FACTORS ASSOCIATED WITH THE BIOSTIMULATORY EFFECT OF BULLS ON RESUMPTION OF OVARIAN CYCLING ACTIVITY AND BREEDING PERFORMANCE OF FIRST-CALF SUCKLED BEEF COWS

by

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TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

ABSTRACT x

1. INTRODUCTION 1

2. LITERATURE REVIEW 3

Endocrinology of the Postpartum Anestrous Cow 3
Gonadotropins 3
Luteinizing Hormone 4
Follicle Stimulating Hormone 5
Ovarian Steroids 5
Estrogen 5
Progesterone 5
Testosterone 6
Role of Hypothalamo-Hypophyseal-Ovarian Axis 7
Biphasic Feedback Effect of Estrogen 8
Postpartum Anestrus and the Negative Feedback Effect of Estrogen 9
Summary of HPO Axis Role in Resumption of Ovarian Cycling Activty 10
Factors Affecting Postpartum Anestrus of the Bovine 10
Nutrition 10
Suckling Stimuli 13
Other Factors 17
The Effect of Bull Exposure on the Postpartum Anestrous Cow 18
Sensory Pathway of Pheromones 25
Pheromone Transport and Perception 26
Pheromone Transport 26
Pheromone Perception 31
Pheromones in Bovine Reproductive Behavior 34
Summary 37

3. STATEMENT OF PROBLEM 39

4. MATERIALS AND METHODS 41

Experiment 1 41
Animals and Treatments 41
Lot Areas 42
Nutrition 43

TABLE OF CONTENTS-CONTINUED

Blood Sampling for Progesterone 43
Estrous synchronization, AI, and Pregnancy diagnosis 45
Statistical Analyses 45
Experiment 2 46
Animals and Treatments 46
Lot Areas 46
Controlled Urine Delivery Device (CUDD) 47
CUDD Attachment 48
CUDD Filling 49
Urine Collection 50
Urine Collection Facilities and Urine Handling 51
Nutrition 52
Blood Sampling for Progesterone 53
Estrous synchronization, AI, and Pregnancy diagnosis 54
Statistical Analyses 54

5. RESULTS 56

Experiment 1 56
Experiment 2 59
AI Pregnancy Rates for Experiment 1 and Experiment 2 Combined 62

6. DISCUSSION 64

Experiment 1 64
Experiment 2 67

LITERATURE CITED 77

APPENDICES 91

Appendix A: Components and Methods Used in the Construction of the Controlled Urine Delivery Device 92
Figure 8. Components and construction of Controlled Urine Delivery Device (CUDD) 93
Figure 8 (continued). Components and construction of Controlled Urine Delivery Device (CUDD) 94
Appendix B: Materials and Methods Used for the Attachment of the Controlled Urine Delivery Device 95
Figure 9. Illustration of Controlled Urine Delivery Device (CUDD) attachment procedure 96
Figure 9 (continued). Illustration of Controlled Urine Delivery Device

TABLE OF CONTENTS-CONTINUED

(CUDD) attachment procedure 97
Appendix C: Materials and Methods Used for the Filling of the Controlled Urine Delivery Device 98
Figure 10. Illustration of Controlled Urine Delivery Device (CUDD) filling procedure 99
Figure 10. (continued) Illustration of Controlled Urine Delivery Device (CUDD) filling procedure 100
Appendix D: Materials and Methods Used for the Collection of Urine 101
Figure 11. Illustration of the urine collection facility and methods used to collect urine 102
Figure 11 (continued). Illustration of the urine collection facility and methods used to collect urine 103
Figure 11 (continued). Illustration of the urine collection facility and methods used to collect urine 104

LIST OF TABLES

Table Page

1. Number of cows per treatment and least square means for calving date, cow BW,calf BW, BCS, calf sex ratio, and dystocia score for first calf suckled beef cows exposed (BE) or not exposed (NE) to bulls at the start of the experiment. 56

2. Number of animals per treatment and percentage of cows exhibiting estrus by 60 h after $PGF_{2\alpha,}$ timed AI (TAI), AI, and overall pregnancy rates for first-calf suckled beef cows exposed to bulls (BE and BENE) or not exposed to bulls (NE and NEBE) before the start of the estrous synchronization protocol (ES). 58

3. Number of cows per treatment and least square means for calving date, cow BW change, calf BW, BCS, BCS change, calf sex ratio, dystocia score and interval from exposure to resumption of cycling activity for first -calf suckled beef cows exposed to bull urine (BUE) for or exposed to steer urine (SUE)..... 60

4. Number of animals per treatment and percentage of cows exhibiting estrus by 60 h after $PGF_{2\alpha,}$ TAI, AI, and overall pregnancy rates for first-calf suckled beef cows exposed to mature bull urine (BUE) or exposed to steer (SUE) before the start of the estrous synchronization protocol 62

LIST OF FIGURES

Figure Page

1. Experimental design, number of animals per treatment, and protocols. BE = bull exposed, NE = no bull exposure....42

2. Pattern of progesterone concentrations used to determine occurrence of resumption of ovarian cycling activity....44

3. Progesterone pattern used to determine the interval from the start of treatment to resumption of ovarian cycling activity....53

4. Percentages of first-calf suckled cows exposed (BE) or not exposed (NE) to bulls that resumed ovarian cycling activity by the end of the the breeding season....57

5. Percentages of first-calf suckled cows exposed to mature bull urine (BUE) or exposed to steer urine (SUE) that were cycling at the start of the breeding season....61

6. Percentages of first-calf suckled cows exposed to bulls or bull urine (BE + BUE) or not exposed to bulls or bull urine (NE +SUE) to bulls that were cycling at the start of the breeding season. 63

7. Graphical illustration of the “Quantal Threshold” hypothesis....74

8. Components and construction of controlled urine delivery device (CUDD)....93

9. Illustration of controlled urine delivery device (CUDD) attachment procedure....96

10. Illustration of controlled urine delivery device (CUDD) filling procedure....99

11. Illustration of the urine collection facility and methods used to collect urine 102

ABSTRACT

The objective of this research was to evaluate factors associated with the biostimulatory effect of bulls on the resumption of ovarian cycling activity and breeding performance of first-calf suckled beef cows. In Experiment 1, we tested the hypotheses that short-term (30 d) bull exposure before the breeding season does not alter: 1) the proportion of cows that resumed cycling activity; 2) the proportion of cows that responded to estrous synchronization (ES); and, 3) AI and overall pregnancy rates. Resumption of ovarian cycling activity was measured by changes in progesterone patterns at 3 d intervals from the start of the experiment to the start of the breeding season. Cows were synchronized for estrus using an ES protocol that included CIDR, $PGF_{2\alpha}$, GnRH and time AI (TAI). Breeding performance was measured by: estrous response after $PGF_{2\alpha}$, and AI and overall pregnancy rates. We found that short-term bull exposure increased the proportion of cows that; were cycling by the end of the exposure period, and were pregnant from AI. Experiment 2 tested the hypothesis that exposure to bull urine does not alter: 1) the interval from exposure to resumption of ovarian cycling activity; 2) the proportion of cows that resumed cycling activity; 3) the proportion of cows that responded to ES; and, 4) AI pregnancy rates. Exposure of cows to bull urine did not alter; the interval from exposure to resumption of ovarian cycling activity, the proportion of cows cycling before the breeding season, and the proportion of cows that responded to ES. However, AI pregnancy rates were improved by exposing cows to bull urine before the breeding season. We conclude that the short-term physical presence of bulls, but not long-term continuous exposure to bull urine, reduced the interval from exposure to the resumption of ovarian cycling activity and increased the proportion of cows cycling before the breeding season. However, short-term exposure to bulls or long-term exposure to bull urine, before the breeding season appeared to enhance breeding performance of first-calf suckled beef cows using an ES protocol that included CIDR, $PGF_{2\alpha}$, GnRH and TAI.

CHAPTER 1

INTRODUCTION

The goal of cow-calf producers is to produce one calf per cow each year. This can be referred to as reproductive efficiency. Postpartum anestrus or the time after calving during which cows do not display estrus and cannot become pregnant decreases reproductive efficiency in beef cow herds. This problem is more pronounced in first-calf suckled beef cows that require 15 to 25 d longer to resume ovarian cycling activity than multiparous cows (Short et al., 1994). Many management strategies have been developed to help circumvent the problem of postpartum anestrus. Unfortunately, these strategies can be costly, labor intensive, unsustainable, and socially unacceptable. The use of the biostimulatory effect of bulls may be an effective management strategy to reduce postpartum anestrus that is cost effective, labor saving, sustainable, and socially acceptable to both producers and consumers. The implementation of the biostimulatory effect of bulls in practical situations and the mechanism by which this effect is mediated is not well understood.

The biostimulatory effect of bulls involves interactions between bulls and cows in which the physical presence of bulls stimulates the resumption of ovarian cyclic activity in multiparous and primiparous suckled beef cows (Zalesky et al., 1990; Custer et al., 1990). Joshi et al. (2002) reported that more cows exposed to bulls starting on d 55 after calving resumed ovarian cycling activity within 20 d of exposure than cows exposed to bulls starting on d 15 or d 35. This result indicates that the ability of cows to respond to the biostimulatory effect of bulls increases as time after parturition increases.

Experiment 1 of this thesis focuses on how and when to apply the biostimulatory effect of bulls, and in it we investigated the ability of cows to respond to short-term bull exposure before the breeding season. A recent study performed at Montana State University (Joshi et al., 2002) indicated that the biostimulatory effect of bulls may be mediated through a priming pheromone which is present in the excretory products of bulls. In Experiment 2, we investigated the capacity of bull urine to mediate the biostimulatory effect of bulls. In Experiments 1 and 2, we examined the effect of short-term bull exposure and continuous exposure to bull urine on the breeding performance of first-calf suckled beef cows using a progestin-based estrous synchronization protocol.

The review of literature will encompass: 1) an overview of the endocrinology of postpartum cows and factors that influence the postpartum interval to resumption of ovarian cycling activity; 2) an in depth review of the biostimulatory effect of bulls on the resumption of ovarian cycling activity of postpartum cows; and 3) a summary of literature related to pheromonal communication in mammals.

CHAPTER 2

LITERATURE REVIEW

Endocrinology of the Postpartum Anestrous Cow

The reproductive endocrine system includes the function of the anterior pituitary gland and the ovaries. The anterior pituitary gland is the source of gonadotropins: hormones that stimulate the function of the gonads. The ovaries have two functions: 1) development, maturation and release of the female gamete; and, 2) synthesis and secretion of protein and steroid hormones. Reproductive events in the female are regulated by a complex system of interactions between hormones secreted by the anterior pituitary and the ovaries. In this section I will review the anterior pituitary gonadotropes and ovarian steroids related to the postpartum anestrous cow.

Gonadotropins

Luteinizing Hormone (LH). Luteinizing hormone is a dimeric glycoprotein secreted by gonadotropes of the anterior pituitary (Hafez and Hafez, 2000). This hormone is the main stimulus for follicular ovulation. During the luteal phase of the estrous cycle, LH is secreted into the general circulation in a temporal pattern characterized as having low frequency and high amplitude pulses. During proestrus, the pulsatile characteristic in LH changes to that of low amplitude and high frequency LH release. This pattern of LH release leads to the preovulatory surge of LH that causes ovulation (Hafez and Hafez, 2000).

Generally, there is an increase in LH pulse frequency before anestrous cows resume ovarian cycling activity (Walters et al., 1982; Humphery et al., 1983; Peters and Lamming, 1990). Furthermore, LH pulse frequency, amplitude (Rawlings et al., 1980; Garcia-Winder et al., 1984; Garcia-Winder et al., 1986; Savio et al., 1990; Wright et al., 1990), and average plasma concentration (Walters et al., 1982; Humphrey et al., 1983; Garcia-Winder et al., 1984; Garcia-Winder et al., 1986; Nett et al., 1988) have been shown to increase with time postpartum. When LH pulse frequency, amplitude, and concentration change to that observed in proestrus, a surge of LH is released from the anterior pituitary which causes ovulation and resumption of normal ovarian cycling activity in postpartum anestrous cows.

Follicle Stimulating Hormone (FSH). Like LH, FSH is a dimeric gonadotropin glycoprotein secreted by gonadotropes of the anterior pituitary (Hafez and Hafez, 2000). Follicle stimulating hormone differs molecularly from LH in the β-subunit. The α-subunit composition is the same for both FSH and LH. This hormone causes the recruitment and development of secondary and tertiary follicles of the ovary (Hafez and Hafez, 2000). Also, follicular selection and dominance is dependent on FSH (Hafez and Hafez, 2000). Follicle stimulating hormone concentrations and fluctuations have been shown to increase shortly after parturition (Moss et al., 1985; Crowe et al., 1998). These fluctuations do not result in ovulation. In a normal cycling cow, FSH secretion is controlled by the negative feedback effects of follicular inhibin. As one follicle begins to dominate, the granulosa cells within this follicle release inhibin, inhibiting the release of FSH from pituitary gonadotropes causing smaller follicles within that wave become atretic. The appropriate temporal LH release pattern stimulates the final maturation and

development of the largest follicle or dominant follicle. Maturation, development, and ovulation of follicles do not occur in anestrous cows because of the inappropriate release of LH that fails to stimulate the growth beyond 7 to 9 mm in diameter (Gong et al., 1995; Gong et al., 1996).

Ovarian Steroids

Estrogen**.** The dominant form of ovarian estrogen in females is estradiol-17β. It is synthesized primarily by the granulosal cells of the ovarian antral follicles. Early after parturition, estradiol-17β is very low because of the absence of large follicles on the ovaries (Arije et al., 1974; Humphrey et al., 1983; Crowe et al., 1998). However, follicular waves begin to develop between 5 to 11 d after parturition (Crowe et al., 1998). From that point onward, estradiol-17β concentrations remain relatively constant and low through the postpartum anestrous period (Carruthers et al., 1980; Chang et al., 1981). Suckling and milk production have no effect on estradiol-17β secretion (Carruthers and Hafs, 1980). A change in estradiol-17β occurs only when the dominant follicle is stimulated by the appropriate LH signal; the follicle becomes larger and secretes high levels of estradiol-17β. This leads to behavioral estrus and signals the preovulatory release of LH in normal cycling cows.

Progesterone**.** Progesterone is a steroid hormone produced by the theca interna of antral follicles and corpus luteum (CL; Hafez and Hafez, 2000). Progesterone concentrations decrease almost immediately after parturition and remain low throughout the postpartum anestrous period. The postpartum cow generally exhibits a small rise in progesterone concentration 3 to 7 days before the first postpartum ovulatory cycle (Arije

et al., 1974; Stevenson and Britt, 1979; Rawlings et al., 1980; Humphrey et al., 1983; Werth et al., 1996). This rise in progesterone concentration is followed by a return to baseline concentrations before the first postpartum estrus and is often referred to as a "short cycle". Short cycles are due to ovulation and formation of a CL (Castenson et al., 1976; Stevenson and Britt, 1979) however, the CL formed after this ovulation is short lived and produces small amounts of progesterone (Corah et al., 1974). Behavioral estrus does not generally accompany this ovulation probably because there is no preovulatory increase in estrogen. Progesterone-priming is thought to be necessary for the full expression of estrus in response to estrogen in the bovine (Kieborz-Loos et al., 2003). The lifespan of this CL is shortened because of a premature release of $PGF_{2\alpha}$ soon after uterine exposure to progesterone, thus shortening the luteal phase of this cycle (Daily et al., 1992). Mann and Lamming (2000) showed the shortened lifespan of the CL is most likely caused by low concentrations of estradiol-17β before ovulation which, in turn, causes the retention of oxytocin receptors in the endometrium of the uterus; this allows for a premature increase in $PGF_{2\alpha}$ and lysis of the CL. Most but not all (88 to 92%) cows express a "short cycle" before resumption of normal ovarian cycling activity (Hafez and Hafez, 2000); this is usually true for primiparous beef cows.

Testosterone. The ovaries produce testosterone throughout the estrous cycle of a cow. Kanchev and Dobson (1976) reported that serum concentrations of testosterone rose sharply to 180 to 200 pg/mL seven days before estrus and returned to baseline concentrations of 5 to 60 pg/mL through the remainder of the estrous cycle. Also, Kesler et al. (1979) reported a marked increase in testosterone concentration 7 d before behavioral estrus in cycling cows. The relationship between changes in testosterone

concentrations and postpartum physiology of cows is not understood because it has not been thoroughly investigated throughout anestrus in cows.

Role of the Hypothalamo-Hypophyseal-Ovarian Axis

The hypothalamo-hypophysial-ovarian (HPO) axis is a complex neuroendocrine-endocrine system that acts as the physiological control mechanism for all reproductive events including postpartum anestrus in the cow. In general the, hypothalamus stimulates the pituitary which, in turn, stimulates the ovary. The following is an overview of the interaction between the hypothalamus, pituitary, and ovary during the postpartum anestrous period.

The hypothalamus, located in the medial-basal aspect of the brain, contains a sub-set of neurons that are vitally important to regulation of reproductive events in both males and females. These neurons are known as neurosecretory neurons or peptidergic neurons because they synthesize peptides that are secreted into the blood, instead of a synaptical space. Thus they are known as neurohormones. Gonadotropin releasing hormone (GnRH) is a 10 amino acid long neurohormone that stimulates pituitary luteotropes and folliculotropes to secrete LH and FSH, respectively. Generally, GnRH neurons intrinsically secrete GnRH in small bursts or pulses; the intrinsic pattern of release is known as the GnRH "pulse generator". For LH, but not FSH, a pulse of GnRH will stimulate a concomitant pulse in LH (Hafez and Hafez, 2000). Thus factors that influence the GnRH pulse generator will in turn affect the pattern of LH release.

Ovarian follicular wave development is reduced before parturition (Savio et al., 1990). Follicle stimulating hormone begins to fluctuate from 5 to 10 days after

parturition; this stimulates the resumption of follicular wave development which produces a dominant follicle (DF) by 11 days after calving (Crowe et al., 1998). The dominant follicle that develops during these early follicular waves does not usually ovulate (Crowe et al., 1998). Therefore, FSH and early follicular wave development is not a major limiting factor that may explain the occurrence of long postpartum anestrus in the cow.

The failure of the DF to ovulate soon after parturition can be explained by the role of LH in follicular development. Luteinizing hormone has direct stimulatory effect on the DF. In a cycling cow, temporal release of LH is characterized by low frequency and high amplitude pulses (Walters et al., 1982; Peters and Lamming, 1990). This temporal release pattern extends the life of the DF and causes it to grow beyond 7 to 9 mm into a pre-ovulation size of greater than 9 mm (Fortune et al., 1991; Savio et al., 1993; Gong et al., 1995; Rhodes et al., 1995; Gong et al., 1996). During the postpartum anestrous period, LH pulses are characterized as less frequent and higher amplitude (Rawlings et al., 1980; Humphery et al., 1983; Garcia-Winder et al., 1984; Garcia-Winder et al., 1986; Savio et al., 1990; Wright et al., 1990). The temporal release pattern of LH is the primary endocrinological cause of postpartum anestrus. Therefore, to understand the physiological mechanisms involved with regulating resumption of ovarian cycling activity in cows, one must understand those mechanisms that limit pulsatile LH release.

Biphasic Feedback Effect of Estrogen. Gonadotropin releasing hormone (GnRH), a critical neurohormone produced by the hypothalamus, stimulates the release of both LH and FSH from the anterior pituitary (Fernandes et al., 1978; Jagger et al., 1987). During the luteal phase of the estrous cycle progesterone secretion is high. Low estrogen

concentrations during this phase causes neurosecretory neurons of the hypothalamus to release infrequent pulses of GnRH which stimulate LH release in a low amplitude and high frequency manner from the anterior pituitary (Rawlings et al., 1980; Humphery et al., 1983; Garcia-Winder et al., 1984; Garcia-Winder et al., 1986; Savio et al., 1990; Wright et al., 1992). During the early proestrous phase of the estrous cycle, as progesterone begins to fall, these neurons release GnRH more frequently, causing release of LH from the anterior pituitary in low amplitude, high frequency manner (Rawlings et al., 1980; Humphery et al., 1983; Garcia-Winder et al., 1984; Garcia-Winder et al., 1986; Savio et al., 1990; Wright et al., 1990). The temporal release of LH in this manner stimulates the continued growth and development of the DF in the ovary, causing the synthesis and secretion of estadiol-17β. When estrogen concentrations reach a threshold, the pre-optic area of the brain, located above the optic chiasm, stimulates the episodic or preovulatory release of LH. This episodic release of LH assures the development and ovulation of the dominant follicle. At the same time the sexual behavior center of the brain detects the rise in estrogen concentration and stimulates behavioral estrus.

Postpartum Anestrus and the Negative Feedback Effect of Estrogen. Estrogen concentration in the blood does not change dramatically throughout postpartum anestrus (Carruthers et al., 1980; Chang et al., 1981). Neurosecretory neurons responsible for the tonic release of GnRH are quite sensitive to low constant levels of estradiol-17β (Acosta et al., 1983). Sensitivity of tonic GnRH release to estradiol-17β causes a decrease in the pulsatile release of LH from the anterior pituitary (Acosta et al., 1983). This effect is called the negative feedback effect of estrogen. As time progresses after parturition sensitivity of neurosecretory neurons to estrogen wanes, allowing GnRH pulses to occur

more frequently. This change in temporal GnRH release stimulates low amplitude, high frequency LH secretion required for ovulation and resumption of ovarian cycling activity.

Summary of HPO Axis Role in Resumption of Ovarian Cycling Activity. After parturition, the hypothalamic GnRH pulse generator is very sensitive to the negative feedback of estradiol-17β. This sensitivity causes the anterior pituitary to release high amplitude, low frequency pulses of LH; which is a signal to the ovary to remain anovulatory. As time progresses postpartum, the sensitivity of the GnRH pulse generator to the negative feedback effect of estrogen decreases causing low amplitude, high frequency release of LH from the anterior pituitary. Low amplitude, high frequency LH release causes an immature DF to ovulate and secrete progesterone for a short period of time allowing a new DF to develop. As this DF matures it synthesizes and secretes estrogen. High concentrations of estrogen trigger behavioral estrus, the episodic release of LH, and ovulation of the DF and CL formation, i.e., resumption of ovarian cycling activity.

Factors Affecting the Postpartum Anestrous Interval of the Bovine

The principal factors that affect the length of time from calving to the resumption of ovarian cycling activity are nutrition, suckling stimuli, and parity. Other factors that influence this interval are breed, environmental stress, dystocia, and social factors (for review, see, Short et al., 1990).

Nutrition

Nutrition and body condition significantly impact the length of postpartum anestrus in the bovine. Basic, essential bodily functions like basal metabolism, growth,

and vital energy reserves take precedence over reproductive functions (Grimard et al., 1997; Guedon et al., 1999). This has been illustrated by research that has shown that low energy intake before and after calving increases the time interval from calving to resumption of ovarian cycling activity (Dunn et al., 1969; Wiltbank, 1970; Falk et al., 1975; Dunn et al., 1985). Furthermore, if cows remain on a low plane of nutrition after calving, they may fail to resume ovarian cycling activity throughout the breeding season (Wiltbank, 1970). On the other hand, the interval to resumption of ovarian cycling activity can be shortened if cows are fed a higher plane of nutrition after calving (Bellows and Short, 1978; Ducker et al., 1985; Henricks and Rone, 1986). As a consequence, cows fed a high plane of nutrition have higher overall pregnancy rates than cows fed a low plane of nutrition (Bellows and Short, 1978; Henricks and Rone, 1986; DeRouen et al., 1993).

Feeding cows a moderate to high plane of nutrition is important for the resumption of ovarian cycling activity postpartum (Dunn et al., 1969; Wiltbank, 1970; Falk et al., 1975; Dunn et al., 1985). However, there is some dispute over when to apply a positive energy balance ration, whether pre- or postpartum, to best reduce the postpartum anestrous interval to resumption of ovarian cycling activity. Houghton et al. (1990) reported that a greater proportion of cows fed a low energy ration prepartum and switched to a high energy ration postpartum (low-high) exhibited estrus by 60 d after calving than cows fed a high-low, high-high, or low-low energy regimen. These results indicate that an increase in postpartum energy intake is more important than prepartum energy intake. These results are contrary to those reported by Dunn and Kaltenbach

(1980) who concluded that prepartum nutrition was more important than postpartum nutrition to reduce postpartum anestrus in cows.

These results led researchers to question how nutrition can affect ovarian processes like follicular development. Henricks and Rone (1986) reported that cows fed a high energy ration had more medium- and small-sized follicles and higher estradiol-17β concentrations than cows fed a low energy ration. Also, an increase in energy intake 2 wk before parturition (Lammoglia et al., 1996), at parturition (Beam and Butler, 1997; DeFries et al., 1998), and at 4 wk after parturition (Khireddine et al., 1998) causes an increase in the number of ovarian follicles. Further evidence has shown that feeding a higher energy ration at parturition prolongs the life of the corpus luteum (Williams, 1989).

Progesterone, the hormone of pregnancy, prepares the uterus for embryonic implantation and sustains pregnancy after implantation. Progesterone concentrations were higher in heifers given a high energy ration than heifers fed a low energy ration (Gombe and Hansel, 1973; Beal et al., 1978). Low progesterone concentrations, in heifers fed a low energy diet, could be explained by poor follicular development (Henricks and Rone, 1986; Perry et al., 1991). In addition, Gombe and Hansel (1973) reported that first-calf cows fed low energy rations postpartum had smaller corpa lutea with lower progesterone concentrations on d 10 of their third ovarian cycle after calving. Therefore, energy intake can be directly related to the ability of the cow to conceive and maintain pregnancy (Dunn et al., 1969).

In addition to energy intake, percent body fat at the time of and after parturition can affect the length of postpartum anestrus in cows (Bartle et al., 1984; Richards et al.,

1986). Generally, cows showing good body condition (BCS = 4 to 6; scale 1 to 9, 1 = emaciated and 9 = obese) at parturition resume ovarian cyclic activity and conceive before the end of the breeding season compared to thin cows (BCS = 1 to 3.5) who generally have longer intervals from calving to resumption of ovarian cycling activity and greater difficulty conceiving before the end of the breeding season (DeRouen et al., 1994; Spitzer et al., 1995; Vizcarra et al., 1998). Body condition score has been shown to positively correlate with early follicular development (Ryan et al., 1994), pituitary luetinizing hormone (LH) concentration (Connor et al., 1990), and LH pulse frequency (Bishop et al., 1994). However, Wright et al. (1990) reported average LH concentration in systemic circulation was not affected by body condition.

Taken together, these results indicate that nutrition is a major factor influencing the postpartum anestrous period in cows. It is clear that good body condition and medium to high energy rations fed pre- or postpartum are necessary for cows to resume ovarian cycling activity, conceive, and maintain pregnancy.

Suckling Stimuli

Suckling stimuli associated with feeding behavior of the calf is another important factor that can influence the length of anestrus in postpartum cows. Suckling stimuli has been shown to be the most important factor contributing to extended postpartum anestrus in beef and dairy cows when nutrition is not a limiting factor (Short et al., 1990; Williams, 1990; Stagg et al., 1998).

Restricting or eliminating suckling stimuli reduces the interval from calving to resumption of ovarian cycling activity. Restricted-suckled cows (suckled once daily),

and non-suckled cows have shorter postpartum anestrous intervals to resumption of ovarian cycling activity than cows suckled twice daily, cows suckled normally, and cows suckled intensively (Smith and Vincent, 1972; Laster et al., 1973; Carter et al., 1980; Odde et al., 1980; La Voie et al., 1981; Randel, 1981; Reeves and Gaskins, 1981; Ramirez-Godinez et al., 1982; Garcia-Winder et al., 1984; Houghton et al., 1990). Additionally, Perez-Hernandez et al. (2002) reported that postpartum anestrous intervals to resumption of ovarian cycling activity in cows that were suckled for 2 h immediately after milking did not differ from those of cows suckled for 2 h, 8 h after milking. These data indicate that delayed suckling does not reduce the interval from calving to resumption of ovarian cycling activity. However, it appears that restricting suckling to 2 h daily, whether immediately after milking or 8 h after milking, influences the length of postpartum anestrus in the same way regardless of when calves are allowed to suckle (Perez-Hernandez et al., 2002). Finally, removing the calf at birth and weaning calves early causes a significant reduction in the length of postpartum anestrus compared to cows suckling calves (Bellows et al., 1974; Walter et al., 1982; Williams, 1990; Short et al., 1990) whereas, intensive suckling can prolong postpartum anestrus in cows (Wettemann et al., 1986; McNeilly, 1988).

The suckling stimulus may serve to regulate the neuroendocrine-endocrine events that control the resumption of ovarian cycling activity. Cows whose calves have been weaned have higher concentrations of LH than cows suckling calves (Walters et al., 1982; Carter et al., 1980). Non-suckled cows and cows restricted to suckling once-daily show a sustained increase in LH concentrations within 20 days after calving, while cows subjected to intensive suckling do not show a sustained increase until 48 days after

calving (Garcia-Winder et al., 1984). Also, LH mean concentration, peak frequency (Carruthers and Hafs, 1980; Whisant et al., 1986), and amplitude (Carruthers and Hafs, 1980; Chang et al., 1981; Whisnant et al., 1986) rise significantly higher within 48 h after weaning compared to non-weaned, suckled cows. Additionally, work reported by Walters et al. (1982) reported that weaning calves from cows caused an increase in LH pulse frequency. Further evidence indicates that suckled cows have lower frequency and amplitude of LH release than milked cows (Carruthers and Hafs, 1980). Therefore, suckling may affect the GnRH neurosecretory neuronal system in the hypothalamus to reduce the tonic release of LH which results in the postponement of ovulation.

It is clear that suckling delays the resumption of ovarian cycling activity by causing a reduction in LH secretion; however, how this happens is unclear. Suckling may cause a decrease in the ability of the hypothalamus to respond to the positive feedback of estrogen (Short et al., 1970). Another proposed hypothesis is that suckling may inhibit the tonic release of LH causing the low constant secretion of estrogen from the ovary. This type of estrogen release enhances the negative feedback effect of estrogen and delays ovulation during the postpartum anestrous period. This idea was supported by Garicia-Winder et al. (1984) who concluded that suckling and ovarian factors interact during the postpartum period to suppress LH secretion and pulse frequency. Furthermore, Acosta et al. (1983) showed that cows treated with estradiol 17-β when nursing had lower LH concentrations than cows not treated with estradiol 17-β. However, after the calves were weaned from the cows, estradiol 17-β stimulated the release of LH above that of cows that did not receive estradiol 17-β treatment. These data indicate that suckling acts in concert with ovarian estrogen to inhibit the release of

LH in a high frequency low amplitude manner. However, the effect of suckling and the negative feedback effects of estrogen diminish as time increases postpartum. Relaxation of these inhibitory influences upon the GnRH pulse generator changes the temporal LH secretion pattern to one characterized by low amplitude, high frequency pulses, causing the resumption of ovarian cycling activity.

The inhibitory effect of suckling stimuli on LH release may involve more than mammary-somatosensory pathways. In other words, the physical presence of the calf may be needed to elicit this effect. Williams et al. (1993) found LH pulse frequency increased within 9 to 13 days after weaning over that of suckled cows. Surprisingly, these investigators also found that mammary denervation did not increase the LH pulse frequency. Therefore in some way, the physical presence of a calf appeared to be a component of the inhibitory effect of the suckling stimulus. Cows recognize calves through olfactory and visual cues; this recognition is called the cow-calf bond (Williams and Griffith, 1995; Lamb et al., 1997). Cows whose calves were continually present but restricted from suckling had shorter postpartum anestrous intervals than cows whose calves were continually present and allowed to suckle (Hoffman et al., 1996). This indicates that the cow-calf bond along with mammary stimulation (Lamb et al., 1997) causes the negative effect of suckling stimulus on the resumption of ovarian cycling activity. Furthermore, the physical presence of the calf stimulus is greatest if cows are suckling their own calves rather than foster calves (Wetteman et al., 1978). Cows suckling their own calves have lower occurrence of ovulation early postpartum than cows suckling foster calves (Williams et al., 1991). Therefore, the unrestricted presence and

mammary stimulation by the original calf is thought to elicit the maximum inhibitory effect of suckling stimuli on extending postpartum anestrus in cows.

Based upon the aforementioned studies, it may be possible to model the mechanism by which suckling stimuli fits into the endocrinology of the postpartum anestrus cow. After parturition, suckling stimuli is high because of the close relationship and physical presence of the calf. Suckling stimuli cause the GnRH pulse generator of the hypothalamus to be more sensitive to the negative feedback effects of estrogen; as a result, high amplitude, low frequency pulses of GnRH are released. This causes high amplitude, low frequency pulses of LH which signals the ovary to remain anovulatory. As the calf ages, it becomes more nutritionally and socially independent, causing a reduction in suckling stimuli. The GnRH pulse generator then becomes less sensitive to the negative feedback effect of estrogen. This results in the release of LH in a high frequency, low amplitude manner. This pattern of LH release is the signal to the ovary to resume ovarian cycling activity.

Other Factors

The following is a summary of other factors contributing to postpartum anestrus reviewed by Short et al. (1990). Multiparous cows have shorter anestrous intervals than do primiparous cows. The reason for this is due to a combination of an increased incidence of dystocia and greater nutrient demands on primiparous cows due to energy requirements for growth, lactation, and production of a calf. Dystocia in both primiparous and multiparous cows increases the length of postpartum anestrus. The greater degree of difficulty during parturition causes more damage to the reproductive

tract and increases amount of physical stress. Stress increases energy demand which, in turn, causes prolonged periods of anestrus after parturition. Stress can come in many forms; studies have shown that stressing the cow with extreme heat or cold will prolong postpartum anestrus.

Breed also plays a role in the length of postpartum anestrus. *Bos taurus* cows have shorter postpartum anestrus intervals than *bos indicus* cows, and cows from Continental breeds have longer postpartum anestrous intervals than cows from British breeds. These differences may be due to variations in the physical make-up of different types of cattle. For example, Continental cattle breeds have higher growth and milking genetic potentials than do British breeds. Increased milking and growth potentials cause an increase in nutrient demands, which may result in longer intervals from calving to resumption of ovarian cycling activity. Therefore, breed, stress, parity, and dystocia are factors which can result in variability in responses when evaluating postpartum anestrus in cattle.

The Effect of Bull Exposure on the Postpartum Anestrous Cow

The effect of exposing cows to bulls and subsequent resumption of cycling activity was first documented in early breeding experiments (Nersesjan, 1962; Sipilov, 1964; Ebert et al., 1972). These early breeding experiments found a higher occurrence of estrus in groups of cows that were present with teaser bulls; however, these studies did not specifically address the resumption of ovarian cycling activity. Results reported in these experiments could be explained by the enhanced ability of observers to detect estrus due to the physical presence of teaser bulls. Subsequently, a large number of studies

have shown that the presence of yearling or mature bulls reduced the interval from calving to the resumption of ovarian cycling activity in both primiparous and multiparous suckled beef cows (Macmillan et. al., 1979; Zalesky et al., 1984; Berardinelli et. al., 1987; Scott and Montgomery, 1987; Custer et. al., 1990; Naasz and Miller, 1990; Stumpf et. al., 1992; Cupp et. al., 1993; Hornbuckle et. al., 1995; Fernandez et al., 1996; Peres-Hernandez et al., 2002; Anderson et al., 2002). This biostimulatory effect of bulls is described as the influence of bulls on the reproductive function of cows that is mutually beneficial for successful reproduction.

Although there are numerous studies that support the biostimulatory effect of bulls, there are a few studies that do not. Bonavera et al. (1990) reported that bulls did not shorten the postpartum anestrous interval from calving to the resumption of ovarian cycling activity. However, a difference between cows exposed and not exposed would have been difficult to detect in this experiment because cows not exposed to bulls resumed ovarian cycling activity within 38 days after calving. Shipka and Ellis (1998; 1999) reported that dairy cows exposed to bulls through fence-line contact showed equal or longer intervals from calving to resumption of ovarian cycling activity than cows not in fence-line contact with bulls. However, Fike et al. (1996) reported that fence-line contact reduced the length of postpartum anestrus in first-calf beef cows. The difference between the results reported by Fike et al. (1996) and those of Shipka and Ellis (1998; 1999) could be explained by the fact that bulls in the latter experiment were in contact with cows only thrice daily when cows were in the milking parlor and were never closer than an alley width (6 to 8 m) from cows, whereas, cows in the study by Fike et al. (1996) were allowed direct fence-line contact with bulls for 24 h each day. Lastly, other data

indicate that the biostimulatory effect of bulls may be influenced by season. Fall-calving cows do not respond to the biostimulatory effect of bulls as do spring-calving cows (Macmillan et al., 1979; Perry et al., 1993). This may mean that the biostimulatory effect of bulls is dependent upon factors associated with changes of the season. More controlled studies will be necessary to evaluate the seasonal effects involved with the biostimulatory effect of bulls.

The biostimulatory effect of bulls interacts with other factors known to influence the length of postpartum anestrus of *bos taurus* (Stumpf et al., 1992; Hornbuckle et al., 1995) and *bos indicus* cows (Rekwot et al., 2000). Hornbuckle et al. (1995) reported cows exposed to bulls grazing higher quality bluestem had shorter postpartum anestrous intervals than cows exposed to bulls that grazed fescue pasture. Furthermore, Stumpf et al. (1992) showed cows exposed to bulls and fed a low plane of nutrition had postpartum anestrous intervals that were 14 d shorter than cows fed a low plane of nutrition and not exposed to bulls. Also, cows fed a high plane of nutrition that were exposed to bulls had postpartum anestrous intervals to resumption of ovarian cycling activity 6 d shorter than non-exposed cows. These data indicate that the biostimulatory effect of bulls shortens postpartum anestrus to a greater extent under nutrient-restricted conditions in cows.

Although the biostimulatory effect of bulls and plane of nutrition appear to reduce the postpartum anestrous period, there is no evidence to suggest the same relationship between the biostimulatory effect of bulls and suckling stimuli. This may be because most experiments that address the bull effect are designed to equalize suckling stimuli over all treatments. Delayed suckling 8 h after milking does not appear to interact with the biostimulatory effect of bulls to reduce postpartum anestrus (Perez-Hernandez et al.,

2002). In a study that evaluated the interaction between suckling and bull exposure, Joshi et al. (2002) found that the interval to resumption of ovarian cycling activity was not different among cows that were suckled 2 h daily or continuously suckled when exposed or not exposed to bulls. However, it is important to note that researchers in this experiment may not have found a restricted suckling effect because calves were housed in pens adjacent to cows; this allowed for the maintenance of the cow-calf bond via close proximity to their own calves. Future investigation is necessary to determine if there is an interaction between calf suckling stimuli and the biostimulatory effect of bulls.

One important aspect of the biostimulatory effect of bulls is timing and/or duration of exposure needed to cause a biostimulatory effect. Long-term continuous physical presence of bulls, greater than 60 d, has been shown to reduce the interval from calving to resumption of ovarian cycling activity (Macmillan et. al., 1979; Zalesky et al., 1984; Berardinelli et. al., 1987; Scott and Montgomery, 1987; Custer et. al., 1990; Naasz and Miller, 1990; Stumpf et. al., 1992; Cupp et. al., 1993; Hornbuckle et. al., 1995; Fernandez et al., 1996; Anderson et al., 2002). Fernandez et al. (1996) reported that continuous bull exposure from 3 or 30 days after calving reduced the interval from calving to resumption of ovarian cycling activity to the same extent. Also, Fernandez et al. (1993) reported that exposing cows to bulls for 2 h every third day for 18 days did not alter the postpartum anestrous interval for cows exposed to bulls in this manner or not exposed to bulls. Furthermore, Sipilov (1966) reported that more cows exhibited estrus and were inseminated within 30 d after calving if cows were exposed to bulls from 4 to 5 d after calving for 3 to 4 h daily. These results indicate that bull exposure for greater than

2h every day or continuous physical presence of bulls may be needed to cause a biostimulatory effect.

Cows may respond differently to bulls as time after calving increases. Based upon this premise, Joshi et al. (2002) tested the ability of cows to respond to bull biostimulation starting at 15, 35, and 55 days after calving. All cows exposed to bulls had shorter postpartum anestrous intervals than cows not exposed to bulls. However, more cows exposed to bulls starting at d 55 after calving resumed ovarian cycling activity within twenty days of exposure than cows exposed to bulls starting on either 15 or 35 d after calving. This indicates that postpartum anestrous cows become more sensitive to the biostimulatory effect of bulls as time after calving increases.

Some evidence indicates the biostimulatory effect of bulls can improve breeding performance of cows. Application of modern estrous synchronization (ES) and artificial insemination (AI) technologies are more successful in cows that have resumed ovarian cycling activity (Lucy et al., 2001). More cows exposed to bulls resume cycling activity than cows not exposed to bulls before the breeding season (Custer et al., 1990; Joshi et al., 2002). Furthermore, Anderson et al. (2002) suggested the possibility that the physical presence of bulls with cows during ES and 5 d after timed AI may improve AI pregnancy rates. Taken together, the biostimulatory effect of bulls may improve breeding performance in beef cattle herds.

The data presented thus far leads to the conclusion that the biostimulatory effect of bulls exists and that at least the continuous physical presence of the bull sometime after calving is needed to mediate this effect. The next question is, “How does the biostimulatory effect of bulls influence the endocrinology of the postpartum anestrous

cow?" Data from previous research showed that an increase in LH pulse frequency occurred directly before postpartum anestrous cows resumed ovarian cycling activity (Walters et al., 1982; Peters and Lamming, 1990). This led researchers to postulate that the biostimulatory effect of bulls could influence the hypothalamo-hypophseal-ovarian axis to release LH. Custer et al. (1990) reported that cows exposed to bulls had the same LH baseline concentration, mean concentration, pulse duration, pulse amplitude, and pulse frequency as cows not exposed to bulls. However, the researchers in this study acknowledged that immediate changes in LH may not have been observed because blood samples were only taken weekly. To address this possibility, Fernandez et al. (1996) intensively collected blood samples every 10 min for 4 h every three days from cows continuously exposed to bulls, intermittently exposed to bulls for 2 h every third day, or not exposed to bulls. Cows continuously exposed to bulls and cows exposed to bulls intermittently had higher mean LH concentrations and greater frequency of LH pulses than cows that were not exposed to bulls. Additionally, Baruah and Kanchev (1993) administered bull urine oronasally to cows and found that mean LH and FSH concentrations increased in the blood within 80 min after urine application. However, Fernandez et al., (1996) and Baruah and Kanchev (1993) reported that exposing cows to bulls intermittently or administering bull urine oronasally to cows did not decrease the length of postpartum anestrus. These data indicate that the quantity of stimulation was not sufficient to cause a biostimulatory effect. Taken together, these studies support the hypothesis that the mechanism whereby the biostimulatory effect of bulls acts involves the hypothalamo-hypophseal-ovarian control axis and allows one to speculate that a urinary pheromone may be involved with this effect.

The mechanism by which the bull stimulates the hypothalamo-hypophseal-ovarian control axis is not well understood. Berardinelli et al. (2004) explored the hypothesis that a social bond may be formed between the bull and cow, causing the biostimulatory effect of bulls. This notion was tested by comparing the length of postpartum anestrus of cows exposed to familiar bulls, then switched to exposure to unfamiliar bulls, and cows exposed to familiar ovariectomized (OVX) cows and switched to exposure of unfamiliar OVX cows. Cows exposed to familiar and unfamiliar bulls had shorter intervals from calving to the resumption of ovarian cycling activity than cows exposed to OVX cows. However, familiarity had no effect on the length of postpartum anestrus. Therefore, social interaction and bonding between bulls and cows may not be an important factor that mediates the biostimulatory effect of bulls. Instead, it appears that the physical presence of bulls acts independent of social interaction and is probably related to a pheromonal factor. Joshi et al., (2002) tested this idea by comparing intervals to resumption of ovarian cycling activity in cows that were: 1) exposed to continuous presence of bulls, 2) exposed to excretory products of bulls for 12 h daily, 3) exposed to their own excretory products for 12 h daily, or 4) not exposed to bulls, excretory products of bulls or cows beginning 35 d after calving. Cows exposed to bulls or excretory products of bulls or their own excretory products had shorter intervals to resumption of cycling activity than cows not exposed to bulls, excretory products of bulls or excretory products of cows. These data indicate that the biostimulatory effect of bulls is mediated through the excretory products of bulls. Burns and Spitzer (1992) showed that length of postpartum anestrus for cows exposed to testosterone-treated cows and cows exposed to bulls did not differ but were shorter than that for control cows. The data from these

experiments indicate that the biostimulatory effect of bulls could be mediated through a pheromone(s) present in bull excretory products, and that this pheromone could be androgen-dependent.

Sensory Pathway of Pheromones

Pheromones are airborne chemical substances released from the urine, feces, or cutaneous glands that are sensed by the olfactory or respiratory systems and cause behavioral and endocrine responses in conspecifics (for review, see, Rekwot et al., 2000). Pheromones can be classified as priming or signaling types. Signaling pheromones cause immediate, short-term, stimulation and response, this can be immediately followed by a period of non-stimulation and non-response. Priming pheromones stimulate a cascade of the neuroendocrine-endocrine events that generally have long-term effects on reproductive function and behavior (Izard, 1983).

The following is a summary of the manner by which pheromones are sensed by mammals and is based upon a review by Dulac (2000). The nasal cavity in mammals may be divided into two distinct regions, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The MOE is located on the posterior recesses of the nasal cavity and detects a large number of small volatile odorants which are processed in the cognitive centers of the brain. The VNO is a bilateral sensory structure that lines the ventral nasal septum and is itself lined with chemosensory neurons that detect pheromones. The VNO expresses neuron receptors that are produced from two unrelated families of genes, V1R genes and V2R genes. Each receptor gene is expressed on a distinct, spatially segregated population of neurons. The V1R receptors are located in the

apical half of the VNO and express 30 to 50 receptor genes. The V2R receptors are located on the basal half of the VNO and express approximately 100 genes. Interestingly, V2R receptors may vary between males and females.

Vomeronasal organ receptor neurons become activated by ligands, or pheromones, for specific, non-overlapping sets of neurons. Amazingly, this activation does not diminish with duration of stimulation or exposure to a specific ligand. The activation response of these neurons can be explained by quantifying ligand binding. Neuron stimulation from the VNO is thought to bypass the olfactory bulb and cognitive parts of the brain (Dulac, 2000). Instead, these specialized neurons probably stimulate a cascade of neuroendocrine responses independent of cognitive recognition. In human fetuses, VNO neurons are directly connected to neurons in the hypothalamus that secrete GnRH; however, as the fetus develops, this connection is severed (Takami, 2002). Currently, there is no report in the literature to establish this type of connection in the bovine. If such a connection is found, it would be the first evidence to suggest that VNO stimulation leads to direct responses in the hypothalamus in the bovine.

Pheromone Transport and Perception

Pheromone Transport

Pheromone communication between individuals is a complex system that relies upon the transport of semi-volatile compounds from an individual into the environment. The way in which transport occurs may be one of the most important keys to understanding the mechanism and control of pheromone action. Pheromones can be transported from individuals in feces, urine, and cutaneous gland excretions (for review,

see, Rekwot et al., 2000). Evidence indicates that bovine pheromones are present in feces and urine (Baruah and Kanchev, 1993; Joshi et al., 2002), or in cervical mucus (Wright et al., 1994). Pheromone transport has been studied extensively in mice and rats. A review of pheromone transport in mice and rats may give us insight into pheromonal communication of bovine.

There is extensive evidence to indicate that pheromones in mice and rats are transported from the blood to urine through the use of small transport proteins called lipocalins (for review, see, Flower, 1996; Cavaggioni and Mucignat-Caretta, 2000; for review, see, Flower et al., 2000; Achiraman and Archunan, 2002; Sharrow et al., 2002). Lipocalins are small proteins approximately equal to 19 kDa in size and are produced by metabolic processes in various tissues throughout the body (Flower, 1996). Lipocalins are a large group of proteins that vary in structure and function within and between species and fall within the calycin protein superfamily (Flower, 2000). Some functions of lipocalins include retinol transport, cryptic coloration, olfaction, enzymatic synthesis of prostaglandins, and pheromone transport. The lipocalins are classified and characterized by highly conserved sequence motifs. Kernel lipocalins share three sequence motifs while outlier lipocalins share only two common sequence motifs. Together these motifs form a highly conserved tertiary structure called the lipocalin fold. The lipocalin fold is characterized by eight anti-parallel β-sheets that are hydrogen-bonded to form a β-barrel with N and C terminal alpha-helices. At each turn of the β-barrel are small hairpin loops that connect the anti-parallel β-sheets. These hairpin loops fold together to form a lid over the ligand binding site inside the β-barrel. The size and amino-acid composition of this lid determines ligand specificity. Perhaps the most

interesting characteristic of lipocalins is their ability to bind a wide variety of small hydrophobic ligands. Ligands are thought to be bound to various binding sites on the protein and held together through the use of hydrogen bond networks (Scott et al., 2004). Two lipocalins have been identified as pheromone transport proteins; major urinary protein (MUP) and α-2u globulin (A2U; Flower, 1996; Cavaggioni and Mucignat-Caretta, 2000; Flower et al., 2000; Achiraman and Archunan, 2002; Sharrow et al., 2002).

Major urinary protein, a 17.8 kDa dimer, is produced mainly in the liver of mice (Flower, 1996). Major urinary protein production is stimulated by androgens, and males produce 5 to 20 times more MUP than females (Achiraman and Archunan, 2002). Major urinary protein has the ability to bind odorant molecules and may release pheromones from the urine into the environment as urine dries and the protein denatures (Flower, 1996; Cavaggioni and Mucignat-Caretta, 2000). A variety of polymorphic genes produce MUP in both males and females (Cavaggioni and Mucignat-Caretta, 2000). This genetic polymorphism causes a variety of MUP isoforms to be produced (Cavaggioni and Mucignat-Caretta, 2000). The endocrine status of the individual; male, orchiectomized male, female, ovariectomized female, or a female exhibiting estrus, controls gene transcription in the liver and determines which isoform is produced (Cavaggioni and Mucignat-Caretta, 2000). The affinity of MUP for specific pheromones also varies with the isoform being produced (Scott et al., 2002). One possible function of MUP is to bind pheromones in the blood and transport them to the urine (Cavaggioni and Mucignat-Caretta, 2000). Major urinary protein has been shown to have high affinity for odorants and is associated with a priming type pheromone action that accelerates the onset of puberty in female mice (Flower, 1996; Cavaggioni and Mucignat-Caretta, 2000). Major

urinary protein binds three ligands known to exhibit pheromone activity in mice; 2-(s-butyl) thiazoline, 2,3-dehydroexobrevicomin, and 4-(ethyl) phenol.

The other known pheromone transport vehicle is α-2u globulin (A2U). Alpha-2u globulin is a close homolog of MUP and comprises 30 to 50% of the total urinary protein excreted from the male rat. This protein is an 18.7 kDa dimer produced by the liver and other tissues. One interesting fact about A2U is that production is regulated by multiple hormones (Flower, 1996). Androgenic steroids increase A2U production with 5α-dihydrotestosterone being the most potent stimulator, whereas, estrogens inhibit the production of A2U (Flower, 1996).

The lipocalins, A2U produced in rats and MUP produced in mice, may give us insight into the type of pheromone transport that might occur in cattle. There has been very little study of pheromone transport in large ruminants. Nevertheless, there is some evidence for this phenomenon. A pheromone produced by the female elephant signals the male that she is exhibiting estrus (Lazar et al., 2004). Lazar et al. (2004) identified elephant serum albumin (ESA) as the pheromone transport protein. This study utilized a polyclonal antibody used for detecting mouse MUP. Interestingly, the researchers in this study found no evidence of MUP in elephant urine; instead they found that ESA binds a signaling type pheromone. At the time of estrus the female urine is at pH 8.4 and the pH of the vormernasal organ in the trunk of the male is 5.5. When the male sucks the urine into its trunk, ESA releases the pheromone, presumably as a result of the change in pH, to an odorant binding protein (OBP) on the nasal mucosa and the male detects that the female is in estrus. It is important to note that this pheromone is a signaling type

pheromone and may be extensively different from priming-type pheromone transport mechanisms.

Data reported by Joshi et al. (2002) indicates that pheromones are involved in bovine reproduction. Taken together with the aforementioned data, we can assume that bovine pheromone transport may involve a protein-pheromone interaction. The pheromone itself is probably a small semi-volatile hydrophobic compound produced by various tissues throughout the body, processed in the liver, and transported to the urine or feces by a lipocalin or serum albumin. Also, the transport mechanism is probably dependent on androgenic steroids; however, the pheromone itself could be dependent on or independent of androgenic steroids. For example, cells under the influence of androgens could produce a specific metabolite or product which is transported to the urine by the use of non-specific carriers such as serum albumin. On the other hand, cells under the influence of androgens could produce a protein that is a specific carrier of pheromones to the urine that would otherwise be destroyed as soon as unbound pheromone entered the blood. Therefore, the production of the pheromone(s) of interest may be independent of androgens but the transport mechanism of the pheromone to the urine could depend upon androgens or vice versa. Pheromones present in the urine could be released into the environment from carrier proteins through a number of mechanisms; 1) release by pH change from blood to urine, 2) release by protein denaturation after urine excretion, 3) release by pH change from urine to VNO, or 4) some other undefined mechanism. Clearly, pheromone transport is a very complex process involving the interaction of many distinct biochemical entities and processes.

Pheromone Perception

The next point of discussion is how pheromones are perceived or "made aware of" by conspecifics. In other words, "How do pheromones get from the environment to a site in the vomeronasal organ or the nasal epithelium where they can be sensed?" The ability of an organism to perceive and react to pheromonal cues is equally or more complex than the transport of pheromones from one organism to another.

Pheromones are small hydrophobic airborne chemicals released into the environment, and once released into the environment, are taken in through the olfactory/respiratory system. The mucosal epithelium of the bovine nasal cavity contains a small lipocalin protein called odorant binding protein (OBP). Odorants and pheromones are thought to be bound in the nasal cavity to OBP (Pevsner et al., 1985; Pevsner et al., 1986; Tirindelli et al., 1989; Pevsner et al., 1990; Bovdjelal et al., 1996; Flower, 1996; Bianchet et al., 1996; Tegoni et al., 1996; Pelosi, 2001). Odorant binding protein is found in the main olfactory epithelium and the vomeronasal organ of the bovine (Guiradie et al., 2003).

The first evidence of a soluble protein that binds odorants was presented by Pevsner et al. (1985) who isolated a 19 kDa dimer in the nasal mucosa that was termed an olfactory receptor protein for pyrazine. This pyrazine binder, later termed odorant binding protein, made up 2% of total nasal mucosa protein and was found to bind a variety of odorants (Pevsner et al., 1986). Tirindelli et al. (1989) determined the sequence of OBP and determined it was 159 amino acids long and part of the retinol binding protein family.

Pevsner et al. (1990) characterized ligand binding of OBP. They reported over 80 ligands for OBP and that OBP had no specific affinity for any single chemical class of binding molecules. Furthermore, they indicated that OBP exists as a dimer of two subunits, 19 kDa in size, and defined the binding kinetics of OBP to be negative cooperative. This means that as binding for one ligand increases, the ability for OBP to bind another ligand decreases. The evidence for negative cooperation supports the idea that OBP delivers ligands to receptor neurons. Furthermore, OBP has been shown to have multiple binding sites for small hydrophobic molecules (Bianchet et al., 1996; Tegoni et al., 1996). These data indicate that odorants and/or pheromones circulating in the air bind to and are delivered by OBP to receptor neurons of the main olfactory epithelium and vomernasal organ (Pevsner et al., 1990; Bianchet et al., 1996; Tegoni et al., 1996). However, for OBP to deliver odorants and pheromones to neurons there must be a membrane receptor associated with these neurons of the nasal cavity. Odorant binding protein is thought to bind to membrane receptors and unload or expose the receptor neurons to the odorant or pheromone. Boudjelal et al. (1996) tested this idea by measuring OBP binding in the nasal epithelium and in various tissues throughout the body. Interestingly, the researchers found a specific membrane receptor for OBP in respiratory tissue, hela cells, cos cells and skin fibroblasts, but not in ciliated olfactory epithelium. From this evidence the authors concluded that OBP does not deliver ligands such as odorants and pheromones to receptor neurons of the main olfactory epithelium and vomernasal organ. This conclusion is contrary to previous experimentation that explained the existence of OBP.

Perhaps the function of OBP can be clarified by an examination of odorant receptor neuron (ORN) transduction mechanisms. The following discussion is based on a review by Schild and Restrepo (1998). Odorant receptor neurons are very sensitive and can be stimulated by a single odorant molecule. Odorants pass through a mucous interface and bind to odorant receptors on neurons. The sensitivity of ORN can be explained by the utilization of second- and third-messenger cascades. In neurons of the main olfactory epithelium, odorants bind to odorant receptors, the receptors activate a G-protein-coupled process that results in an increase of adenosine 3'-5'-cyclic monophosphate (cAMP). However, activation of vomernasal neurons occurs through the production of cAMP without the use of a G-protein-coupled process. Instead, vomeronasal neurons could use nitric oxide or IP3 second-messenger activation pathways. The increase in cAMP causes the opening of cation exchange channels gated by cAMP. In physiological conditions Ca^{2+} has the highest permeability to this channel. Consequently, intracellular Ca^{2+} increases and causes the activation of Cl ion current which depolarizes the ORN. Also, the increase in intracellular Ca^{2+} causes a Ca^{2+} gated third messenger cascade. An increase in Ca^{2+} leads to the opening of Ca^{2+} regulated conductances and greatly amplifies action potentials. Action potentials then convey chemosensory information to the olfactory bulb from neurons in the main olfactory epithelium, while VNO neurons may bypass the olfactory bulb and directly stimulate other centers in the brain (Dulac, 2000).

The following system is a hypothesis of how the system may function in mammals based upon the discussion of the pheromone perception. Odorant binding protein or a similar small protein binds ligands or pheromones in the nasal cavity. After

binding occurs, OBP must deliver the pheromone and activate VNO receptor neurons. The mechanism of VNO activation is very sensitive (Schild and Restrepo, 1998) and could happen in a number of ways: 1) the receptor could recognize the pheromone-OBP complex, 2) the ligand could be transferred to the receptor with the assistance of OBP; and/or, 3) the ligand could be spontaneously freed (Pelosi, 2001). Freeing of ligands could occur by the binding of some other small hydrophobic molecule that displaces the ligand(s) present on OBP binding sites, or by changes in pH from the nasal cavity to the vomernasal organ. This displacement could then allow the interaction of the pheromone with its receptor neuron population. As a subset of neurons begin to activate, this causes a hypersensitivity of other neurons within a specific subpopulation. Vomernasal organ neurons have the ability to encode stimulus strength (Keverne, 1999); therefore, it is logical to assume a stimulation threshold must be reached for the entire subpopulation of neurons to reach action potential threshold and conduct a strong electrochemical signal to the brain. This signal could directly stimulate the hypothalamus to exhibit an appropriate neuroendocrine-endocrine response unique to the specific subpopulation of neurons stimulated in the vomernasal organ (Takami, 2002). It is likely that the activation of an entire subpopulation of neurons may be needed to cause a neuroendocrine response in the hypothalamus of the affected conspecific.

Pheromones in Bovine Reproductive Behavior

There is some evidence for the existence of female pheromones in bovine reproduction. Two specific compounds have been isolated in estural urine of cows, di-n-propyl phthalate and 1-iodoundecane could possibly be estrus specific compounds that

may act as signaling type pheromones for the bull to detect estrus (Kumar et al., 2000). In this study, researchers conducted a gas chromatograph profile from the urine of cows in estrus and compared the profile to the urine of cows not in estrus. The authors did not conduct a live animal experiment to confirm these estural specific compounds as signaling type pheromones. Indirect evidence for the existence of female bovine pheromones is that of a putative priming type involving a female-female interaction in postpartum cows. Wright et al. (1994) reported that exposing isolated postpartum cows to cervical mucous of estrual cows shortened the postpartum anestrous interval. Furthermore, cows exposed to their own excretory products for 12 h daily had shorter postpartum anestrous intervals than cows not exposed to their own excretory products (Joshi et al., 2002). These data support the existence of priming type female-female pheromones in the bovine.

There is some evidence that indicates that bulls produce pheromones. Baruah and Kanchev (1993) administered bull urine oronasally to dairy cows 7 d after calving and found that LH and FSH concentrations increased in the blood within 80 min after urine application. In this experiment, treatment was applied for one day on d 7 postpartum making it difficult to be certain whether this pheromone had a long-term effect. Additional evidence for bull to cow priming type pheromones was suggested by Joshi et al. (2002) who placed cows in small pens for 12 h each day where bulls had been the previous 12 h. They reported that cows exposed to excretory products of bulls in this manner had shorter postpartum anestrous intervals than cows not exposed to bulls.

The reduction in postpartum anestrous interval caused by cervical mucous of cows reported by Wright et al. (1994) could have been due to an increase in testosterone

concentration 7 d before estrus in cycling cows (Kanchev and Dobson 1976; Kesler et al., 1979) which may have caused the production of an androgen-dependent pheromone. Joshi et al. (2002) reported that the interval of postpartum anestrus was reduced in cows exposed to their own excretory products. These data indicate the possibility that a biostimulatory effect is dependent upon a threshold level of pheromone stimuli. In this experiment cows exposed to their own excretory products were forced to be in close proximity to each other 12 h daily while cows not exposed to excretory products were housed in larger pens. In both treatments some cows began to cycle, however, cows exposed to their own excretory products were forced to be in close proximity to cycling cows that may have produced an androgen-dependent pheromone present in their excretory products. When more and more cows began to cycle the pheromone signal may have become strong enough to result in biostimulation of anestrous pen mates. Cows housed in larger pens were not forced to be in close proximity to the androgen-dependent pheromone, thus biostimulation of anestrous pen mates did not occur. Additional evidence for an androgen-dependent bovine pheromone was illustrated by data that showed that testosterone-treated cows reduced the postpartum interval to resumption of ovarian cycling activity the same as the physical exposure to bulls (Burns and Spitzer, 1992). The observation that bull excretory products produce the same biostimulatory effect as the physical presence of bulls indicates the existence of an androgen-dependent bovine pheromone.

Summary

The length of postpartum anestrus in the bovine is controlled by the reproductive neuroendocrine-endocrine system. Resumption of ovarian cycling activity is characterized by the release of GnRH by the hypothalamus in low amplitude high frequency temporal release pattern. As a result, the anterior pituitary releases low amplitude, high frequency pulses of LH. This stimulates ovulation and resumption ovarian cycling activity.

Many factors act upon the hypothalamo-hypophyseal-ovarian axis and contribute to the length of postpartum anestrus. The most important factor is nutrition. Insufficient energy intake, before and/or after calving, prolongs postpartum anestrus. If nutrition is adequate, suckling stimuli is the next most influential factor. Cows suckling calves have longer postpartum anestrus than cows that are restricted-suckled or not suckled. Also, multiparous cows have shorter postpartum anestrous periods than primiparous cows, and other influential factors include breed, environmental stress, and dystocia.

The physical presence of bulls reduces the interval from calving to resumption of ovarian cycling activity in cows. The effect is termed biostimulation. The biostimulatory effect of bulls interacts with nutrition and could interact with suckling stimuli to reduce the duration of postpartum anestrus. The ability of the cow to respond to the biostimulatory effect of bulls increases with time postpartum. Clearly, long-term physical presence of bulls causes a biostimulatory effect. However, the exact timing and duration of bull exposure needed to elicit a biostimulatory effect are not known.

The exact mechanism of the biostimulatory effect of bulls is not clear. However, it is known that this effect is not dependent upon the development of a bull-cow social bond. The evidence indicates that the mechanism is mediated through a priming type pheromone(s). The pheromone(s) appears to be androgen-dependent and present in either the feces or urine of bulls. This pheromone(s) is released from these excretory products, where it probably acts upon the vomeronasal organ receptor neurons of the postpartum anestrous cow to stimulate a neuroendocrine-endocrine cascade that reduces the interval of anestrus in postpartum cows.

CHAPTER 3

STATEMENT OF PROBLEM

Prolonged postpartum anestrus is the major cause of cows failing to rebreed or breeding late in the breeding season. This is a particularly important problem in first-calf suckled cows because they require 15 to 25 d longer to return to estrus than multiparous cows. For this reason, extended postpartum anestrus can present a challenge for the successful implementation of modern estrous synchronization (ES) and artificial insemination technologies in primiparous beef cows.

Gonadotropin releasing hormone (GnRH)-based estrous synchronization (ES) protocols are more successful if postpartum cows have resumed cycling activity. Thus, the biostimulatory effect of bulls could be used to increase the proportion of cows that resume ovarian cycling activity before the breeding season and improve estrous synchronization (ES) response and AI pregnancy rates. There is preliminary evidence that indicates exposing cows to bulls may increase timed AI (TAI) pregnancy rates if bulls remained with cows throughout the ES protocol and for 5 d after TAI.

The objectives of Experiment 1 were to determine if short-term bull exposure of first-calf suckled beef cows increases the number of cows cycling before the breeding season and breeding performance of cows exposed before, during, and after an estrous synchronization protocol that included controlled intravaginal drug release (CIDR) device, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and GnRH. We tested the hypotheses that exposing first-calf suckled beef cows to bulls does not affect, 1) proportions of cows cycling before the start of an ES protocol, 2) estrous synchronization response, and 3) AI and overall

pregnancy rates among cows that received bull exposure before, during, and after ES+AI; bull exposure before but not during or after ES+AI; no bull exposure before, during, and after ES+AI; or bull exposure during and after but not before ES+AI.

Little is known of the biological mechanism by which the biostimulatory effect of bulls initiates resumption of ovarian activity in postpartum anestrous females. The biostimulatory effect of bulls may be mediated through the excretory products of bulls. It appears that bulls excrete a pheromone into the urine, feces or cutaneous glands that may initiate a neuroendocrine-endocrine cascade which results in the resumption of ovarian cycling activity.

The question asked in Experiment 2 was, "Does bull urine cause the same biostimulatory effect as the physical presence of bulls?" The objectives of this experiment were to determine if bull urine exposure alters the resumption of ovarian cycling activity before the breeding season and breeding performance of first-calf beef cows. We tested the hypotheses that: 1) the interval from exposure to resumption of cycling activity; 2) proportion of cows cycling before the breeding season; 3) response to estrous synchronization, and 4) AI pregnancy rates, did not differ between cows exposed to mature bull urine or cows exposed to steer urine.

CHAPTER 4

MATERIALS AND METHODS

Experiment 1

Animals and Treatments

Fifty-three spring-calving two-yr-old first-calf suckled Angus Hereford crossbred beef cows and four mature, epididymectomized Angus x Hereford bulls were used in this experiment conducted at the Montana State University Livestock Teaching and Research Center, Bozeman. Animal care, handling, and protocols used in this experiment were approved by the Montana State University Institutional Animal Care and Use Committee.

Cows and calves were maintained in a single pasture and had no contact with bulls or their excretory products during pregnancy and from calving until the start of the experiment. Average calving date for these cows was Feb. 16. At the start of the experiment (d -40) cows averaged 63 d (± 14 d; ± SE) postpartum; d 0 of the experiment was day of TAI. One week before the start of treatment cows were stratified by calving date, cow BW, calf birth weight, calf sex, dystocia score, and BCS. Once cows were stratified they were assigned randomly to one of two treatments; exposure to mature bulls (BE; n = 26) or no bull exposure (NE; n = 27) from the start of the experiment until the start of ES. At the start of the ES protocol (d -10) one-half of the BE cows were assigned randomly to no bull exposure (BENE; n = 13) and one-half of the NE cows were assigned randomly to bull exposure (NEBE; n = 13). Cows remained in treatments from d -10 until 8 d after breeding when cows in all treatments were combined and maintained in a

single pasture. Figure 1 shows the design of the experiment, the number of animals per treatment, and the protocols used in this experiment.

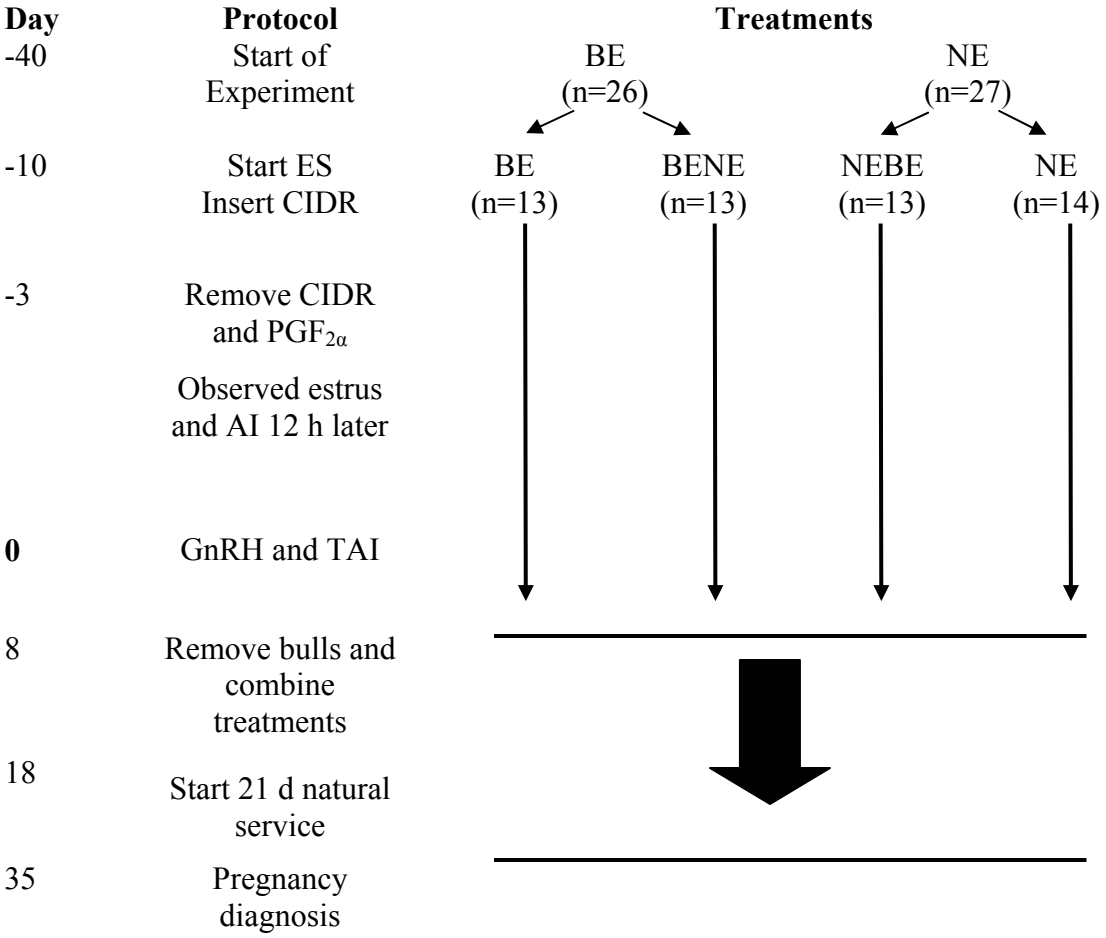


Figure 1. Experimental design, number of animals per treatment, and protocols. BE = bull exposed, NE = no bull exposure.

Lot Areas

Two lots were used for this experiment, designated north and south by their geographic location. Each lot contained four pens (41 m x 18 m) that were identical in east-west configuration, bunk space, aspect, slope, and connection to open-shed shelters.

Lots were approximately 0.35 km apart, and the arrangement was such that the prevailing wind blew from south to north. Animals housed in one lot were not able to see or smell animals housed in the other lot; however, there was a possibility that sounds made by animals in one lot could be heard by animals in the other lot. These lots and arrangement have proven to be effective in previous experiments involving bull-cow interactions (Fernandez et al., 1993; 1996). Pens in the south lot had not held bulls for more than six years. Pens in the north lot had not held bulls for ten months.

Nutrition

Cows had free access to good quality, chopped mixed-grass alfalfa hay, and any pasture grasses that were available before the start of the experiment. Once cows and calves were moved into pens they were given free access to the same hay, 0.5 kg/hd/d cracked barley, water, and a trace mineral-salt supplement. The TDN of the diet exceeded the NRC requirement for lactating beef cows with a mature weight of 545 kg by approximately 18% (NRC, 1996). Bulls were fed the same ration as cows.

Blood Sampling for Progesterone

Blood samples were collected from each cow by jugular venepuncture at 3-d intervals from the start of the experiment to the start of the breeding season. Serum was assayed for progesterone concentration using solid-phase RIA kit (Diagnostic Systems Laboratories Inc., Webster, TX), we validated this assay for bovine serum. Intra- and interassay CV's for a serum pool that contained 0.8 ng/mL were 4.8 and 11%, respectively; and for a pool that contained 7.0 ng/mL were 20 and 5.4%, respectively. Progesterone concentrations patterns were used to assess the resumption of ovarian

cycling activity. A rise in progesterone concentration of > 0.5 ng/ml in three consecutive samples that exceeded 1 ng/mL provided evidence that cows resumed ovarian cycling activity during the experimental period.

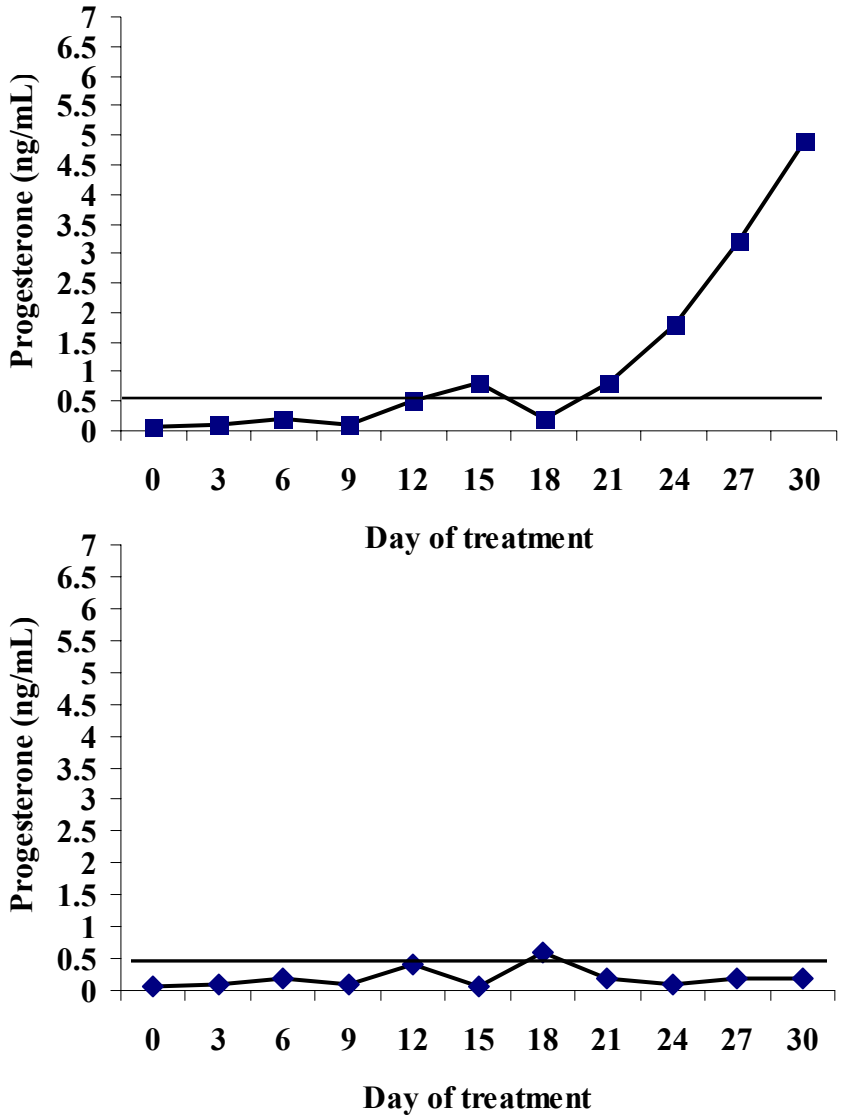


Figure 2. Pattern of progesterone concentrations used to determine occurrence of resumption of ovarian cycling activity. Panel A represents a cow that resumed cycling activity. Panel B represents a cow that did not resume ovarian cycling activity.

Graphic representation of the pattern of progesterone concentrations used to determine the occurrence of resumption of ovarian cycling activity is illustrated in Figure 2.

Estrous Synchronization, AI and Pregnancy Diagnosis

Thirty days after the start of the experiment, each cow was given exogenous progesterone via a controlled intravaginal drug release (CIDR) device (Figure 1; d -10). Seven days later CIDR's were removed and cows were given $PGF_{2\alpha}$ (25 mg/hd) intramuscularly. Cows were visually observed for estrus thrice daily after $PGF_{2\alpha}$ injection (0730, 1200, and 1800 h). Cows that exhibited estrus within 60 h after $PGF_{2\alpha}$ injection were bred by AI 12 h later. Cows that did not exhibit estrus by 60 h were given GnRH (100 μg/hd) intramuscularly, and bred by time AI 72 h after CIDR removal. Artificial insemination was accomplished using semen from one bull and a single AI technician. Cows were exposed to natural service 18 d later for 21 d. Pregnancy was determined by transrectal ultrasonography of the uterine contents of each cow 35 and 107 d after timed AI.

Statistical Analyses

Comparisons of calving date, calf birth weight, calf sex ratio, dystocia score, and body condition score were analyzed by analysis of variance for a completely random design using PROC GLM of SAS (SAS, Cary, NC). The model included treatment. Means were separated by PDIFF procedure of SAS. Proportions of cows that were cycling at the start of the experiment, resumed ovarian cycling before the start of estrous

synchronization, exhibited estrus after $PGF_{2\alpha}$ injection, and were pregnant to AI were analyzed by chi-square analyses using the PROC FREQ of SAS

Experiment 2

Animals and Treatments

Thirty-eight two-yr-old Angus X Hereford first-calf suckled beef cows, four Angus x Hereford epididymectomized bulls, and four 1-yr-old Angus Hereford crossbred steers were used in this experiment conducted at the Montana State University Livestock Teaching and Research Center, Bozeman. Animal care, handling, and protocols used in this experiment were approved by the Montana State University Institutional Animal Care and Use Committee.

Cows and calves had no contact with bulls or their excretory products from calving until the start of treatment. Average calving date was Feb. 9. Two wk before the cows were exposed to urine they were stratified by calving date, cow BW, calf BW, calf sex, dystocia score, and BCS and fitted with a controlled urine delivery device (CUDD). Cows were assigned randomly to either exposure to steer urine (SUE) or exposure to bull urine (BUE). The average length of exposure time before the start of estrous synchronization was 64 days.

Lot Areas

Two lots were used for this experiment, designated north and south by their geographic location. Each lot contained four pens (41 m x 18 m) that were identical in east-west configuration, bunk space, aspect, slope, and connection to open-shed shelters.

Lots were approximately 0.35 km apart, and the arrangement was such that the prevailing wind blew from south to north. Animals housed in one lot were not able to see or smell animals housed in the other lot; however, there was a possibility that sounds made by animals in one lot could be heard by animals in the other lot. These lots and arrangement have proven to be effective in previous experiments involving bull-cow interactions (Fernandez et al., 1993; 1996). Pens in the south lot had not held bulls for more than seven years. Pens in the north lot had not held bulls for ten months. Cows exposed to bull urine (BUE) were housed in the two eastern-most pens the north lot and cows exposed to steer urine (SUE) were housed in the two western-most pens of the south lot.

Controlled Urine Delivery Device (CUDD)

Continuous exposure to urine was accomplished through the use of a Controlled Urine Deliver Device (CUDD). A CUDD consisted of a neoprene bag, 30 cm long, 17.5 cm wide, with an inner pouch and outer nylon fenestrated barrier, and a closure-flap which spanned from the inner pouch to the outer fenestrated barrier. The bottom of the bag was pleated, allowing for the expansion and contraction of the bag contents. The inner pouch contained a human urinary leg bag (Hollister Incorporated, Libertyville, IL) which held 750 mL of liquid and served as a "reservoir" for urine. The outer, fenestrated barrier contained a chemical resistant cellulose sponge, 17 cm x 10.8 cm x 4.5 cm (L x W x D). A lumen was made along the entire length of the middle of the sponge to accommodate a urine flow-control valve. Flow-control valves were approximately 12.5 cm long and 1.25 cm in diameter, and made of polypropylene tubing. A flow-control valve was fit into the lumen of each sponge and connected to the reservoir by a 0.953 x

0.635 cm elbow shaped hose barb made of nylon. The elbow was placed through the neoprene dividing the inner and outer pouches at the opposite end of the flap. The barbed end of an elbow was pressure fitted into the reservoir, and the threaded end of the elbow was connected to the flow-control valve using a small hose clamp. A # 00 rubber stopper capped the end of each valve distal to the elbow. A small outlet hole was made in each control valve with a 23 ga. needle, 2 cm from the capped ends, to allow urine to flow from the valves and soak the sponges. The lumen of each valve was packed with 3 g of polyester fiber above the elbow to a distance of 7.5 cm. This was necessary to regulate the flow of urine from the reservoir into the sponge for an extended period of time. The polyester fiber was then packed tightly to a distance of 5 cm below the outlet hole and packed loosely for 2.5 cm above the elbow. Urine flowed from the reservoir, through the elbow, into the valve, out of the 23 ga. outlet hole, and soaked the sponge in the outer fenestrated barrier of the CUDD. Laboratory tests, using distilled water as the media, confirmed that fluid flowed from the reservoir and into the sponge, keeping the sponge moist for a period of 3 d. Construction details for a CUDD are illustrated in Appendix A.

CUDD Attachment

Two wk before the start of treatment, each cow was fitted with a 7.5 cm dia. ring placed through the loose skin in the mid-line of the dulap, 7.5 cm anterior to the sternum. The ring was plastic-coated steel cable, 5 mm in dia. The loose skin and tissue next to the brisket were shaved, and 5 to 7 mL of lidocaine hydrochloride was injected s.c. to desensitize the area for cable insertion. A 1 cm long incision was made through the skin and a trochar was inserted into the incision and through the soft tissue and out the

opposite side of the dulap. A 9 mm cannula was fitted through the lumen created by the trochar. The cable was then inserted through the center of the cannula and the cannula was removed from the dulap leaving the piece of cable extended on each side of the dulap. The cable ends were then crimped together to form a ring. This ring served as a point of attachment for the ventral mount of each CUDD. At the start of treatment, CUDD were attached to each cow using an elastic band (5 cm wide) attached to the dorsal end of the CUDD near the flap and tied around the neck of the cow; the ventral end of the CUDD was attached to the ring, using 7.5 cm-long plastic zip-ties. The elastic strap allowed the CUDD to move laterally on each side of a cows' neck with the bottom of the bag firmly attached to brisket ring keeping the bag parallel to the anterior aspect of the neck and sternum. Details of the CUDD attachment procedure are illustrated in Appendix B.

CUDD Filling

Urine was collected in 2 L i.v. bags. Each collection bag was sealed and placed on ice overnight in a cold room (4º C). A white precipitant formed in each bag during storage. Urine from the collection bag was transferred into a clean i.v. bag to prevent clogging of the urine flow-control valve by the precipitant. Transfer bags were suspended 2 m in the air. A 2.5 m length of latex tubing was fitted to the effluent opening of the transfer bag and closed with a plastic hose clamp. Each cow was then restrained in a standard chute/head-catch system. Once the cow was in position, the CUDD closure-flap was opened, exposing the urine reservoir. The latex tubing was then attached to the CUDD affluent opening of the urine reservoir. Urine flowed from the

collection bag through the latex tubing and into the reservoir of the CUDD filled with approximately 750mL of urine. After the reservoir was filled to capacity the CUDD flap was closed and the filling procedure was complete. Collection and transfer bags and all latex tubing were emptied of any remaining urine and rinsed three times in hot tap water followed by two rinses in distilled water. Bags and tubing for bull urine and steer urine were washed, rinsed, dried, and stored at two separate locations to prevent cross-contamination. This procedure was repeated every three days throughout the experiment. At the time of filling, each CUDD was inspected for non-functioning parts for repair. Details for the filling procedure are illustrated in Appendix C.

Urine Collection

Urine was collected from four mature, epididymectomized Angus x Hereford bulls and four 1-yr-old crossbred steers beginning 1 d before the start of treatment and every 3 d thereafter until the end of treatment. Bull and steer urine was collected within the same facility but on different days. Collection periods varied from 4 to 8 h on any given day for bulls and steers, dependent upon the quantity of urine needed to fill the reservoirs of CUDD and the metabolism of the males to excrete urine. These collections provided the urine necessary to fill CUDD every third day throughout the treatment period. After each collection period the facility was thoroughly washed with soap and a high-pressure washer system.

Urine Collection Facilities and Urine Handling

The urine collection facility consisted of four stalls raised 26 cm off a cement floor. Raising the stalls was accomplished by placing the stalls on top of two railroad ties for each stall. The railroad ties measured 2 m x 24 cm x 26 cm (L x W x D). Each stall was 1 m wide by 1.5 m long and 1.15 m tall. Each bull or steer had the ability to protrude their head and neck out the front of the stall; this allowed them to eat and drink while urine was collected. A watering and feeding trough was placed in front of the stall that ran the entire length of the four stalls. At the start of each collection, the trough was filled with grain and hay. After the bulls or steers consumed their allotment of feed, water was allowed to flow continuously through the trough.

The stall floors consisted of two planks 25 cm wide and 5 cm thick placed at the front and back of the stall to support the weight of the bull or steer. The planks were arranged so that no planks were in a position directly under the prepuce of the animal. Plastic coated, 1.25 cm thick grating was placed on top of, and secured to, the planks. Urine collection pans, 75 cm x 62 cm, were then placed directly beneath the plastic grate under the prepuce of the animals. The collection pans, polyethylene waste container lids, were held in place by two pieces of angle iron attached to the side of the railroad ties underneath the stall floor. A separate set of pans was used for collecting bull and steer urine. The pieces of angle iron were arranged in such a way that insertion and extraction of collection pans was time and labor efficient, without placing undo stress on the animals. Also, one piece of angle iron was placed 3 cm lower than the other, causing the urine to pool on one side and edge of the pan. A 2.75 cm hole was drilled into the pan at the side where pooling occurred. A polyethylene connector, 2.5 cm in dia., was glued

inside the hole. A faucet screen was then placed into the lumen of the connector to hinder the flow of dirt and hay into a piece of latex tubing, 1.5 m long, connected to the other side of the spout. A 2 L i.v. bag, or collection bag was then connected to the opposite end of the tubing.

The urine flowed from the prepuce of the bull or steer, through the plastic grated floor into the urine collection pan, screen, connector, and latex tubing into the collection bag. When the collection bags were filled with urine they were replaced with empty collection bags. Each full collection bag was then placed on ice in a chest cooler and stored in a cold room at 4º C until the next day when CUDD were required to be filled. Details of the collection facility and method of collection are illustrated in Appendix D.

Nutrition

Cows had free access to good quality, chopped mixed-grass alfalfa hay, and any pasture grasses that were available before the start of the experiment. Once cows and calves were moved into pens, they were given free access to the same hay, 0.5 kg/hd/d cracked barley, water, and a trace mineral-salt supplement. The TDN of the diet exceeded the NRC requirement for lactating beef cows with a mature weight of 545 kg by approximately 18% (NRC, 1996). Bulls had ad libitum access to fair quality, chopped barley hay. During collection periods, bulls were fed 0.5 kg of cracked barely and good quality, chopped mixed-grass alfalfa hay. Steers were fed a finishing ration that consisted of 70% concentrate and 30% roughage throughout the experiment.

Blood Sampling for Progesterone

Blood samples were collected from each cow by jugular venepuncture at 3-d intervals from the start of the experiment to the start of the breeding season. Serum was assayed for progesterone concentration using a solid-phase RIA kits (Diagnostic Products Corp., Los Angeles, CA) validated for bovine serum in our laboratory (Custer et al., 1990). Intra- and interassay CV for a serum pool that contained 2.55 ng/mL were 0.4 and 7.4%, respectively; and for a pool that contained 7.49 ng/mL were 11 and 3.4%, respectively. Progesterone concentrations patterns were used to assess the resumption of ovarian cycling activity. As in experiment 1, a rise in progesterone concentration of > 0.5 ng/ml in three consecutive samples that exceeded 1 ng/mL was used as the criteria to determine the occurrence of resumption of ovarian cycling activity.

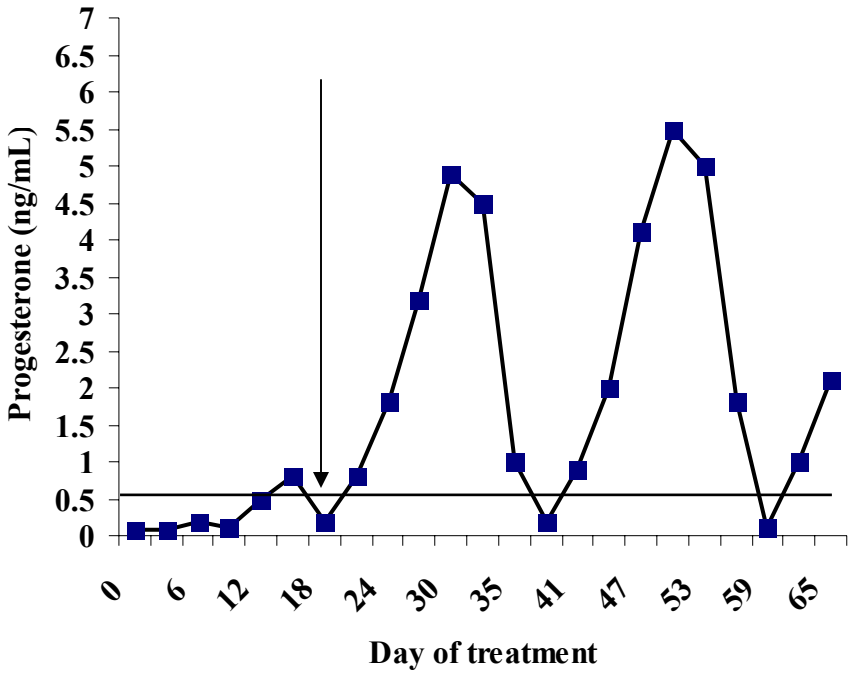


Figure 3. Progesterone pattern used to determine the interval from the start of treatment to resumption of ovarian cycling activity. Arrow indicates the lowest inflection point before a rise in three consecutive samples of > 0.5 ng/mL that exceeded 1 ng/mL.

Intervals from the start of treatment to resumption of ovarian cycling activity were determined by changes in the temporal release of progesterone. The day of the lowest inflection point before a rise in progesterone concentration over a nine day period was determined to be the day of resumption of ovarian cycling activity. The number of days from the start of treatment to the lowest inflection point was the interval from the start of treatment to resumption of ovarian cycling activity. Cows not showing a rise in progesterone over three consecutive blood samples were assigned an interval from the start of treatment to the end of treatment.

Estrous Synchronization, AI and Pregnancy Diagnosis

Each cow was given exogenous progesterone via a controlled intravaginal drug release (CIDR) device starting on May 18 (d -10). Seven d later (d -3) CUDD and CIDR were removed and cows were given $PGF_{2\alpha}$ (25 mg/hd) intramuscularly. Cows were visually observed for estrus thrice daily after $PGF_{2\alpha}$ injection (0730, 1200, 1800 h). Cows that exhibited estrus within 60 h after $PGF_{2\alpha}$ injection were bred by AI 12 h later. Cows that did not exhibit estrus by 60 h were given GnRH (100 μg/hd) intramuscularly, and bred by AI 72 h after CIDR removal (d 0). Artificial insemination was accomplished using semen from a proven bull and a single AI technician. Cows were exposed to natural service bulls 18 d later for 21 d. Pregnancy was determined by transrectal ultrasonography of the uterine contents of each cow 35 d after timed AI.

Statistical Analyses

Comparisons of calving date, cow BW, calf birth weight, calf sex ratio, dystocia score, and BCS were analyzed by separate ANOVA for a completely randomized design

using PROC GLM of SAS (SAS Inst. Inc., Cary, NC). The model included treatment. Means were separated by the PDIFF procedure of SAS. Interval from the start of treatment to resumption of ovarian cycling activity was analyzed by ANOVA for a completely randomized design using PROC GLM of SAS. Proportion of cows cycling at the start of estrous synchronization, proportion of cows that exhibited estrus after $PGF_{2\alpha}$ injection, and AI pregnancy rates were analyzed by chi-square analyses using the PROC FREQ of SAS.

CHAPTER 5

RESULTS

Experiment 1

Average calving date, cow BW, calf birth weight, calf sex ratio, dystocia score, and BCS did not differ between BE and NE cows (Table 1).

Table 1. Number of cows per treatment and least square means for calving date, cow BW, calf BW, BCS, calf sex ratio, and dystocia score for first calf suckled beef cows exposed (BE) or not exposed (NE) to bulls at the start of the experiment

	Treatment			
Variable	BE	NE	SEM[a]	*P* value
n	26	26		
Calving date[b]	47	46	14.37	0.93
Cow BW (kg)	518	509	41.72	0.40
Calf BW (kg)	37	37	3.89	0.66
BCS	4.7	4.8	1.11	0.98
Calf sex ratio[c]	0.53	0.59	0.49	0.96
Dystocia score[d]	1.08	1.15	0.39	0.91

[a]SEM = Standard error for means.
[b]Day of year.
[c]Calf sex ratio = ratio of male to female calves.
[d]Dystocia Score: 0 = No assistance to 5 = Caesarean section.

Proportions of BE (50%) and NE (44%) cows cycling at the start of the experiment did not differ ($P = 0.68$; Figure 2). However, a greater proportion ($P < 0.05$) of cows exposed to bulls (BE; 100%) were cycling by the end of the 30-d exposure period than cows not exposed to bulls (NE; 70.4%; Figure 2).

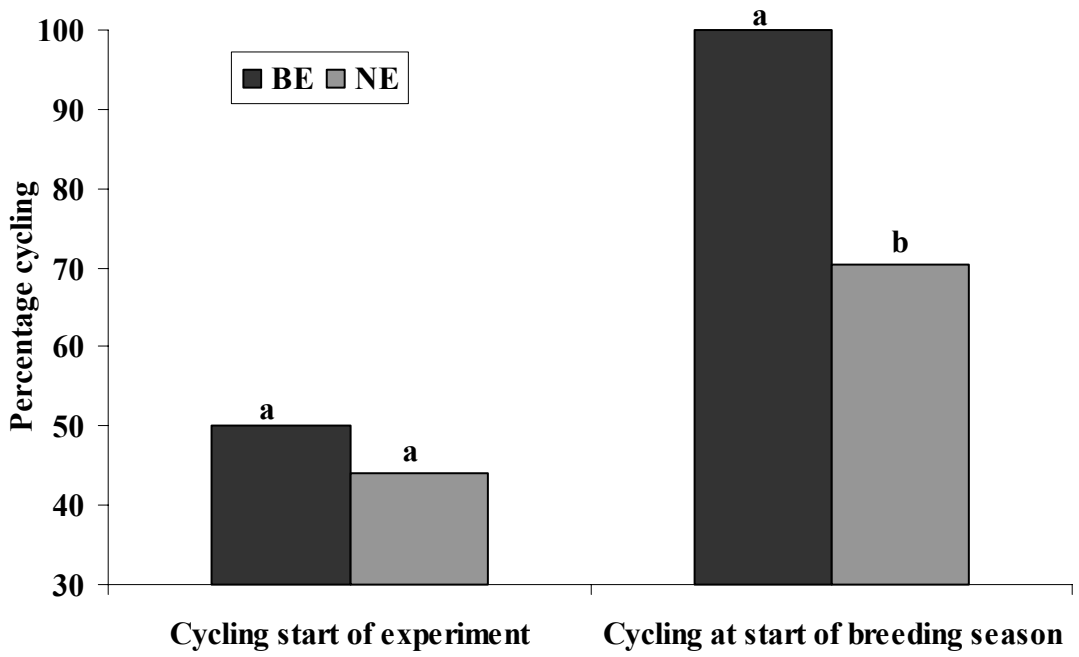


Figure 4. Percentages of first-calf suckled cows exposed (BE) or not exposed (NE) to bulls that were cycling at the start of the experiment and cycling after the 30-d exposure period. Bars that lack common letters within each cycling classification differ, $P < 0.05$; cycling start of experiment, $X^2 = 1.02$, d.f. = 1; cycling at start of breeding season, $X^2 = 9.1$, d.f. = 1.

Proportion of cows that exhibited estrus by 60 h after $PGF_{2\alpha}$ did not differ ($P = 0.53$) among BE, BENE, NE, and NEBE cows (30%, 54%, 36%, and 42%, respectively). Data were pooled for cows exposed to bulls (BE and BENE) or not exposed to bulls (NE and NEBE) before the start of the breeding season. The proportion of BE and BENE cows that exhibited estrus did not differ ($P = 0.50$) from that of NE and NEBE cows (Table 2). The proportions of BE and BENE cows and NE and NEBE that were cycling at the start of estrous synchronization (ES) that exhibited estrus by 60 h after $PGF_{2\alpha}$ did not differ ($P = 0.98$). There were no anestrous cows in the BE and BENE treatments at

the start of ES. The proportion of anestrous cows in the NE and NEBE treatments was 37.5%.

Table 2. Number of animals per treatment and percentage of cows exhibiting estrus by 60 h after $PGF_{2\alpha,}$ timed AI (TAI), AI, and overall pregnancy rates for first-calf suckled beef cows exposed to bulls (BE and BENE) or not exposed to bulls (NE and NEBE) before the start of the estrous synchronization protocol (ES)

	Treatment			
Variable	BE and BENE	NE and NEBE[a]	X^2	*P* value
n	26	25		
% in estrus by 60 h after $PGF_{2\alpha}$	42.3%	36.0%	0.21	0.64
Estrus AI pregnancy rate	100%	66.7%	4.3	< 0.05
TAI pregnancy rate	73.3% [c]	50.0% [c]	1.78	0.18
AI pregnancy rates[b]	84.6% [c]	56.0% [d]	5.03	< 0.05
Overall pregnancy rates	88.5% [c]	92.3% [c]	0.22	0.63

[a]Data for two cows in this treatment were not included in this analysis due to medical treatment during the ES protocol.

[b]AI pregnancy rates equal cows bred 12 h after estrus and cows bred at TAI.

[c,d]Percentages within rows that lack a common superscript differ.

Artificial insemination pregnancy rates among BE, BENE, NE, and NEBE cows did not differ (*P* = 0.12; 76.9%, 92.3%, 53.9%, and 58.3%, respectively). Overall breeding season pregnancy rates among BE, BENE, NE, and NEBE cows did not differ (*P* = 0.13; 76.9%, 100%, 85.7%, and 100%, respectively). Therefore, AI and overall breeding season pregnancy rate data were pooled for cows exposed to bulls (BE and BENE) or not exposed to bulls (NE and NEBE) before the start of the ES protocol. Timed AI pregnancy rates did not differ (*P* = 0.18) between cows exposed to bulls (BE

and BENE) or not exposed to bulls (NE and NEBE) before the start of the ES protocol (Table 2). Whereas, AI pregnancy rate (cows bred 12 h after estrus and cows bred at TAI) was greater ($P < 0.05$) for cows exposed to bulls (BE and BENE) than that of cows not exposed to bulls (NE and NEBE) before the ES protocol (Table 2). Overall breeding season pregnancy rates did not differ ($P = 0.63$) between cows exposed to bulls (BE and BENE) and cows not exposed to bulls (NE and NEBE) before the ES protocol (Table 2).

Experiment 2

Calving date, cow body weight, calf birth weight, calf sex ratio, dystocia score, and body condition score did not differ between cows exposed to bull urine (BUE) and cows exposed to steer urine (SUE) (Table 3).

Cow BW and BCS change from the start of treatment until after ES did not differ ($P = 0.22$, and 0.98, respectively) between BUE and SUE cows. There was no difference ($P = 0.17$) in the intervals from treatment to the resumption of ovarian cycling activity between BUE and SUE cows (Table 3). There was no difference in the interval from the start of treatment to resumption of ovarian cycling activity between BUE or SUE cows ($P = 0.40$ and $P = 0.75$ respectively).

Table 3. Number of cows per treatment and least square means for calving date, cow BW at the start of treatment, cow BW change, calf BW at the start of treatment, BCS, BCS change, calf sex ratio, dystocia score and interval from exposure to resumption of cycling activity for first-calf suckled beef cows exposed to bull urine (BUE) or exposed to steer urine (SUE)

	Treatment			
Variable	BUE	SUE	SEM[a]	*P* value
n	19	19		
Calving Date[b]	39	39	9.22	0.98
Cow BW (kg)	557	550	40.9	0.59
Cow BW Change[c]	-37.2	-22.3	21.5	0.22
Calf BW (kg)	35	39	9.1	0.92
BCS	5.1	5.2	0.34	0.60
BCS Change[c]	-0.04	-0.04	0.35	0.98
Calf sex ratio[d]	0.47	0.60	0.51	0.44
Dystocia Score[e]	1.00	1.05	0.16	0.34
Interval (d)[f]	62.5	55.8	14.6	0.17

[a]SEM = Standard error for means.

[b]Day of year.

[c]Cow BW and BCS change are differences from the start of treatment to the end of treatment.

[d]Calf sex ratio = ratio of male to female calves.

[e]Dystocia Score: 0 = No assistance to 5 = Caesarean section.

[f]Days from the start of exposure to resumption of cycling activity.

There was no difference ($P = 0.25$) in the proportion of cows cycling by the end of the exposure period between BUE and SUE cows (Figure 5).

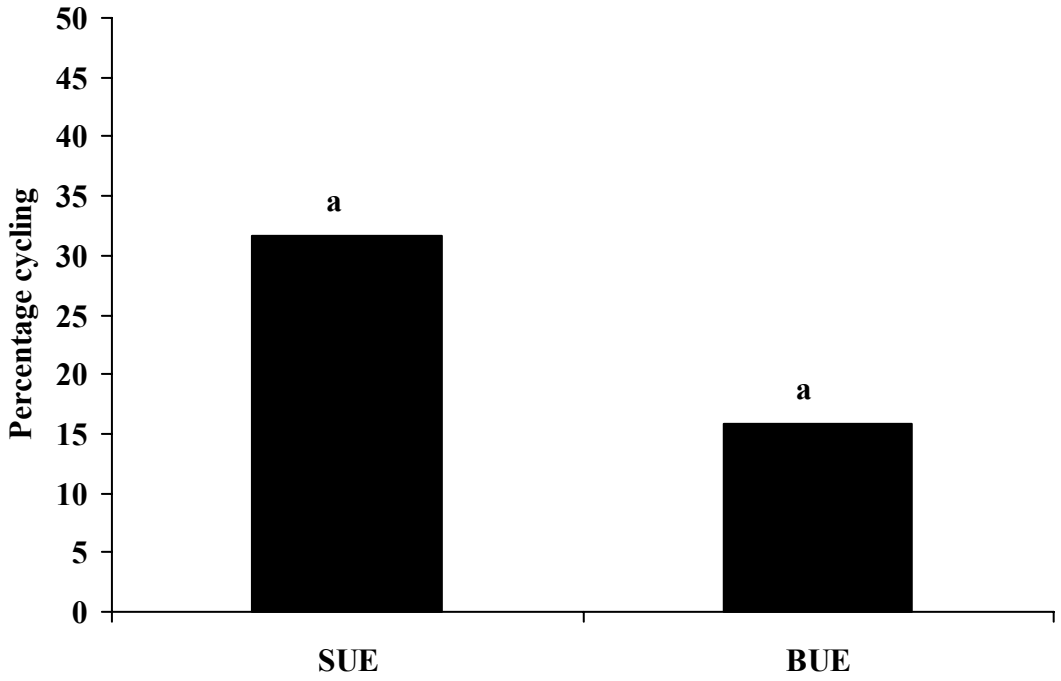


Figure 5. Percentages of first-calf suckled cows exposed to mature bull urine (BUE) or exposed to steer urine (SUE) that resumed ovarian cycling activity by the beginning of the estrus synchronization protocol. Bars with common letters do not differ, $P = 0.25$, $X^2 = 1.3$, d.f. = 1.

Proportions of cycling and anestrous cows that exhibited estrus by 60 h after $PGF_{2\alpha}$ did not differ ($P = 0.34$ and 0.24, respectively) between cows BUE and SUE cows (Table 4). Therefore, data were pooled for cycling and anestrous cows that exhibited estrus by 60 h after $PGF_{2\alpha}$ for cows BUE and SUE cows. The proportion of cows that exhibited estrus by 60 h after $PGF_{2\alpha}$ did not differ ($P = 0.09$; Table 4) between BUE and SUE cows.

Table 4. Number of animals per treatment and percentage of cows exhibiting estrus by 60 h after $PGF_{2\alpha,}$ TAI, AI, and overall pregnancy rates for first-calf suckled beef cows exposed to mature bull urine (BUE) or exposed to steer (SUE) before the start of the estrous synchronization protocol

	Treatment			
Variable	BUE	SUE	X^2	*P* value
n	19	19		
% in estrus by 60 h after $PGF_{2\alpha}$	80.0%[b]	52.6%[b]	2.9	0.09
TAI pregnancy rate	100%[b]	66.7%[b]	1.7	0.19
AI pregnancy rates[a]	89.5%[b]	57.9%[c]	4.9	< 0.05
Overall pregnancy rates[d]	100%[b]	89.5%[b]	2.1	0.15

[a]AI pregnancy rates equal pregnancy rates for cows bred 12 h after estrus and cows bred at TAI determined at 35 d after TAI.

[b,c]Percentages in rows that lack a common superscript differ.

[d]Overall pregnancy rates = AI and natural service over 35 d period at 107 d after TAI.

Timed AI pregnancy rates did not differ ($P = 0.19$) BUE and SUE cows. Therefore, data for cows bred by TAI and cows bred 12 h after estrus were pooled within treatments for overall AI pregnancy rates. A greater proportion ($P < 0.05$) of BUE cows than SUE cows were pregnant from AI (Table 4). Overall pregnancy rates did not differ ($P = 0.15$) between BUE and SUE cows (Table 4).

AI Pregnancy Rates for Experiment 1 and Experiment 2 Combined

There was no interaction for AI pregnancy rates between Experiment 1 and Experiment 2 for cows exposed to bulls and bull urine (BE + BUE) or cows not exposed to bulls or bull urine (NE + SUE). Therefore AI pregnancy rate data for Experiment 1

and Experiment 2 were pooled. Artificial insemination pregnancy rate for BE + BUE was greater ($P < 0.05$) than that for NE + SUE cows (Figure 4).

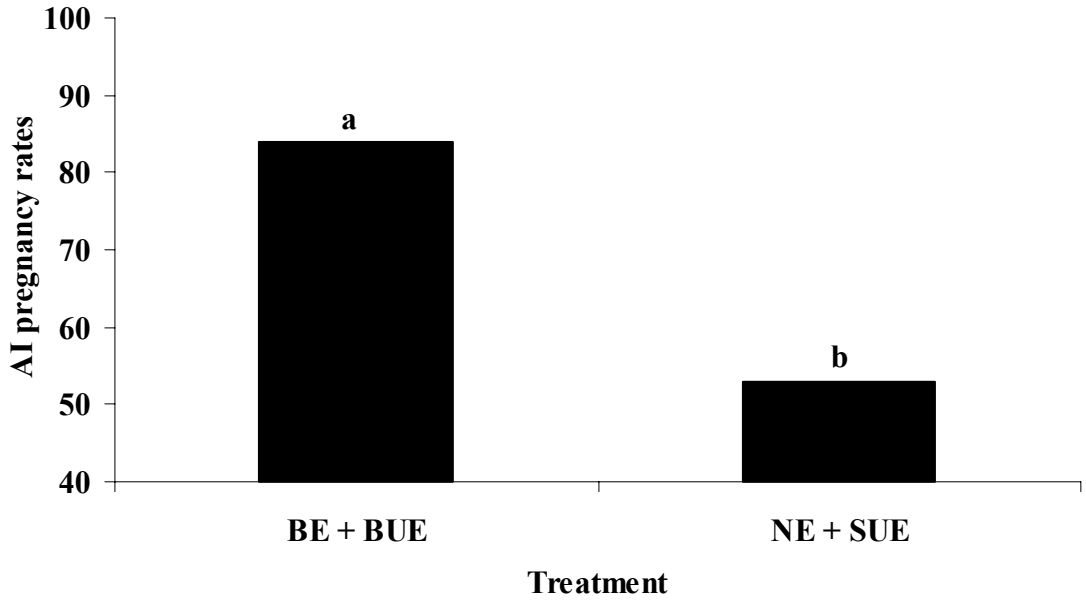


Figure 6. AI pregnancy rates of cows exposed to bulls or bull urine (BE + BUE) or not exposed to bulls or bull urine(NE + SUE). Bars that lack common letters differ, $P < 0.05$; $X^2 = 10.4$, d.f. = 1.

CHAPTER 6

DISCUSSION

Experiment 1

Long-term bull exposure (> 60 d) reduces the postpartum interval to resumption of ovarian cycling activity in first-calf suckled beef cows (Custer et al., 1990, Fernandez et al., 1993). The first objective of our experiment was to determine if short-term bull exposure (30 d) increased the number of cows cycling before the start of the breeding season. In this experiment cows exposed to mature bulls for 30 d before an ES increased the proportion of that started to cycle by 24% relative to cows that were not exposed to bulls before ES. Macmillan et al. (1979) reported a greater proportion of cows exposed to bulls than cows not exposed to bulls for 21 d before the breeding season showed estrus during the 60 d breeding season. Similarly, Scott and Montgomery (1987) reported that more cows exposed to bulls for 20 d before the breeding season began cycling than cows not exposed to bulls. These results indicate that short-term bull exposure has the same effect as long-term exposure. Short-term bull exposure may be an effective strategy to increase the proportion of first-calf suckled cows that are cycling at the beginning of the breeding season.

The second objective of this experiment was to determine if short-term bull exposure alters the breeding performance of first-calf suckled beef cows using a progestin-based estrous synchronization protocol that included $PGF_{2\alpha}$, GnRH, and timed AI. Specifically, we asked the question, “Does bull exposure influence the proportion of cows that exhibit estrus after ES?” In this experiment all cows exposed were exposed to

bulls before, during and after the ES protocol. The proportions of cows that exhibited estrus by 60 h after $PGF_{2\alpha}$ injection was independent of bull exposure. These results are similar to those reported by Anderson et al. (2002) and Berardinelli et al. (2004) for cows exposed or not exposed to bulls who used a modified CO-Synch synchronization protocol (Geary and Whittier, 1998).

Additionally, we asked, "Does short-term bull exposure for 30 d before, during and after ES influence the proportion of cows that become pregnant as a result of AI?" Anderson et al. (2002) suggested the possibility that the physical presence of a bull not only before but during, and after estrous synchronization and AI (ES+AI) may be necessary to enhance AI pregnancy rates. We tested this notion by exposing cows to bulls before, during, and after ES+AI, exposing cows to bulls before but not during and after ES+AI, not exposing cows before but during and after ES+AI, or not exposing cows to bulls. Pregnancy rates to AI were not different among treatments. It seems that bull exposure is not effective in increasing breeding performance if cows are exposed to bulls only during and after a GnRH-based ES+AI protocol that includes a progestin. However, the numbers of cows exposed to bulls during and after ES were limited in this experiment. Therefore, there is a possibility that exposing cows to bulls during and after ES may improve breeding performance; however, results from this experiment indicate the contrary.

Pregnancy rate to AI for cows exposed to bulls before the breeding season was greater than that for cows not exposed to bulls before the breeding season. This result indicates that bull exposure before the breeding season improves breeding performance in primiparous beef cows. The reason for this result is that AI pregnancy rate for cows bred

12 h after estrus were greater in cows exposed to bulls than that of cows not exposed to bulls for 30 d before the breeding season. This result is not consistent with that reported by Berardinelli et al. (2001). The difference between Berardinelli et al. (2001) and the present experiment is the use of a progesterone-impregnated CIDR. Progestin was not used by Berardinelli et al. (2001); and recently, Stevenson et al. (2003) reported that progestin treatment concurrent with a GnRH-based ES protocol improves pregnancy rates in suckled beef cows after timed AI. Results from the present experiment indicate that bull exposure used in conjunction with a progestin-based ES protocol may improve AI pregnancy rates.

Overall pregnancy rates were not affected by bull exposure. This result is consistent with results reported by Custer et al. (1990), Fernandez et al. (1993), and Berardinelli et al. (2001).

We conclude that short-term bull exposure of first-calf suckled beef cows increased the number of cows cycling before the beginning of the breeding season, but did not alter the estrous synchronization response. Furthermore, bull exposure during and after an estrous synchronization protocol that included progestin (CIDR), $PGF_{2\alpha}$, and GnRH did not alter breeding performance for those cows not exposed to bulls before the start of the ES protocol. Whereas, AI pregnancy rates of first-calf suckled beef cows was enhanced by exposing cows to bulls before implementation of this protocol.

Experiment 2

The question asked in Experiment 2 was, "Does bull urine contain a urinary pheromone that mediates the biostimulatory effect of bulls?" Cows under the influence of androgens, or gonadal steroids, can elicit the same biostimulatory effect as bulls (Burns and Spitzer, 1992). Therefore, to control for the possible negative or positive effect of urine exposure on the resumption of ovarian cycling activity and breeding performance of first-calf suckled beef cows we decided to use bovine urine devoid of the influence of gonadal steroids, i.e. steer urine.

The objectives of this experiment were to determine if bull urine exposure alters the proportion of cows that resume of ovarian cycling activity before the breeding season and breeding performance of first-calf beef cows. Exposing cows to mature bull urine had no effect on the interval to resumption of ovarian cycling activity and did not increase the proportion of cows that started to cycle before the breeding season. We observed a trend ($P = 0.09$) for more cows exposed to bull urine (BUE) than cows exposed to steer urine (SUE) to respond to estrous synchronization. Lastly, we found that approximately 32% more BUE cows became pregnant from AI than SUE cows.

A negative control for the effect of urine exposure on postpartum anestrus was not include in this experiment; however, the percentage of SUE cows cycling by the end of the exposure period was comparable to the percentage of cows cycling that were not exposed to bulls in previous years (Berardinelli et al., 2001; Joshi et al., 2002; Berardinelli et al., 2004;), but lower than the proportion of cows that resumed ovarian cycling activity in Experiment 1. Only 15% of BUE cows were cycling by the end of the exposure period. This number is quite low considering that cows were 92 to 111 days

postpartum by the end of the exposure period. These data differ from those of Joshi et al. (2002) who reported that more cows exposed to the excretory products of bulls resumed cycling activity than cows exposed to their own excretory products and cows not exposed to bulls or their own excretory products. The results indicate that continuous exposure of postpartum anestrous cows to mature bull urine does not alter the occurrence of resumption of ovarian cycling activity.

In regard to breeding performance 15 out of 19 BUE cows and only 10 of 19 SUE cows exhibited estrus within 60 h after $PGF_{2\alpha}$. Although this difference is not statistically significant it is not congruent with previous experiments that found no indication for an improvement in response to estrous synchronization between cows exposed to bulls or cows not exposed to bulls (Berardinelli et al., 2001; Anderson et al., 2002; Berardinelli et al., 2004).

Overall AI pregnancy rate was 31% higher for BUE than SUE. It seems that bull urine exposure was an effective way to improve breeding performance of first-calf beef cows. This result is not consistent with that of Anderson et al. (2002) who reported that AI pregnancy rates did not differ among; cows exposed to the excretory products of bulls, cows exposed to their own excretory, cows exposed to the physical presence of bulls, and cows not exposed to bulls or the excretory products of cows. Results from Experiment 1 indicate that the use of CIDR in conjunction with bull exposure can improve AI pregnancy rate. As in Experiment 1 CIDR were used in this experiment, thus bull urine exposure used with this ES protocol improved AI pregnancy rate. It appears that bull exposure, whether urine or physical presence, used in conjunction with an estrous

synchronization protocol that included progestin (CIDR), $PGF_{2\alpha}$, GnRH and timed AI can improve AI pregnancy rates in primiparous beef cows.

At first glance these data appear inconsistent and confusing; we would expect to observe lower AI pregnancy rates for BUE than SUE cows because fewer cows were cycling by the end of the exposure period. From the results of this experiment, it appears that the biostimulatory effect of bulls may not be mediated by a pheromone secreted in the urine of bulls. However, before making such a generalization, it is necessary to examine more clearly the known mechanisms for pheromonal transmission and perception.

Transmission and perception of pheromones are very complex process. Pheromones can be transported into the urine and perceived in the vomernasal organ through the use of small lipocalin proteins. Perhaps the procedures used during collection and handling of urine destroyed the pheromones ability to be perceived. For instance, the pheromone may have been damaged from storage on ice in a room at 4° C overnight, or perhaps a change in the pH of the urine occurred from the white precipitant that formed in the collection bags. Another possibility may be that the equipment used in association with the manufacturing of the CUDD was not appropriate to promote the release of the pheromone into the environment. The putative pheromone could have reacted with the CUDD reservoir (human urinary leg bag) or could have bound to the cellulose sponge located in the outer fenestrated pouch of the CUDD. However, if one assumes that any one of the aforementioned procedural factors caused the pheromone to be destroyed, or that the pheromone was not transmitted from the urine; we still cannot explain the enhanced AI pregnancy rate of cows exposed to mature bull urine. A flaw in

any procedure associated with the transmission of the pheromone from the urine to the environment cannot explain the positive results in breeding performance observed in this experiment. This type of flaw would significantly reduce pheromonal stimulation and breeding performance would not have been improved.

Instead, the results may be clarified by examining the manner by which a pheromone(s) from bull urine may have been perceived by cows. In this experiment cows were exposed to bull urine continuously for 24 h daily for an average of 64 days. One possible explanation of the results may be that we "overloaded" the pheromonal perception system of cows by exposing them continuously to bull urine. This type of exposure could have caused an overload in the perception system and after time the cows became "conditioned" to the bull pheromone. This conditioning may have caused the pheromone perception system to shut-down or senesce, resulting in the inhibition of cycling activity. However, this explanation is not congruent with known attributes of pheromonal perception in mammals. From the literature review we know that once molecules of pheromones are transported to the vomernasal organ (VNO) very few can cause a stimulatory response in distinct receptor neuron populations located in the VNO (Dulac, 2000; Takami, 2002). Also, this stimulatory pathway does not diminish as time of ligand exposure to receptor neurons increases (Dulac, 2000; Takami, 2002). In fact, stimulation can be measured by simply calculating the occurrence of receptor-ligand complex formation (Dulac, 2000; Takami, 2002). In light of this information it is not likely that an overstimulation or overload of the pheromone perception system occurred in cows exposed continuously to mature bull urine. Furthermore, if we assume that an overload of the pheromone perception system causes this system to shut-down, then

pheromone exposure would have resulted in zero stimulation of the pheromone signal. This explanation does not explain enhanced AI pregnancy rate of cows continuously exposed to bull urine.

It is possible that the stimulation signal from the VNO to the hypothalamus was not appropriate to cause resumption of ovarian cycling activity. If we assume bull urine contains a pheromone that stimulates VNO neurons, these neurons would be activated by the constant signal being reinforced by continuously exposing cows to bull urine. Therefore, we must consider that this type of pheromonal exposure, continuous (24 h daily) to bull urine and the subsequent neuroendocrine-endocrine response may hinder the onset of ovarian cycling activity in first-calf beef cows. From the literature review, intermittent physical presence of bulls does not induce the resumption of ovarian cycling activity in postpartum anestrous cows (Fernandez et al., 1996); however, the continuous physical presence of bulls does cause this effect (Custer et al., 1990). The continual physical presence of bulls may not be the same as continual pheromonal stimulation. Pheromonal stimulation may require cows to be in close proximity to bulls or their excretory products periodically and repeatedly throughout the day. If we assume that when cows interact with bulls or their excretory products they perceive a bull pheromone, and this pheromone causes the immediate stimulation of the VNO neurons, then we can also assume that when cows leave the close proximity of bulls or their excretory products VNO neuron activation wanes. Therefore, repeated contact could cause periodic stimulation of VNO neuron activity: the frequency of periodic stimulation may be the method by which biostimulation occurs. This may be analogous to the pulse frequency modulation of GnRH that is necessary to trigger the appropriate LH pattern for

resumption of cycling activity in postpartum anestrous cows. Thus, VNO neuron activation could directly stimulate the hypothalamo-hypophyseal-ovarian (HPO) axis or some other control mechanism in a pulsatile manner to influence the HPO axis.

Mora and Sanchez-Criado (2004) have suggested that pheromones of male rats stimulate the hypothalamo-hypophyseal-adrenal axis which then allows for these pheromones to act upon the HPO axis and stimulate the resumption of ovarian cycling activity in female rats. They proposed that the male pheromone acts upon the vomernasal organ which then stimulates the hypothalamus to release corticotrophin releasing hormone (CRH) that acts upon the anterior pituitary to release ACTH. The ACTH then stimulates the release of progesterone and cortisol from the adrenal gland. This increase in progesterone and cortisol serves as positive feedback re-enforcement of the pheromonal signal received by the vomernasal hypothalamus communication complex. This type of re-enforcement allows for stimulated neurons in the vomernasal organ to act upon the HPO axis and stimulates the resumption of ovarian cycling activity. This system in rats could explain the results observed in this experiment. It is possible that cortisol released from the adrenal gland could serve as an "on" switch for the pheromonal mechanism that stimulates the hypothalamus to release GnRH. However, this switch may need to be turned "on" and "off" to allow the resumption of ovarian cycling activity. Fernandez et al. (1996) reported that intermittent bull exposure caused an increase in the pulse frequency of luteinizing hormone (LH) from the anterior pituitary. These data support the hypothesis that bull exposure stimulates the perception of pheromones which cause a relatively immediate stimulation of the HPO axis and the release of LH. However, cows exposed to bulls intermittently (2 h every 3 d for 21 d starting on d 30

postpartum) did not resume ovarian cycling activity faster than cows not exposed to bulls (Fernandez et al., 1993) while cows continually exposed to the physical presence of bulls had shorter postpartum anestrous intervals to cycling activity than cows not exposed to bulls. These data indicate that pheromonal stimulation causes a relatively immediate neuroendocrine-endocrine response for LH release in postpartum anestrous cows. However, this response must then be reinforced by pheromonal stimulation periodically and repeatedly throughout the day for a period of time after initial exposure. The period between re-enforcement events and how often these events must be re-enforced is not known.

There is evidence to suggest that bovine pheromones must reach some threshold level before biostimulation occurs. We can assume that the biostimulatory effect of bulls is androgen-dependent (Baruah and Kanchev, 1993; Joshi et al., 2002). Additionally, Joshi et al. (2002) reported that cows exposed to their own excretory products resumed cycling activity sooner after exposure began than cows not exposed to their own excretory products; however, cows exposed to their own excretory products did not resume cycling activity as fast as cows exposed to the excretory products of bulls. As cows exposed to their own excretory products began to cycle they may have stimulated anestrous cows to resume cycling activity by excreting pheromones. Therefore, cycling cows under the influence of a rise in testosterone 7 to 8 days before estrus (Kanchev and Dobson, 1976; Kesler et al., 1979) may emit pheromones during proestrus/estrus that cause a biostimulatory effect. These data support the hypothesis that in the bovine, both males and females produce a pheromone(s) that must reach some threshold intensity before biostimulation can occur. Bulls appear to be more effective because they are

constantly under the influence of androgens, and constantly produce this pheromone(s) allowing it to reach threshold levels more rapidly.

From the foregoing discussion, we propose a pheromonally mediated simulation hypothesis by which the biostimulatory effect of bulls operates. This hypothesis may be known as the quantal pheromone threshold fluctuation hypothesis and is illustrated below in Figure 5.

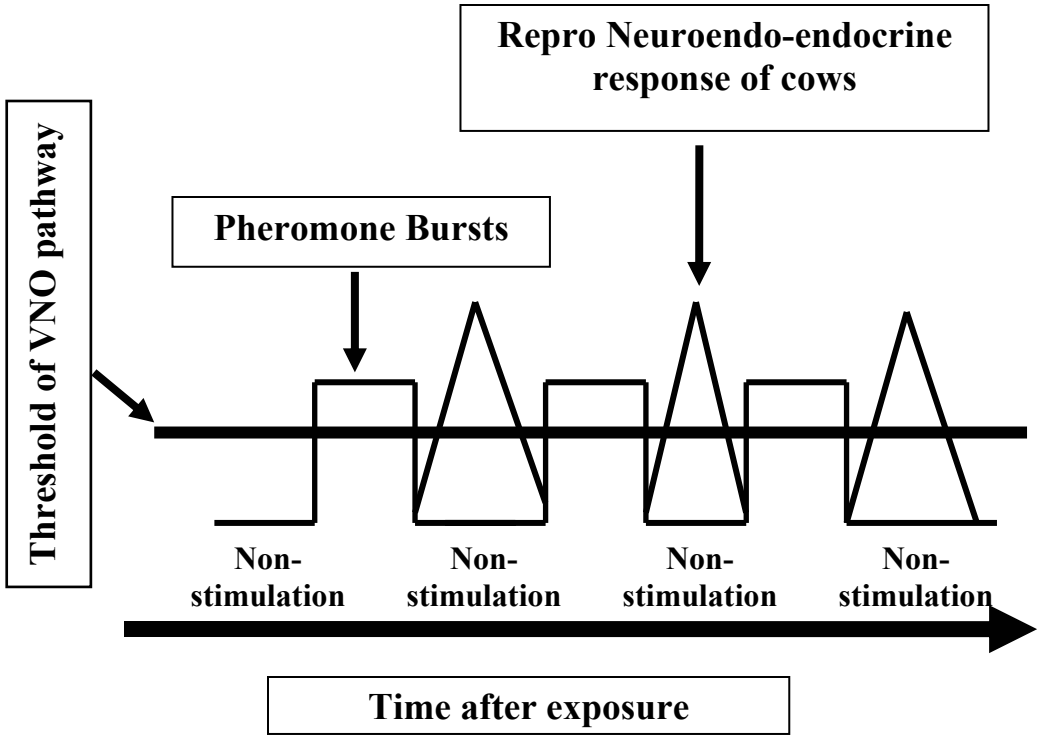


Figure 7. Graphical depiction of the "Quantal Threshold" hypothesis. Large arrow indicates time after exposure. Horizontal line above the large arrow indicates some threshold level of pheromone stimulation. Pheromone bursts exceed threshold level followed by a period of non-stimulation. During this period of non-stimulation reproductive neuroendocrine-endocrine responses can occur.

This hypothesis states that pheromone stimuli must occur in bursts. These bursts much acquire some threshold level of stimulation. Each burst is followed by a period of

non-stimulation during which reproductive neuroendocrine-endocrine changes can occur. This pattern of stimulation must occur over an unspecified period of time before the induction of a biostimulatory effect.

The "Quantal Threshold" hypothesis could also provide an explanation for why cows are more sensitive to the biostimulatory effect of bulls later in the postpartum anestrous period. Recall from the literature review that suckling stimuli requires mammary stimulation and the presence of the calf to prolong postpartum anestrus. Cows respond faster to the biostimulatory effect of bulls as the calf becomes older and more socially independent. The increased ability to respond to the biostimulatory effect of bulls could be due to competitive binding between calf pheromones and bull pheromones on pheromone binding proteins, like odorant binding protein (OBP), found in the nasal cavity. Therefore, the calf "smell" or pheromone could occupy binding sites on OBP eliminating or reducing the ability of bull pheromone(s) to stimulate VNO receptor neurons that are responsible for transmitting the appropriate biostimulatory signal to the HPO axis. When bulls are introduced to cows shortly after parturition, and cow-calf interaction is greatest, cows do not respond to the bull pheromone and the subsequent biostimulatory effect of bulls. This could be explained in part by pheromones that may be produced by calves that inhibit bull pheromone stimulation. In other words, the stimulation threshold necessary for biostimulation to occur may be difficult to attain during the time that cow-calf bonding is highest. Consequently, stimulation of the hypothalamus does not occur and LH is not released in a manner that stimulates the resumption of ovarian cycling activity. As the calf becomes more independent the cow-calf "weakens", and less of its pheromone may compete with the bull pheromone and

threshold stimulation levels are acquired periodically and repeated throughout the day; this type of pheromone stimulation could promote the occurrence of the biostimulatory effect of bulls.

It is important to stress that the above hypothesis is based on speculation, experimental evidence, and observations in bovine and in small mammals such as mice and rats. However, the hypothesis could give us insight as to what to expect when investigating the bovine pheromone system. Why would a complex system, like the one described above, be beneficial to bovine? When females in herding type situations, as those seen with wild ruminants, start to cycle this system could ensure that females, who would not normally cycle in the absence of pheromone stimulation, return to cycling activity. Moreover, males may produce pheromones to cause more females to cycle resulting in more copulation events per breeding male in a shorter period of time.

In conclusion, continuous bull urine exposure of first-calf suckled beef cows did not decrease the interval from calving to the resumption of ovarian cycling activity and did not increase the proportion of cows cycling before the beginning of the breeding season. Furthermore, continuous bull urine exposure of first-calf suckled beef cows did not improve response to estrous synchronization. However, continuous exposure to mature bull urine before an estrous synchronization protocol that included progestin (CIDR), $PGF_{2\alpha}$, and GnRH increased AI pregnancy rate. Therefore, it is possible that bull urine contains a pheromone(s), and while continuous exposure to this pheromone(s) did not alter the resumption of ovarian cycling activity it did improve the breeding performance of first-calf suckled beef cows.

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APPENDICES

APPENDIX A

COMPONENTS AND METHODS USED IN THE CONSTRUCTION OF THE CONTROLLED URINE DELIVERY DEVICE

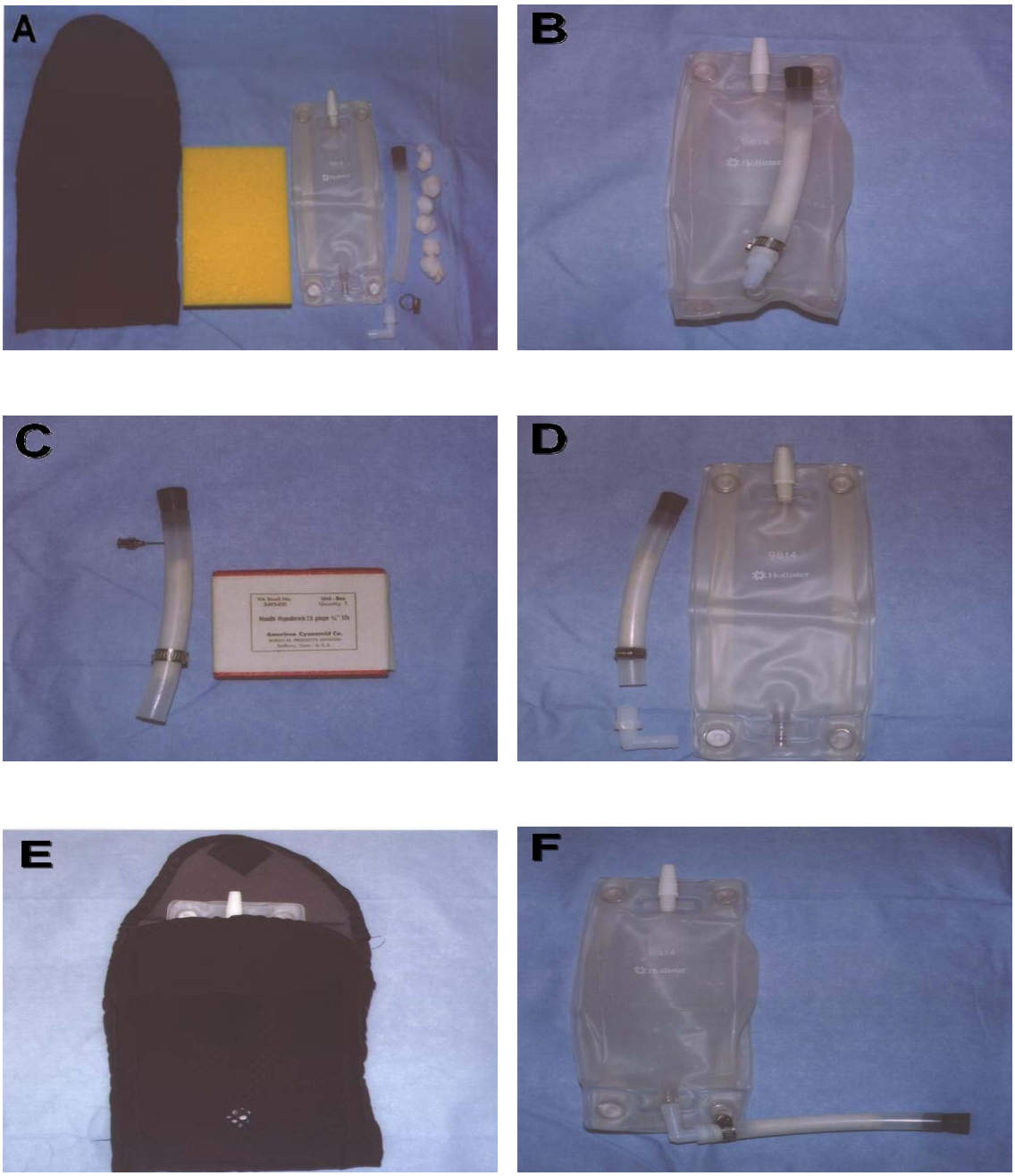


Figure 8. Components and construction of controlled urine delivery device (CUDD). Panels: A) CUDD components; B) reservoir attached to flow control valve; C) flow control valve with 23 gauge hole placed 2 cm from plug; D) attach flow control to reservoir using polyethylene elbow with small hose clamp E) place reservoir inside neoprene bag; F) once reservoir is inside bag attach flow control valve.

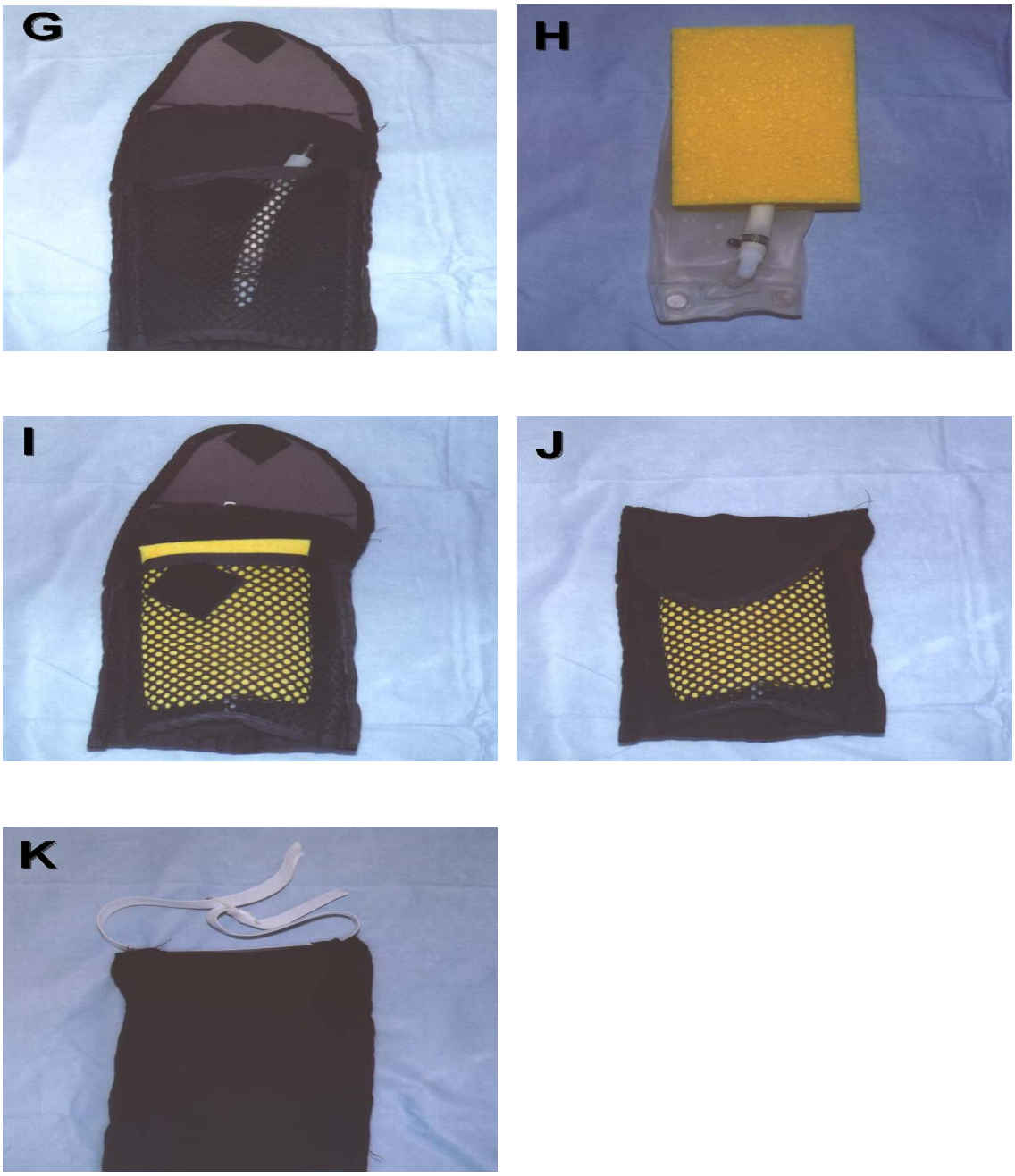


Figure 8 (continued). Figure 8. Components and construction of controlled urine delivery device (CUDD). Panels: G) reservoir in inner pouch and flow control valve in outer pouch; H) place sponge around flow control valve; I) reservoir, valve, and sponge placed inside neoprene bag; J) close upper flap on CUDD; K) finished product with elastic strap through the top of CUDD.

APPENDIX B

MATERIALS AND METHODS USED FOR THE ATTACHMENT OF THE CONTROLLED URINE DELIVERY DEVICE



Figure 9. Illustration of controlled urine delivery device (CUDD) attachment procedure. Panels: A) equipment for CUDD attachment crimpers, pan with disinfectant, trochar, and cable; B) restrain animal within chute/head catch; C) shave dulap area; D) measure 7 cm anterior to brisket; E) numb area with lidocaine; F) make incision with electro-surgical tool.



Figure 9 (continued). Illustration of controlled urine delivery device (CUDD) attachment procedure. Panels: G) insert trochar through incision; H) insert cable through the hole made by the trochar; I) crimp ends of cable together; J) dulap ring successfully in place; K) attach CUDD to ring via zip ties and to the neck using an elastic strap around the neck of the cow.

APPENDIX C

MATERIALS AND METHODS USED FOR THE FILLING OF THE CONTROLLED URINE DELIVERY DEVICE

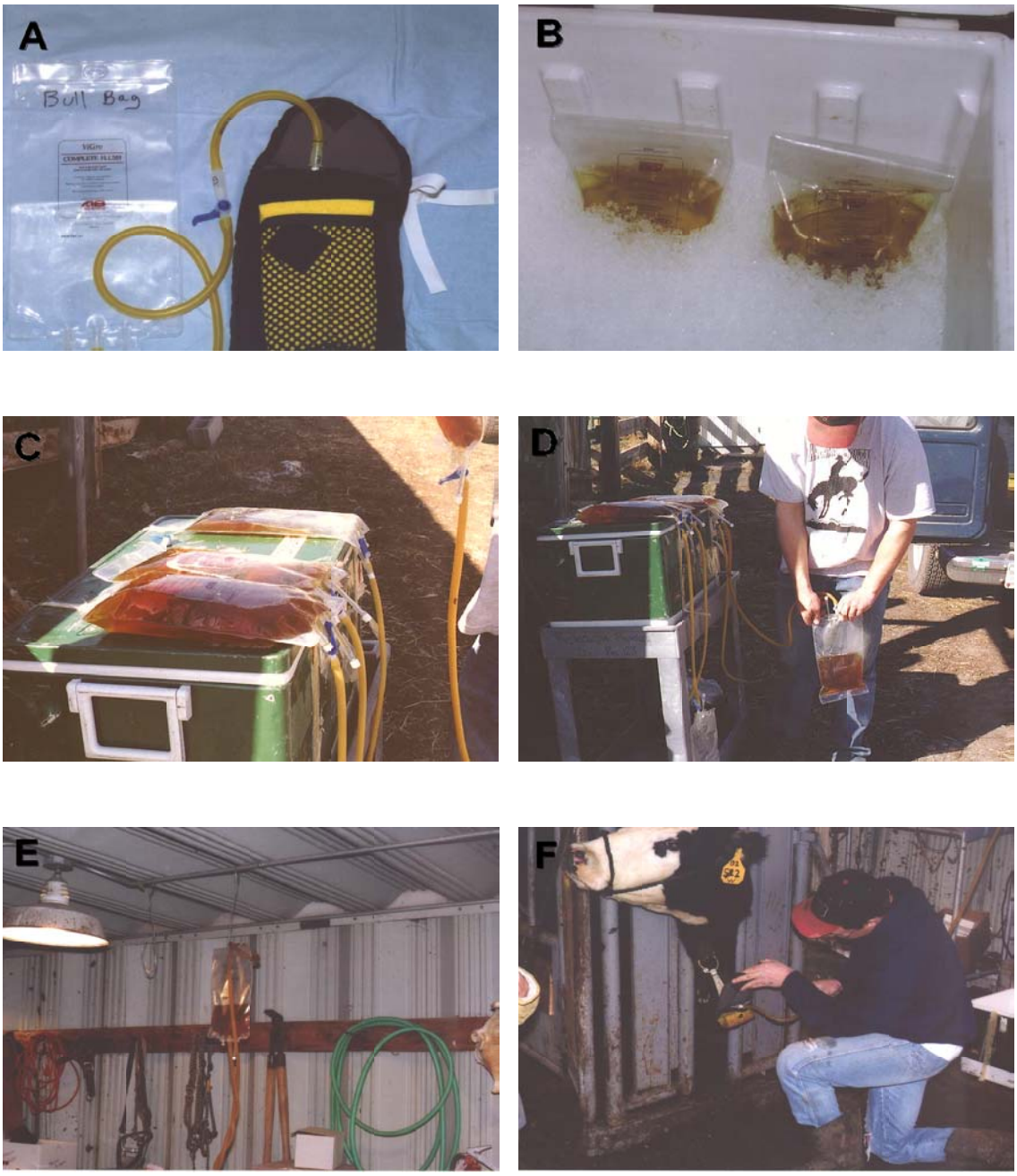


Figure 10. Illustration of controlled urine delivery device (CUDD) filling procedure. Panels: A) tube and bag connections; B) urine from previous collection C) place urine on flat surface; D) Transfer to a clean bag to remove precipitant; E) suspend collection bag; F) attach tubing to urine reservoir and fill CUDD.



Figure 10 (continued). Illustration of controlled urine delivery device (CUDD) filling procedure. Panels: G) rinse bags after filling procedure is complete.

APPENDIX D

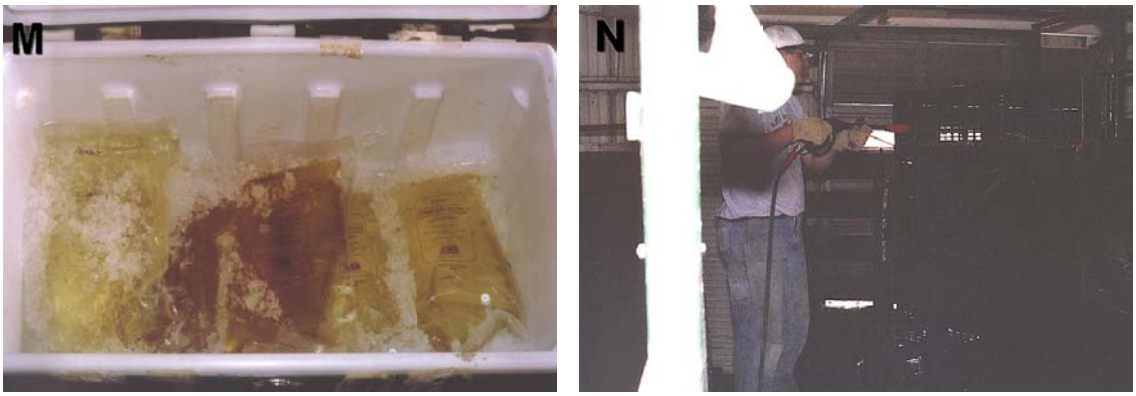
MATERIALS AND METHODS USED FOR THE COLLECTION OF URINE



Figure 11. Illustration of the urine collection facility and methods used to collect urine. Panels: A) mature A X H bulls; B) 1 yr. old steers; C) urine collection stalls; D) feed and water trough; E) bulls and steers had the ability to protrude head out the front of stalls; F) floor of collection stall.



Figure 11 (continued). Illustration of the urine collection facility and methods used to collect urine. Panels: G) urine collection pan; H) hole (2.75 cm diameter) with spout and screen in place; I) tubing connected to urine collection bag; J) tubing connected to pan and collection pan; K) collection pan placed beneath prepuce of bull or steer; L) location of tubing and collection bags on facility floor underneath feeding trough.



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Figure 11 (continued). Illustration of the urine collection facility and methods used to collect urine. Panels: M) full collection bags placed on ice; N) facility washed with soap and pressure washer.