate that not only the suckling stimulus, but also the presence of mammary gland without the suckling stimulus can extend postpartum anestrus. In accordance, another study reported that NS cows resume their cyclicity within 10 to 14 DPP (Williams, 1990).

The physiological mechanisms controlling the negative effect of suckling despite of the nutrition demand for lactation in cyclicity are complex and conflicted. Different patterns of FSH and LH responsiveness when challenged by GnRH were found within suckled and non-suckled cows. There is however, evidence that opioids play a role on the control of cyclicity. This proposed model can be explained by the increase in LH secretion seen when naloxone, an opioid antagonist, is administered to suckled beef cows (Whisnant et al., 1986). In fact, when cows are weaned, the patterns of GnRH secreting hormones are impaired and LH production pattern is unable to promote final follicular development and maturation (Montiel and Ahuja, 2005).

The cow-calf bond has been recognized to play an important role in the negative effect of suckling on reproduction. Cows that have their own calves weaned and are suckled by unrelated calves, have their LH pulse frequency and production increased.

The cow can recognize her own calf by both olfactory and vision ways as in both blind cows and anosmic cows, after being separated from their calves, can recognize their own calves and maintain the pattern of LH production as observed when suckled. Cows that are both blind and anosmic behave as weaned cows and have the LH pulse and frequency increased (Montiel and Ahuja, 2005).

Adoption of ES protocols may be an interesting strategy to overcome the detrimental effects of suckling on fertilitiy. The GnRH injection 20 to 30 DPP resulted in greater LH release in S when compared to NS cows (Williams, 1990). Despite of this positive effect of GnRH inducing ovulation of suckled cows, the ES protocol fails to completely overcome the effects of suckling. Calf removal between PGF2α injection and TAI resulted in greater E2 concentrations, as well as greater ovulation proportion and pregnancy rates when compared to cows who did not have their calves removed for the same period (Marquezini et al., 2013).

Parity

Fertility is greatly affected by nutrient partitioning and some categories of beef females will have their fertility more detrimentally affected than others. Primiparous females for example will have a greater challenge when compared to multiparous females, since the first has not yet reached adult body development and the energy competition for growth and lactation are great and pose a challenge.

This effect can be illustrated when the proportion of primiparous cows expressing estrus activity post-partum is lower when compared to multiparous cows. In one study, 64% of multiparous cows had resumed cyclicity before the beginning of the breeding season, whereas only 55% of primiparous had cyclicity resumed in the same period (Stevenson et al., 2003). In other study, DPP of cows averaged 91.6 days for primiparous and 53.4 days for cows that were 5 years or older (Wiltbank, 1970). In

addition, there was a greater proportion of primiparous cows with luteal activity and expressing estrus that were in a BCS of 6 when compared to primiparous cows with a BCS of 5 or 4 at 20, 40 and 60 DPP. Pregnancy rates to the breeding season were also increased in greater BCS animals (Spitzer et al., 1995). These results indicate that feeding cows to achieve a greater BCS at the beginning of the breeding season is an interesting strategy to overcome the intensified detrimental effects of suboptimal nutrition in this particular category of cows.

Temperament and Handling

Temperament in cattle is defined as the behavioral response to human handling and can be assessed through temperament scores. Temperament scores are often calculated as the arithmetic mean of chute scores and exit velocity scores. Chute score is determined by the animal's behavior while being handled in the chute, on the basis of a 5-point scale, where 1 = calm with no movement, 2 = restless movements, 3 = frequent movement with vocalization, 4 = constant movement, vocalization, shaking of the chute, and 5 = violent and continuous struggling. Exit velocity scores are assessed by determining the speed of the cow exiting the squeeze chute by measuring rate of travel over a 1.9-m distance with an infrared sensor (FarmTek Inc., North Wylie, TX). Further, within location, animals are divided into quintiles according to their exit velocity and are assigned a score from 1 to 5, with exit score 1 = animals within the slowest quintile and exit score 5 = animals within the fastest quintiles, as described by (Cooke et al., 2011). Such evaluation is performed in order to temperament scores to be generated using standardized values (1 to 5, for exit velocity). The threshold for temperament classification (as adequate or excitable) is then determined by the evaluator. Other methods for temperament evaluation exist and most are based on observing animal behavior, such as measuring the number of movements of a calf while

restrained in the chute without having the head captured and exposed to a human in front of the chute for 10 seconds (Benhajali et al., 2010). Other studies have also demonstrated that taking photos of the eye of the animal while restrained in the chute for further eye white percentage evaluation through a software is an accurate technique to evaluate temperament (Core et al., 2009).

Several studies have demonstrated that excitable temperament has a detrimental effect on production traits, such as hot carcass weight and marbling scores (Francisco et al., 2012), growth rate (Grandin, 1998) and reproduction (Cooke et al., 2009). The excitable temperament a cow expresses when unable to cope with human handling can be referred as stress, with excitable cows having greater amounts of circulating cortisol when compared to calm cows (Cooke et al., 2012a).

The effects of temperament and stress on fertility have been previously studied. To demonstrate these effects, one study can be explored. This experiment evaluated the temperament of *Bos taurus* cows at the beginning of the breeding season, followed by TAI and 50 days of natural sire exposure. Results indicate excitable cows having greater circulating concentrations of cortisol, decreased pregnancy and calving rates and a tendency to decreased weaning rates ($P = 0.09$) when compared to calm cows (Cooke et al., 2011).

The genetic influence on the excitable phenotype observed might be severe. Heifer calves born from low docility expected progeny differences (EPDs) sires and exhibiting excitable temperament at weaning, showed excitable temperament, lowered estrus expression and lowered fertility later, at breeding, when compared to calm heifers (Kasimanickam et al., 2018). The adoption of semen from sires providing high docility EPDs may be an interesting strategy for producers to overcome the detrimental effect of temperament on fertility. The genetic heritability of temperament has been

demonstrated to be poor to moderate, indicating that the environment plays a more important role in the variation of animal stress responses. Genetic selection however, remains an important tool for a long-term solution to decrease the proportion of excitable animals in the herd (Valente et al., 2017).

Several environmental factors will have an influence on herd's temperament, such as handling management, handling facility design (Grandin, 1998), prey exposure, social environment and previous experience to the exposure of the environment (Cooke et al., 2013). Improving the understanding of animal behavior and adapting the handling facilities to simulate the animal's natural behavior (Grandin, 1998), as well as acclimation to human handling are interesting tools to minimalize the detrimental effect of the environment on temperament.

A strategy to acclimate animals to handling and improve fertility of animals has been explored. An Angus x Hereford replacement heifer herd was exposed to a 28-day period of human acclimation within 45 days after weaning resulted in improved temperament traits, reduced plasma cortisol concentrations and greater distribution of pubertal heifers following the end of acclimation period when compared to non-acclimated heifers (Cooke et al., 2012a).

Summary and Research Goals

Reproduction is controlled in a dynamic manner and understanding of the estrous cycle has allowed the development of ES protocols. Estrous synchronization protocols have the main goal of synchronizing ovulation and allowing insemination without detection of estrus. Furthermore, ES allows insemination of anestrous cows and cyclicity induction. The works completed in this thesis will explore how estrus expression and temperament of beef females influence the success of an ES protocol.

The research that follows will: 1) test the hypothesis that animals that express estrus to the protocol have improved fertility when compared to animals that fail to. Also, that delaying insemination for animals that fail to, will deliver the sperm closer to ovulation and improve fertility of this particular group; and 2) test the hypothesis that excitable heifers have impaired fertility to the ES protocol. Also, that the handling events of the protocol will enhance the stress response of heifers, which in turn will result in further impaired fertility.

Chapter 3

Effects of delayed insemination on pregnancy rates of suckled beef cows enrolled in the 7-d CO-Synch + CIDR estrous synchronization protocol and that were not detected in estrus by the time of fixed-time AI.

Abstract

We determined the effects on pregnancy rates of delaying the time of AI of beef cows enrolled in an estrus synchronization protocol that were not detected in estrus by the time of fixed-time AI. At five locations, a total of 1676 suckled beef cows were enrolled in the 7-day CO-Synch+CIDR estrus synchronization protocol. Briefly, 100 μg injection of GnRH and CIDR insertion (day -10); 25 mg injection of PGF2α, CIDR removal and estrus alert patch attachment (day -3); on day 0 (approximately 66 hours after CIDR removal) cows with activated estrus alert patches received AI, but no injection of GnRH (ESTRUS; n = 866); and cows with non-activated patches were randomly assigned to receive either 100 μg injection of GnRH and AI immediately (GnRH+AI; n = 413); or 100 μg injection of GnRH and delayed AI 8 hours later (GnRH+8AI; n = 397). Pregnancy status was determined by transrectal ultrasonography between days 35 to 45. Pregnancy data were analyzed using GLIMMIX procedure of SAS with cows as the experimental unit, including the fixed effects of treatment, location, age of cow, DPP, BCS, and the random effects of sire and AI technician. There were no differences among treatments on BCS (5.6 ± 0.63; $P = 0.579$), DPP (73 ± 15 d; $P = 0.906$), and cow age (5.4 ± 2.6 yr; $P = 0.192$). A total of 295 cows (54%) had activated estrus alert patches on d 0. Overall pregnancy rates differed ($P < 0.001$) among locations, ranging from 30.7% to 66.9%, and no treatment × location interaction was detected ($P = 0.454$). Average delayed between GnRH injection and delayed AI was 7 ± 1.6 h. Pregnancy rate to TAI differed ($P = 0.018$) among treatments. Cows in

the TAI treatment had the greatest pregnancy rate (59.1 ± 0.03%) compared to cows in GnRH+TAI (43.3 ± 0.04%) group, and GnRH+8AI (50.9 ± 0.05%) was intermediate. We conclude that suckled beef cows enrolled in the 7-day CO-Synch+CIDR estrus synchronization protocol that are detected in estrus prior to the time of AI have greater pregnancy rates compared to cows that are not detected in estrus and receive an injection of GnRH and AI at 66 hours after CIDR removal. Furthermore, delaying insemination after GnRH injection failed to increase pregnancy rates of cows not detected in estrus.

Introduction

Advances in biotechnology and the understanding of the dynamic events of the estrous cycle have made possible the development of hormonal protocols that manipulate the estrous cycle and control ovulation by administration of natural or synthesized hormones, such as GnRH, FSH, LH, PGF2α and progestins (Lamb et al., 2010). The adoption of ES protocols has facilitated the widespread of AI technology and can greatly impact the economic viability of cow-calf operations by enabling the use of improved genetics, allowing improved progeny performance by facilitating genetic enhancement, reducing the risk of spreading sexually transmitted diseases (Ax et al., 2000), and allowing anestrous females to resume cyclicity (Larson et al., 2006), as well as concentration of parturition in the calving season and, as a result, enhancing Kg of calf weaned per cow exposed (Rodgers et al., 2012).

Pregnancy rates to ES protocols may vary, but several studies have shown that animals that fail to express estrus to the protocol prior to AI, yield reduced pregnancy rates when compared to animals that successfully express estrus in the same period (Bishop et al., 2016; Sá Filho et al., 2011). Cows detected in estrus have greater concentration of E2 in the blood when compared with cows not expressing estrus (Perry

and Perry, 2008). A study analyzing 58 targeted genes between cows that failed or succeeded to express estrus, enrolled to an E2 and P4 based ES protocol, demonstrated improved gene expression for critical genes involved in the maternal immune suppression and endometrium receptivity, as well as partial inhibition of PGF2α mRNA machinery. These are key events for pregnancy establishment, resulting in enhanced conceptus quality for the animals that succeeded to express estrus behavior (Davoodi et al., 2016). These results demonstrate that E2 concentration prior to AI plays an important role in signaling important factors for pregnancy establishment.

Preovulatory concentrations of E2 are important in modulating the uterine environment for pregnancy, capacitating and transporting the sperm through the female reproductive tract and in preparing follicular cells for luteinization (Perry and Perry, 2008). In addition, cows expressing estrus behavior have greater circulating concentrations of E2 (Perry and Perry, 2008) and have shown improved fertility associated parameters such as, larger follicle diameter at both CIDR removal and TAI when compared with cows that failed to express estrus behavior, as well as larger CL diameters, producing greater amounts of P4 seven days after TAI (Sa Filho et al., 2011). The preovulatory concentrations of E2 are also essential for changes in the uterine environment and sperm performance, as ejaculated sperm loses the ability of synthesis of bioenergetics balance and cell damage repair, relying solely on the environment for its performance (Hammerstedt, 1993). Estradiol-induced changes in the uterus include a decrease in the pH and a decreased sperm pH results in decreased metabolism and motility which enhance longevity and sperm viability (Perry and Perry, 2008).

The STAI protocol consists of an ES protocol where animals that successfully express estrus behavior prior to AI are inseminated, and animals that failed to express estrus behavior have their insemination delayed. This differs from conventional TAI

protocols where animals are inseminated at a determined time regardless of estrus expression. The delayed insemination is proposed as a way to increase the number of animals expressing estrus, and thus, potentially improving pregnancy outcomes.

Previous studies have shown that implementation of STAI has successfully increased overall estrus response when administration of second GnRH and AI were delayed, as well as tendency for increased pregnancy rates to AI, when compared to a conventional TAI protocol (Bishop et al., 2016; 2017). We hypothesized that animals expressing estrus to an ES protocol would have enhanced fertility compared to animals failing to express estrus behavior. In addition, delaying the insemination time for the animals that failed to express estrus would deliver the semen closer to ovulation time, minimalizing the detrimental effect on sperm viability and thus, result in greater pregnancy rates. The present study has the objective of adapting the ES protocol design to base the time of insemination on expression of estrus, with animals not detected in estrus having insemination delayed, and to evaluate the effects of such delayed insemination on fertility of cows in this group of animals.

Materials and Methods

All experimental procedures were approved by the Virginia Polytechnic Institute and State University Animal care and Use Committee.

Experimental Design

Estrus was synchronized in 1676 Angus based crossbred multiparous cows across 13 locations within the Virginia Department of Corrections for 2 consecutive breeding seasons (spring and fall). All cows were enrolled in the 7-day CO-Synch + CIDR protocol. In brief, cows received a 100-µg injection of GnRH (Factrel; gonadorelin hydrochloride; Zoetis Animal Health, Parssipany, NJ) and a CIDR (EAZI-BREED CIDR; 1.38g of P4; Zoetis Animal Health) insert on day −10, a 25-mg injection

of PGF2α (Lutalyse; dinoprost tromethamine; Zoetis Animal Health) at CIDR removal on day −3. Estrus detection aids (Estrotect, Rockway Inc., Spring Valley, WI, USA) were applied at PGF2α on day -3, with estrus status recorded at day 0. Timing of insemination was determined based on expression of estrus within 66 hours after PGF2α, with cows in estrus being defined as having at least 50% of the paint rubbed off the estrus detection patch. Cows expressing estrus (n = 813; **ESTRUS)** were artificially inseminated and did not receive an injection of GnRH. Cows that failed to exhibit estrus by 66 hours after PGF2α were randomly assigned to one of two treatments: 1) **GnRH+AI**; (n = 413) cows received a 100-µg injection of GnRH and were artificially inseminated immediately (66 hours after PGF2α); or 2) **GnRH+8AI**; (n = 397) received a 100-µg injection of GnRH and had AI delayed by approximately 8 hours (74 hours after PGF2α). Cows were exposed to fertile bulls beginning 15 days after AI for the reminder of the 90 days of breeding season (Figure 1).

Transrectal ultrasonography (Ibex portable ultrasound, 5.0-MHz linear multi-frequency transducer, Ibex, E.I. Medical Imaging, Loveland, CO) was performed between d 35 and d 45 after AI to determine pregnancy diagnosis.

Statistical Analysis

The SAS (version 9.4; SAS/STAT, SAS Inst. Inc., Cary, NC, USA) statistical package was used for all statistical analyses. Cow was considered the experimental unit. Pregnancy data were analyzed using the GLIMMIX procedure of SAS. The model included the fixed effects of treatment, season, location, age of cow, DPP, BCS, and the random effects of sire and AI technician. Statistical differences were considered significant at $P \leq 0.05$ and tendencies considered at $0.05 < P \leq 0.10$.

Results and Discussion

Days-post-partum and BCS

Estrus response, DPP and BCS for all cows enrolled to the protocol are summarized in Table 1. Overall 51.6% cows enrolled in the protocol successfully expressed estrus prior to AI, whereas 49.4% cows enrolled to the protocol failed to express estrus during the same period.

In the present study, there was no difference ($P > 0.05$) for mean DPP among cows expressing or not estrus, and averaged 77 days. In addition, no differences in BCS between groups were detected, with the mean of BCS of 5.8, 5.9 and 5.8 (SEM = 0.03) for ESTRUS, GnRH + AI and GnRH + 8AI groups, respectively.

Previous studies have reported that animals in adequate BCS resume cyclicity, despite of a silent ovulation, in the post-partum period within approximately 30 days and present the first estrus expression along with the second ovulation, 9-11 days after the first ovulation (Crowe, 2008). Other experiments have also reported an increase of peak and duration of estrus activity as the BCS increases. Cows with poor BCS express short duration and peak of estrus activity when compared to adequate BCS (Madureira et al., 2015). Similarly, a greater proportion of cows with adequate BCS expressed estrus when compared to poor BCS cows (Sa Filho et al., 2011). Based on these evidences, in the present study there should be no influence of BCS and DPP on the proportion of cyclic cows, with the capacity to express estrus at the initiation of the protocol between treatments. Conversely, a study performed a meta-analysis examining factors influencing expression of estrus found no impact of DPP on estrus expression ($P = 0.22$), but also identified an influence of BCS on estrus expression with cows in a BCS ≤ 4 having decreased expression of estrus when compared to cows in a BCS > 4 (in a 1 to 9 scale; Richardson et al., 2016). In the present study, no animals were in a BCS < 4 at the initiation of the protocol and, disregarding location, only 36 animals were in a BCS = 4. The number of cows, DPP, BCS and pregnancy rates within

locations are summarized in Table 2. Mean DPP and mean BCS differed within location ($P < 0.05$) and ranged from 68.7 to 85.1 and 5.2 to 6.5 respectively.

Fertility

The objective of the present study was to design an alternative ES protocol, able to increase fertility of cows that fail to express estrus to the protocol, based on timing of semen delivery. Pregnancy rates are summarized in Figure 2. Pregnancy rates were greater ($P < 0.05$) for animals in the ESTRUS group (60.7%) when compared to GnRH + AI group (52.9%) with the GnRH + 8AI group being intermediate (57.6%). Our results on the influence of estrus expression on pregnancy rates are in accordance to previous findings (Sa Filho et al., 2011; Madureira et al., 2015; Bishop et al., 2017; Perry et al., 2005; Perry et al., 2007). The objective of the present study was to design an alternative ES protocol, able to increase fertility of cows that fail to express estrus to the protocol, based on timing of semen delivery.

Further evidence of estrus on fertility to an ES can be found in a study utilizing *Bos indicus* suckled beef cows. Animals were enrolled in an equine chorionic gonadotropin (eCG) E2 and P4 based protocol with or without an injection of GnRH at TAI. Animals that successfully expressed estrus behavior prior to TAI had greater pregnancy to AI (P/AI) when compared to non-estrus cows (63%, 60.7%, 44.7% and 37.8% for estrus/no GnRH, estrus/GnRH, no estrus/GnRH and no estrus/no GnRH, respectively; Sa Filho et al., 2011). In the present study, no fertility parameters other than estrus expression and pregnancy rates were analyzed, but the increased pregnancy rate observed in the ESTRUS group can be explained by the mentioned study where fertility associated parameters were enhanced in estrus expressing cows when compared to non-estrus cows, with animals expressing estrus behavior showing larger follicles at

CIDR removal and TAI, greater ovulation rate (100% vs. 70.6%) and larger CL producing greater concentrations of P4 seven days after AI.

The positive effects on fertility observed in estrus expressing cows can be explained by the several important roles of E2 on the reproductive performance. It has been reported that key proteins for sperm capacitation, fertilization and early embryonic development are estrogen-dependent (Binelli et al., 2014). In fact, circulating concentrations of E2 have been reported to be correlated with fertility, where pregnant cows had greater E2 concentrations prior AI when compared to open cows (Ribeiro et al., 2012). Estradiol has also been correlated with the size of the dominant follicle, where small follicles produce lesser concentrations of E2 when compared to large follicles. In fact, ovulation of larger follicles results in a larger CL during the diestrus phase, producing greater amounts of P4 (Perry et al., 2005). Moreover, induction of ovulation of small follicles resulted in reduced pregnancy rates and increased pregnancy losses. The reduced pregnancy rate and characteristic of estrus behavior observed in the present study for animals failing to express estrus is perhaps a result of smaller dominant follicles formed by the protocol. Perhaps in the present study, the dominant follicle may not have developed sufficiently to induce the onset of estrus and may have required a longer interval between CIDR removal and AI.

The effects of E2 on fertility also include an enhanced environment for semen quality. After final maturation, sperm cells lose the ability of maintenance of the bioenergetics balance and biosynthetic damage repair resulting on environmental dependence for performance and the rate of metabolism will interfere in motility and longevity of sperm (Hammerstedt, 1993). Estrus expression and E2 concentrations have been correlated with a decreased uterine pH (Perry et al., 2007) and decreasing sperm pH results in decreased metabolism and motility and this reversible reduction of

motility plays a role on avoiding unnecessary expenditure of energy, decreasing oxidative damage rate and thus increasing longevity and maintenance of sperm quality (Jones and Bavister, 2000).

In the present study, cows expressing estrus prior to AI (ESTRUS) were inseminated without an injection of GnRH. This was proposed because we hypothesized that these animals did not need the GnRH stimulus to ovulate and furthermore, that the GnRH injection would force the ovulation of the dominant follicle, whereas if cows were allowed to naturally ovulate would result in the ovulation of a more mature and larger follicle, resulting on enhanced fertility. In addition, it has been previously reported that cows expressing estrus to an ES protocol do not require the GnRH ovulation stimulus (Bishop et al., 2016).

Based on these findings it is reasonable that, cows failing to express estrus would not have a decreased uterine pH providing an enhanced longevity to the sperm and, that delaying the insemination for 8 hours would approximate the time of sperm delivery to ovulation, resulting on enhanced fertility for this particular group of cows. In addition, the 8-hour delay would facilitate the handling event for producers that have TAI in the morning period, allowing animals not detected in estrus to remain in the handling facility and receive AI in the afternoon. In the present study, delaying insemination for 8 hours in non-estrus cows failed to improve pregnancy rates to the protocol.

Another strategy is to enhance the time of uterine exposure to E2. It has been reported that a complete induction of luteolysis, resulting in an extended proestrus period and thus, a longer period of reproductive organs exposure to E2 was related to increased fertility and decreased pregnancy loss. In fact, a study analyzing the effects of inducing luteolysis on day 5 along with CIDR removal, and delaying AI to 72 hours

after PGF2α injection and thus, providing a longer proestrus and estrus period, resulted in greater pregnancy rates when compared to the the 7-d CO-Synch + CIDR protocol (Bridges et al., 2010). The development of a strategy to induce a more complete luteolysis and extend the proestrus period could result in larger follicles, producing greater concentrations of E2 and therefore, greater proportion of animals expressing estrus to the protocol, a longer influence of E2 on the reproductive tract and thus, improved fertility. Perhaps in the present study, delaying not only the insemination but also the second GnRH injection would provide an extra 8 hours for dominant follicle development resulting in the ovulation of a more mature follicle and longer key period of reproductive tract exposure to E2. In fact, when GnRH is administered, it triggers the LH surge and results in ovulation 30 hours after injection (Liu et al., 2018), not allowing the further expression of estrus (Lucy and Stevenson, 1986). In a previous study, estrus expression resulted on enhanced fertility and delaying the second GnRH injection increased the proportion of cows detected in estrus during the delayed period, despite of failing to improve pregnancy rates (Bishop et al., 2016). Further investigation of the timing of induction of ovulation in cows failing to express estrus to an ES protocol is required.

Another strategy to increase E2 concentrations prior to TAI is to adopt E2 and P4 based protocols. It was observed an increase in P/AI on dairy cows enrolled to an E2 and P4 based protocol when compared to a 5 day CoSynch protocol and although circulating E2 was not measured in this study, there was a greater proportion of cows exhibiting estrus to the first protocol (62.8%), when compared to the latter (43.4%; Pereira et al., 2016). In a different study, suckled beef cows treated with estradiol ciprionate (eCP) at CIDR removal had an early increase and later decrease, as well as

higher and longer peak of E2 concentrations by 30 hours after administration when compared to no E2 administration (Uslenghi et al., 2016).

In summary we conclude that, cows expressing estrus prior to TAI in an ES protocol yield greater pregnancy rates when compared to cows that fail to express estrus and that delaying the insemination for 8 hours failed to enhance fertility for this group of animals.

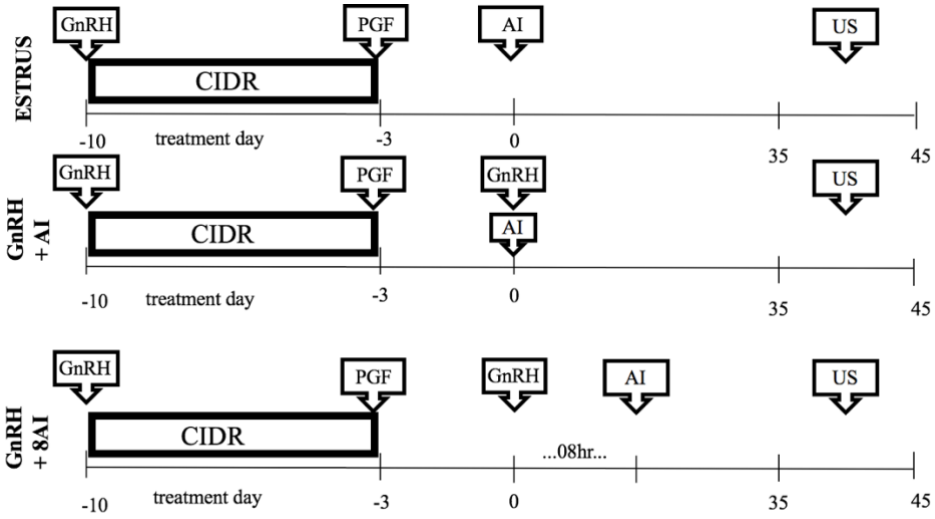

Figure 3.1. Experimental timeline and treatment description. All cows were estrous synchronized using a 7-d CO-Synch + controlled internal drug release (CIDR) protocol in which cows received an injection of gonadotropin releasing hormone (GnRH) on day -10 and CIDR device was inserted followed by injection of prostaglandin F2α (PGF2α), a CIDR device removal and an estrus detection patch on day -3. On day 0, Cows that had > 50% of the paint of the estrus detection patch rubbed off (ESTRUS) received artificial Insemination (AI), whereas cows that had < 50% of the paint of the estrus detection patch rubbed off received an injection of GnRH and were assigned to either receive AI at time of GnRH injection (GnRH + AI) or to receive AI 8 hours later (GnRH + 8AI). Pregnancy diagnosis were performed by ultrasonography between day 35 and 45.

Table 3.1. Number of animals, DPP and BCS according to treatment[1].

	Treatment				
	ESTRUS	GnRH+TAI	GnRH+8AI	SEM	*P*-value*
n	866	413	397	--	--
DPP, d	79	75	79	3.5	0.152
BCS	5.8	5.9	5.8	0.03	0.233

[1] Cows were estrous synchronized using a 7-d CO-Synch + controlled internal drug release (CIDR) protocol in which cows received an injection of gonadotropin releasing hormone (GnRH) on day -10 and CIDR device was inserted followed by injection of prostaglandin F2α (PGF2α), a CIDR device removal and an estrus detection patch on day -3. On day 0, Cows that had > 50% of the paint of the estrus detection patch rubbed off (ESTRUS) received artificial Insemination (AI), whereas cows that had < 50% of the paint of the estrus detection patch rubbed off received an injection of GnRH and were assigned to either receive AI at time of GnRH injection (GnRH + AI) or to receive AI 8 hours later (GnRH + 8AI). Pregnancy diagnosis were performed by ultrasonography between day 35 and 45.

Table 3.2. Overall and by location number of animals, Days-post-partum (DPP), Body condition score (BCS) and pregnancy rates[1].

Location	n	DPP, d	BCS	Pregnancy Rate, %
1	98	83.3	6.1	56.1
2	344	77.6	6.0	56.1
3	144	85.1	5.2	50.6
4	153	73.0	6.5	52.2
5	89	81.9	5.2	35.9
6	45	73.2	5.6	62.2
7	166	76.4	5.9	56.0
8	158	77.3	5.8	54.4
9	68	76.9	6.0	66.1
10	84	75.8	5.6	59.5
11	142	84.8	5.5	67.6
12	58	68.7	5.5	65.5
13	127	79.7	5.5	67.7
Overall	1676	77.6	5.6	56.1

[1] Cows were estrous synchronized using a 7-d CO-Synch + controlled internal drug release (CIDR) protocol in which cows received an injection of gonadotropin releasing hormone (GnRH) on day -10 and CIDR device was inserted followed by injection of prostaglandin F2α (PGF2α), a CIDR device removal and an estrus detection patch on day -3. On day 0, Cows that had > 50% of the paint of the estrus detection patch rubbed off (ESTRUS) received artificial Insemination (AI), whereas cows that had < 50% of the paint of the estrus detection patch rubbed off received an injection of GnRH and were assigned to either receive AI at time of GnRH injection (GnRH + AI) or to receive

AI 8 hours later (GnRH + 8AI). Pregnancy diagnosis were performed by ultrasonography between day 35 and 45. BCS was evaluated in a scale from 1 to 9.

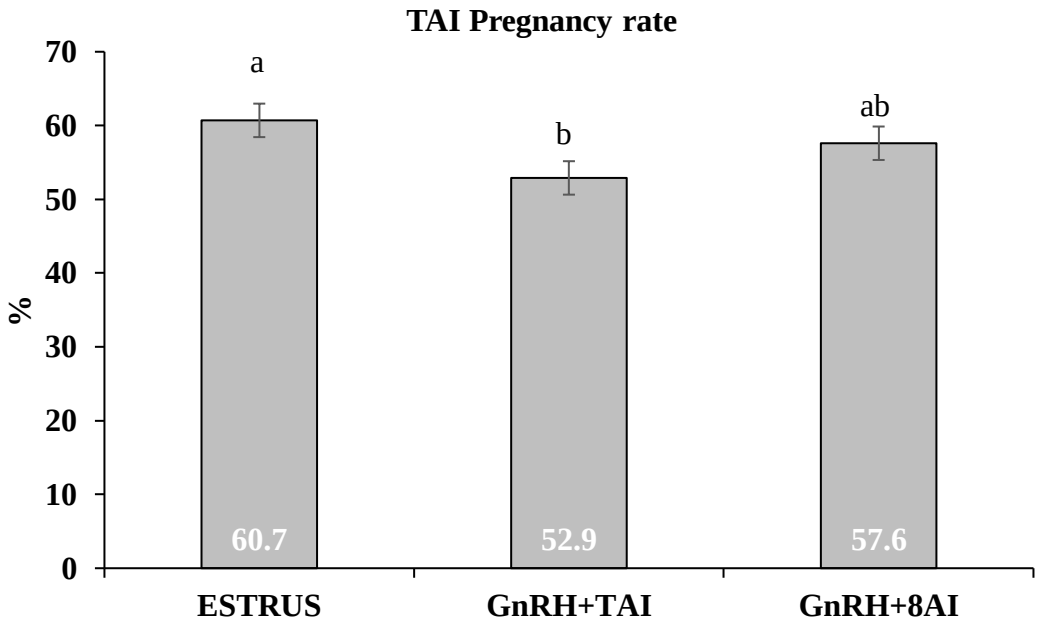

Figure 3.2. Overall pregnancy rates by treatment. In brief, cows were estrous synchronized using a 7-d CO-Synch + controlled internal drug release (CIDR) protocol in which cows received an injection of gonadotropin releasing hormone (GnRH) on day -10 and CIDR device was inserted followed by injection of prostaglandin F2α (PGF2α), a CIDR device removal and an estrus detection patch on day -3. On day 0, Cows that had > 50% of the paint of the estrus detection patch rubbed off (ESTRUS) received artificial Insemination (AI), whereas cows that had < 50% of the paint of the estrus detection patch rubbed off received an injection of GnRH and were assigned to either receive AI at time of GnRH injection (GnRH + AI) or to receive AI 8 hours later (GnRH + 8AI). Pregnancy diagnosis were performed by ultrasonography between day 35 and 45. [a-b] Means with different superscripts differ at $P < 0.05$.

Chapter 4

Effects of temperament on reproductive performance of *Bos taurus* heifers enrolled in a 7 d CO-synch + CIDR protocol

Abstract

It has been previously reported that cattle temperament significantly impact production traits such as reproduction. The objective of the present experiment was to assess the effects of temperament on pregnancy rates to fixed-timed artificial insemination (TAI) in *Bos taurus* beef heifers. A total of 297 Angus influenced heifers from 3 different locations were evaluated for temperament based on chute score and exit velocity on the first day of the estrus synchronization protocol (day -9) and at TAI (day 0). Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable). Pregnancy status was determined by rectal ultrasonography approximately 40 days after TAI. Hair from the tail switch was collected at day -9 and at TAI for cumulative cortisol evaluation. Overall, 71% of heifers were classified as calm whereas 29% as excited. Pregnancy rates to TAI were reduced ($P = 0.042$) in excitable heifers compared to calm heifers (36% vs. 55%, respectively). Mean concentration of cortisol in the hair was reduced ($P < 0.001$) from d-9 (3.5 ± 0.3 pg/mg of hair) to day 0 (1.74 ± 0.3 pg/mg of hair) in all heifers, independently of temperament. In addition, no differences for mean cortisol concentrations were found between calm (2.91 ± 0.2 pg/mg of hair; $P = 0.647$) and excitable heifers (2.67 ± 0.2 pg/mg of hair; $P = 0.412$). We conclude that heifer temperament has negative effects in pregnancy rates to TAI programs. However,

according to hair cortisol concentrations, there was no chronic stress response due to cattle handling during the estrus synchronization protocol. In fact, hair cortisol concentration was reduced between the initiation and completion of the protocol, indicating that heifers were acclimated to handing.

Introduction

Temperament influences fertility in beef cattle, with excitable cattle showing reduced fertility when compared to calm cohorts (Cooke et al., 2009; Cooke et al., 2011; Cooke et al., 2012b). In fact, not only reproduction is affected by temperament, but also growth (Nkrumah et al., 2007), health (Burdick et al., 2011), and carcass quality (Cafe et al., 2011). Temperament in cattle is defined as the behavioral response the animal presents when handled by humans (Burrow and Corbet, 2000) and can be assessed through chute and exit velocity scores (Cooke et al., 2009). Cattle with excitable temperament have neuroendocrine stress responses activated through the hypothalamic-pituitary-adrenal (HPA) axis, resulting in greater blood cortisol concentrations (Cooke et al., 2012a), which in turn results in impaired metabolism (Sanchez et al., 2016).

The phenotype observed in excitable cattle and its influences on fertility have both environmental and genetic influences. The genetic influence on the excitable phenotype observed might be severe where heifer calves born from low docility EPDs sires and exhibiting excitable temperament at weaning showed excitable temperament, less estrus expression and less fertility later at breeding when compared to calm heifers (Kasimanickam et al., 2018). The environmental influence on the excitable phenotype observed plays an important role with several factors having an influence on temperament of the herd, such as handling management, handling facility design (Grandin, 1998), prey

exposure and social environment (Cooke, 2014). Improving the understanding of animal behavior and adapting the handling facilities to simulate the animal's natural behavior (Grandin, 1998), as well as acclimation to human handling are effective tools to minimalize the detrimental effects of the environment on temperament (Cooke et al., 2012a).

The objective of the present study was to evaluate the effects of temperament of Angus influenced heifers enrolled to a 7-day CO-Synch+CIDR protocol to fertility. Moreover, to evaluate the effects of the handling events of the protocol on temperament and therefore, on fertility. We hypothesized that the handling events of the protocol would increase the stress response of the animals, which in turn would be detrimental to fertility. To our best knowledge, no previous attempts to investigate the effects of the handling events of an ES protocol on temperament of cattle have been performed. Comprehension of these effects will help to understand the applicability of such technology.

Materials and methods

The animals utilized were cared for in accordance with acceptable practices as outlined in Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (IACUC). This experiment was conducted over the fall of 2017 in three Virginia farms.

Animals and Reproductive Management

A total of 297 Angus crossbred heifers within 3 locations were assigned to the experiment. All heifers were assigned to the 7-day CO-Synch+CIDR protocol where they received a 100-µg injection of GnRH (Factrel; gonadorelin hydrochloride; Zoetis Animal Health, Parssipany, NJ) and a CIDR (EAZI-BREED CIDR; 1.38 g of P4; Zoetis Animal Health) insert on day −9, a 25-mg injection of PGF2α (Lutalyse; dinoprost tromethamine; Zoetis Animal Health) at CIDR removal on day −3, and a 100-µg

injection of GnRH and TAI 54 ± 2 hours later on day 0. All heifers were exposed to fertile bulls 15 days after TAI for the reminder of the breeding season. Transrectal ultrasonography (Ibex portable ultrasound, 5.0-MHz linear multi-frequency transducer, Ibex, E.I. Medical Imaging, Loveland, CO) was performed between day 35 and day 45 after AI to determine pregnancy diagnosis.

Temperament Evaluation

On day -9, individual heifer temperament was assessed by chute score and exit velocity as previously described by (Cooke et al., 2011). Chute score was assessed on the basis of a 5-point scale, where 1 = calm with no movement, 2 = restless movements, 3 = frequent movement with vocalization, 4 = constant movement, vocalization, shaking of the chute, and 5 = violent and continuous struggling. Exit velocity was assessed immediately by determining the speed of the heifer exiting the squeeze chute by measuring rate of travel over a 1.9-m distance with an infrared sensor (FarmTek Inc., North Wylie, TX). Further, within location, heifers were divided in quintiles according to their exit velocity and were assigned a score from 1 to 5 (exit score; 1 = cows within the slowest quintile, 5= cows within the fastest quintile). Individual temperament scores were calculated by averaging heifer chute score and exit score. Heifers were classified according to the final temperament score (temperament type) as adequate temperament (temperament score ≤3) or excitable temperament (temperament score >3) at a 5-point scale.

Sampling

At location 3, blood samples were collected at days -9; -3 and 0 via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin (148 USP units), placed immediately on ice for no longer than 4 hours, and centrifuged at 2,400 × *g* for 15 min

at room temperature for plasma collection and were stored at -20 °C until further analysis. Plasma concentrations of cortisol were determined in duplicates by using a chemiluminescence assay (Immulite 2000 Xpi, Siemens Medical Solutions). The intra and interassay CV were 5.3% and 4.8% respectively.

In addition, individual hair samples were collected from the tail switch from all heifers, in all locations, on days -9 and 0. Samples were collected from two different regions of the tail switch for each day. Hair was collected as close to the skin as possible using scissors, and the hair material closest to the skin (2.5 cm of length, 300 mg of weight) was stored at -20 °C until processed for cortisol extraction (Burnett et al., 2014). Briefly, samples were cleaned with warm water (37 °C) for 30 minutes and dried at room temperature for 24 hours. Hair samples were then washed twice with isopropanol, dried for 120 hours, and ground in a 10-mL stainless steel milling cup with a 12-mm stainless steel ball (Retsch Mixer Mill MM400 ball mill; Retsch, Hannover, Germany) for 5 min at a frequency of 30 repetitions/seconds. Twenty mg of ground hair and 1 mL of methanol were combined into a 7-mL glass scintillation vial, sonicated for 30 minutes, and incubated for 18 hours at 50 °C and 100 rpm for steroid extraction. Upon incubation, 0.8 mL of methanol was transferred to a 2-mL micro centrifuge tube and evaporated at 45 °C. Samples were reconstituted in 100 μL of the PBS supplied with a salivary cortisol ELISA kit (Salimetrics Expanded Range, High Sensitivity 1-E3002, State College, PA), and stored at -80 °C. Samples were analyzed for cortisol concentrations using the aforementioned ELISA kit.

Statistical Analysis

All data were analyzed using heifer as the experimental unit and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. All models' statements included the effect of cow temperament type (adequate or

excitable). Quantitative data such as plasma and hair cortisol concentrations were analyzed with the MIXED procedure of SAS (version 9.4, SAS Inst., Inc., Cary, NC) with heifer (temperament type × location) as random variable. Binary data such as pregnancy rates was analyzed with the GLIMMIX procedure of SAS with heifer (temperament type × location) as random variable, in addition to sire (location) and AI technician (location) as random variables for pregnancy rates TAI. Statistical differences were considered significant at $P \leq 0.05$ and tendencies considered at $0.05 < P \leq 0.10$.

Results and Discussion

It has been previously reported that cattle with excitable temperament have impaired performance such as growth (Nkrumah et al., 2007), health (Burdick et al., 2011), metabolism (Sanchez et al., 2016), carcass quality (Cafe et al., 2011) and reproduction (Cooke et al., 2011; Cooke et al., 2012b; Dobson and Smith, 2000). The goal of the present study is to evaluate the effects of temperament on fertility of beef heifers enrolled to an ES protocol. In addition, to evaluate the effects of the handling events of the protocol on temperament, and therefore on fertility.

Temperament measurement and number of animals by location is summarized in Table 1. Overall 71% of the heifers analyzed in the present study were classified as calm, whereas 29% as excitable. This distribution of temperament is similar to previous studies where the overall proportion of cattle defined as excitable were 25.05% (Cooke et al., 2012a) and 28.4% (Cooke et al., 2011).

Pregnancy rates by location and temperament by location are summarized in Figures 1 and 2, respectively. Supporting our main hypothesis, overall excitable heifers had decreased pregnancy rates to TAI when compared to adequate temperament heifers (36% vs. 55%, respectively; $P = 0.042$) and are shown in figure 3. This detrimental

effect of temperament on fertility is in accordance with previous studies where pregnancy rates to the breeding season of excitable animals were decreased when compared to adequate temperament animals (Cooke et al., 2012a).

Cattle with excitable temperament have more activated HPA axis as evidenced by greater physiological concentrations of cortisol in the blood (Cooke et al., 2012a); (Sanchez et al., 2016). In summary, the paraventricular nucleus in the hypothalamus contains numerous inputs from other hypothalamic nuclei and thus, is sensitive to a wide range of stimuli from both physiological and environmental sources. These stimuli result in the production of two neuropeptides, corticotropin-releasing hormone (CRH) and vasopressin (AVP), which in turn are released through the median eminence into the hypothalamic pituitary portal system and reach the anterior pituitary. The anterior pituitary then produces the adrenocorticotropic hormone (ACTH) that reaches the systemic circulation and acts on the adrenal gland promoting cortisol production and secretion. Cortisol in turn promotes breakdown of glycogen, fat and proteins, producing energetic metabolites to be used by the immunological defense mechanisms to cope with the stressor (Mormède et al., 2007). In addition, cattle with excitable temperament also present greater blood concentrations of non-stratified fatty acids (Cafe et al., 2011); (Sanchez et al., 2016), urea and glucose as well as greater physiological concentrations of insulin, decreased insulin sensitivity and increased rectal temperature (Sanchez et al., 2016) when compared to adequate temperament cattle. Although energy and metabolism play important roles on reproduction, the detrimental effects of temperament on reproduction are not limited to the impaired homeostasis.

The hypothalamus has a critical role on orchestrating various metabolic and environmental inputs on metabolic function. It has been demonstrated that stress results in expression of the RF-amide-related peptide (RFRP) in the dorsomedial nucleus of

rats, region known to express glucocorticoid receptors and contains neuronal projections that make connections with GnRH neurons (Breen et al., 2014). The RFRP has been identified in cows and is known to inhibit GnRH production and release (Tsutsui et al., 2010). Further evidence can be seen when both LH amplitude and pulses are reduced in cattle after exposure to acute stressors, as well as after ACTH treatment (Dobson and Smith, 2000). These effects however, are not limited to the hypothalamus. An in vitro study was conducted utilizing mouse cell lines, which contain great homology across mammals including the cow, showed that the activated glucocorticoid receptor is recruited and physically binds to the 5' promoter region of the LHβ gene and interferes with crucial response elements, resulting in impaired GnRH induced LH production (Breen et al., 2014).

These evidences indicate that the effects stress is exerting on fertility are through an ovulation impairment. Indeed, it has been shown a reduced LH response after GnRH challenge in cattle treated with ACTH when compared to cattle treated with saline (Li and Wagner, 1983). Based on these findings, one can speculate that the hormones administered during the ES protocol would help to overcome these detrimental effects of cortisol on ovulation, more specifically the GnRH administration at TAI, which would increase downstream intracellular second messengers, pushing the equilibrium and enhancing competition with the glucocorticoid receptor to the promoter region of LHβ gene.

Blood cortisol data for location 3 are summarized in Figure 4. Adequate temperament heifers had reduced plasma cortisol concentrations when compared to excitable heifers in all handling events. More interestingly, independent of temperament, cortisol concentration was greater on day -9 when compared to day -3, which in turn was greater than day 0. This is in conflict with our original hypothesis that

the handling events of the protocol would increase the stress response of the animals. Nevertheless, the managements of the protocol seem to have acclimated the heifers to handling, lowering plasma cortisol concentrations of excitable heifers from intense fear (>40 ng/ml; Grandin, 2017) to mild anxiety as the protocol progresses. Thus, an adoption of an ES protocol may be an interesting strategy for producers to decrease cortisol concentrations at the time of ovulation and thus, improve fertility. In fact, heifers characterized as excitable that were pregnant had lesser blood cortisol concentration than non-pregnant heifers with the same temperament (Figure 5), providing evidence to the inhibitory effect of cortisol on ovulation and fertility.

The positive effects of acclimation on temperament and thus, in fertility have been reported in a previous study, where heifers that were enrolled to an acclimation protocol within one month after weaning had greater proportion of puberty achievement after the end of the study when compared to non-acclimated heifers (Cooke et al., 2012a). The hair cortisol profile analyzed in the present study is in agreement with the proposed acclimation effect of the protocol, and is summarized in figure 6. Overall, there were no differences in hair cortisol concentrations between calm and excitable heifers on day -9 and on day 0. However, despite of temperament classification, all animals had decreased hair cortisol concentrations at the completion of the protocol when compared to the initiation of the protocol.

The measurement of cortisol in the hair is proposed to evaluate the accumulated cortisol in the animal without the induced stressful effect of blood collection. The blood substances enter the hair through passive diffusion, making the hair an interesting biological tissue for hormone evaluation (Moya et al., 2013). In the present study, hair collection was performed in the most effective method as previously described (Burnett et al., 2014). There were however, no differences in hair cortisol between temperaments

and interpretation of data must be made carefully. It is unclear whether the decrease in hair cortisol concentration observed herein is due to acclimation to the protocol or whether the 9-day period of the protocol was insufficient for the hair to grow from the follicle to the skin surface and therefore such difference is unrelated to acclimation. A previous study has reported that it takes approximately 2 weeks for the hair to reach the surfice of the skin (Burnett et al., 2014), although this study was performed with dairy cattle and hair growth in cattle is extremely influenced by individual, breed, nutritional status and season (Carter and Dowling, 1954). There is lack of evidence in the literature on the pattern of hair growth of Angus heifers during the fall in the northern hemisphere. Based on the unknown period which the hair collected in the present experiment is reflecting to, one could speculate that the increased hair cortisol concentrations of both temperaments at the initiation of the protocol compared to the completion of the protocol reflects, perhaps, to a period of limited feed available or a period when animals were transported, rather than physiological concentrations, and that the sample taken at the completion of the protocol reflects a second period when this stress generated by these eventual stressors is already lowered.

We conclude that temperament is detrimental to fertility of beef heifers. Moreover, the handling events of the ES protocol, based on cortisol blood profile from location 3, seem to be acclimating the heifers to management and may be an interesting strategy for producers to overcome the detrimental effects of temperament on fertility and thus, improve the efficiency of a cow-calf operation.

Table 4.1. Mean and overall chute score, exit velocity and temperament score of heifers by location[1].

		Temperament Measurement		
Location	n	Chute Score	Exit Velocity	Temperament Score
1	48	2.8 ± 0.5	2.4 ± 0.8	2.3 ± 0.5
2	206	2.0 ± 0.6	1.3 ± 0.6	2.6 ± 0.5
3	43	2.1 ± 0.5	1.8 ± 0.5	2.5 ± 0.5
Overall	297	2.3 ± 0.5	1.8 ± 0.6	2.4 ± 0.5

[1]Heifers were evaluated for temperament based on chute score and exit velocity on the first day of the estrus synchronization protocol (day -9). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on d -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable).

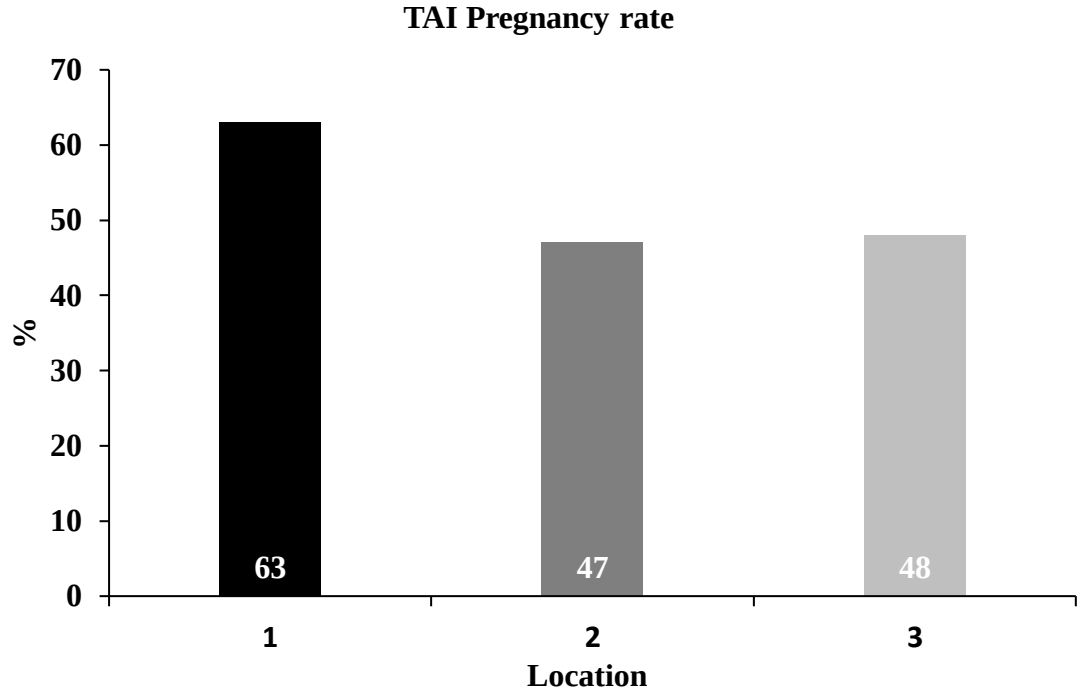

Figure 4.1. Overall pregnancy rates by location. Heifers were evaluated for temperament based on chute score and exit velocity on the first day of the estrus synchronization protocol (day -9). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day-9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on day -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable).

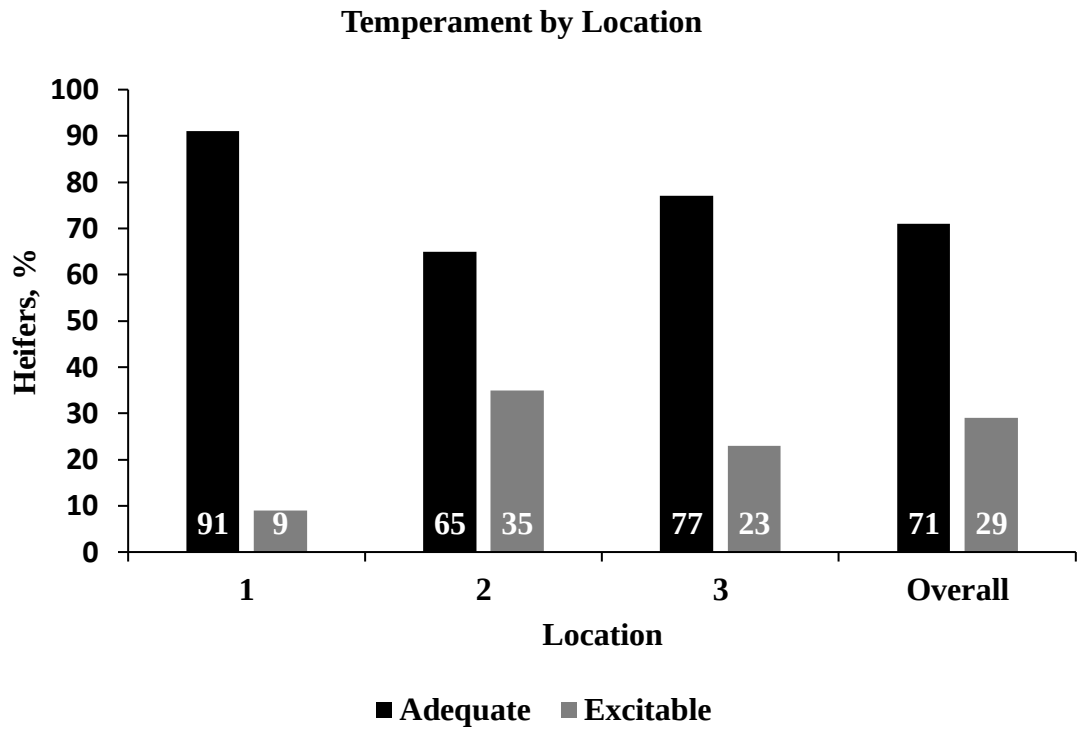

Figure 4.2. Overall and by location temperament distribution. Heifers were evaluated for temperament based on chute score and exit velocity on the first day of the estrus synchronization protocol (day -9). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on day -3 followed by TAI on d 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable).

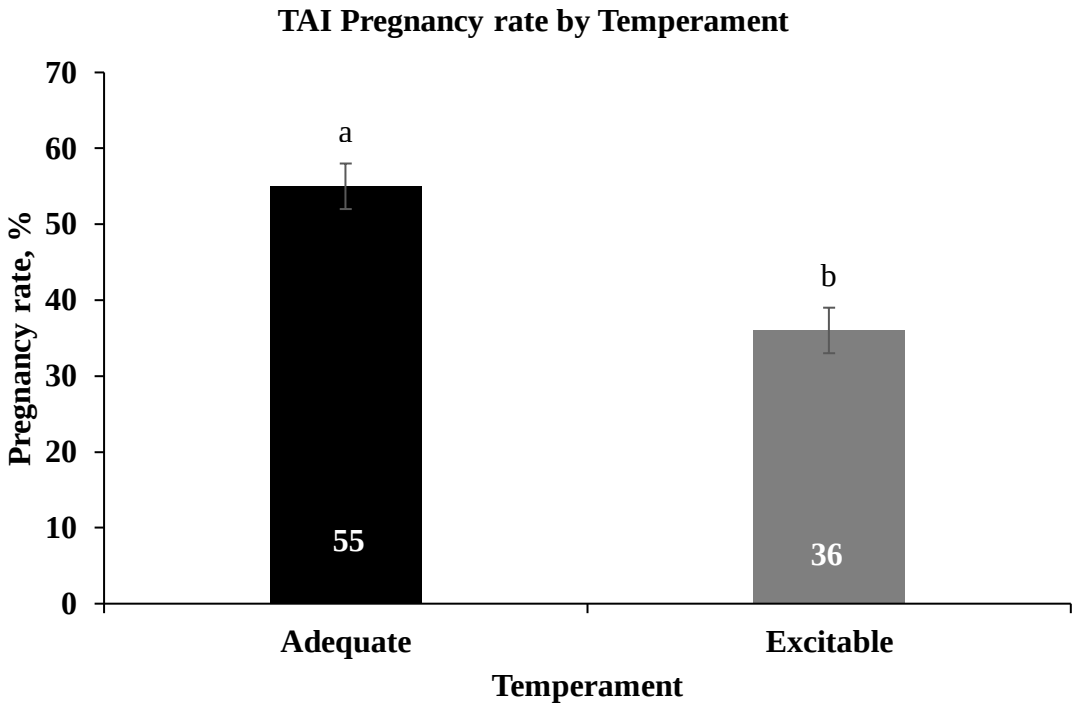

Figure 4.3. Pregnancy rates according to temperament. There was an effect of temperament on pregnancy rates ($P = 0.042$). In summary, heifers were evaluated for temperament based on chute score and exit velocity on the first day of the estrus synchronization protocol (day -9). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on d -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable). [a-b] Means with different superscripts differ at $P < 0.05$.

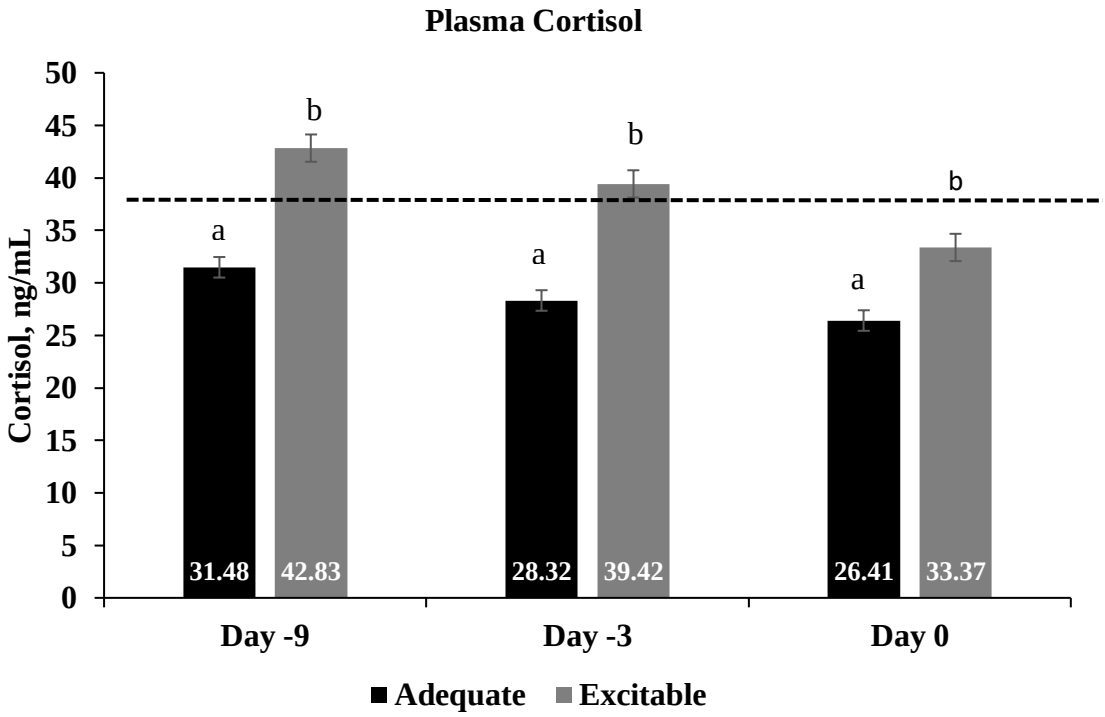

Figure 4.4. Plasma cortisol for heifers in location 3 according to experimental day. Plasma cortisol concentrations were affected by day (P = 0.031), temperament (P = 0.015) and the interaction of temperament and day (P = 0.046). The dotted line represents the threshold for intense fear (Grandin, 2017). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on d -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable). Heifers in location 3 had blood collected from the jugular vein in all handling events of the protocol for plasma cortisol evaluation. [a-b] Means with different superscripts differ at $P < 0.05$.

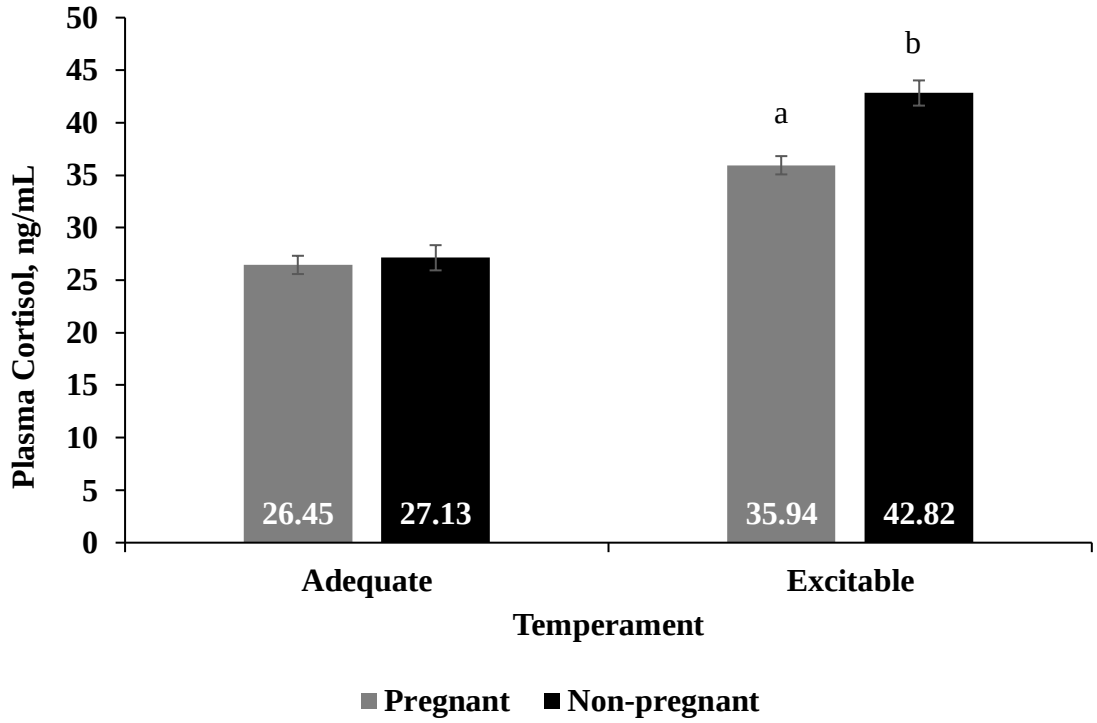

Figure 4.5. Plasma cortisol concentrations of pregnant and non-pregnant heifers between temperaments. There was a difference on plasma cortisol concentration between excitable pregnant heifers and excitable non-pregnant heifers ($P < 0.001$). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on d -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable). [a-b] Means with different superscripts differ at $P < 0.01$.

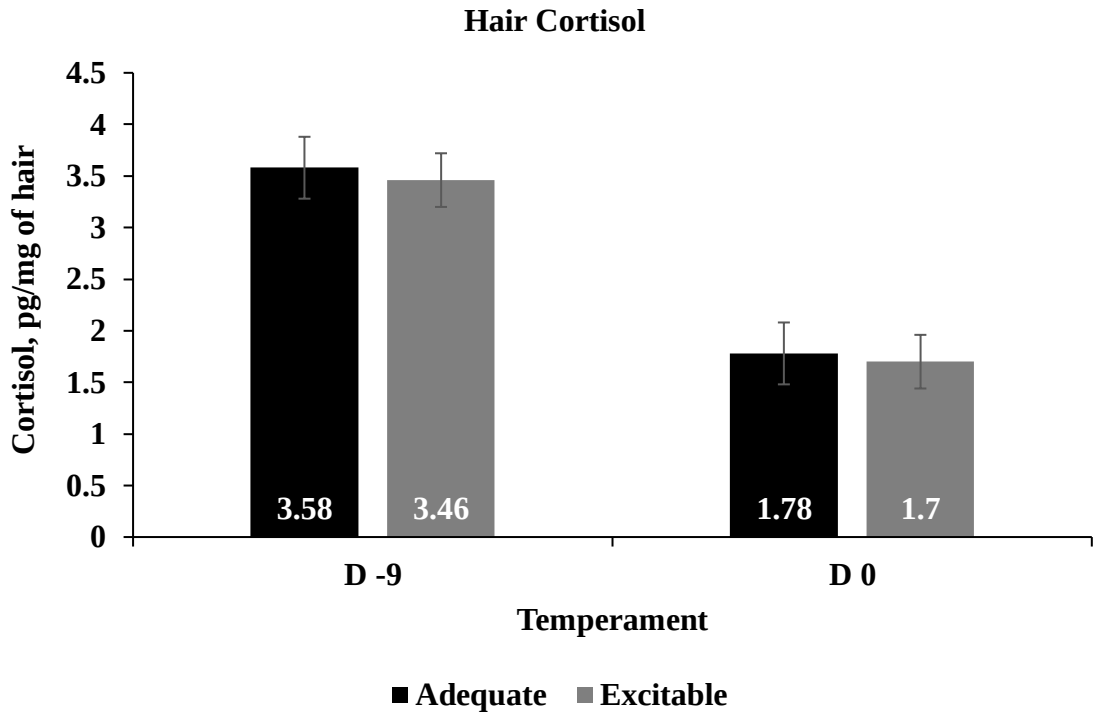

Figure 4.6. Mean hair cortisol concentrations of calm and excitable heifers for the initiation and completion of the protocol. There was an effect of day on hair cortisol concentrations ($P < 0.001$). Heifers were estrous synchronized using a 7-d CO-Synch +CIDR protocol in which heifers received an injection of GnRH on day -9 and CIDR device was inserted followed by injection of PGF2α and CIDR device removal on d -3 followed by TAI on day 0. Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (≤ 3 = calm; > 3 = excitable).

Chapter 5

Summary and conclusions

Fertility is the main issue cow-calf producers face in order to reach maximum profit. Various environmental and metabolic factors contribute to fertility of cattle. The altered metabolic state a cow will face after parturition, commonly accompanied by poor nutritional status and environmental influences will disrupt the physiological control of reproduction, leading to impaired fertility. Adoption of progestin based ES protocols has allowed the pharmacological control the estrous cycle, allowing timing insemination relative to ovulation (Lamb et al., 2010). Also, these protocols can induce cyclicity in anestrous cows (Meneghetti et al., 2009). These and other benefits of ES protocols make them a valuable tool to improve fertility and profitability on beef operations in Virginia and elsewhere in the United States.

The progestin based ES protocols indeed assist animals to overcome the environmental factors, such as nutrition, the suckling stimulus and temperament, that ultimately result in anestrus, and allows ovulation to occur (Meneghetti et al., 2009). These environmental factors however, still play a role in reproductive efficiency of animals enrolled in the protocol and diminishing these factors that induce anestrus and improving the environment of animals under the protocol are important strategies to improve success and widespread of this technology.

The present work has shown the difference in fertility of animals that fail or succeed to express estrus when enrolled in the ES protocol. Further, a strategy to increase fertility of animals that fail to express estrus, when challenged to the protocol, by delaying time of insemination was explored. Further studies are required to develop 2 strategies: 1) increase the number of animals expressing estrus to the protocol, and 2) determine the time of induction of ovulation and insemination for animals that fail to

express estrus. Perhaps delaying not only insemination, but also the ovulatory stimulus of GnRH will provide longer period for dominant follicle development, leading to longer period of E2 produced and period of uterine exposure to E2, resulting in increased fertility for this group of animals.

The present work has also shown the effects of temperament of heifers enrolled to the ES protocol and the effects of the handling events of the protocol on temperament of these heifers. Adoption of ES protocols may be an interesting tool for producers to overcome the detrimental effects of temperament on fertility, since the ES protocol may potentially improve fertility of heifers in two ways: 1) the handling events of the protocol may be acclimating the heifers to handling, resulting in reduced stress response and thus, diminishing the effects of stress on reproduction, and 2) providing a GnRH booster as ovulation stimulus, as excitable heifers may have impaired GnRH and LH production. Enhancing the environmental conditions in order to increase the proportion of animals with adequate temperament and selecting sires for temperament improvement are important strategies to improve reproductive efficiency of a herd.

Beef exports are expected to increase by the year of 2024 (USDA) in order to sustain the increasing global demand for animal protein (FAO, 2018). In order to increase beef production, more animals need to be introduced in the system and the success of cow-calf operations is key for this goal. Estrous synchronization protocols can improve fertility in many ways, but adapting the protocols to achieve greater pregnancy rates is key to allow the widespread of this technology and allow beef production to supply the global demands. Altogether, this work provides exploration of factors that influence fertility of animals enrolled in an ES protocol, as well as, strategies to overcome these effects and increase fertility to the protocol.

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