THE BIOSTIMULATORY EFFECT OF BULLS ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AND -OVARIAN AXES AND ON TEMPORAL ASPECTS OF RESUMPTION OF OVARIAN CYCLING ACTIVITY IN PRIMIPAROUS, POSTPARTUM, ANESTROUS, SUCKLED, BEEF COWS

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ABSTRACT

Exposing cows to bulls or excretory products of bulls stimulates resumption of ovarian cycling activity in postpartum, suckled, anestrous cows. This biostimulatory effect may be mediated by pheromones produced by bulls that stimulate physiological changes in the hypothalamic-pituitary-ovarian (HPO) and/or -adrenal (HPA) axes of cows. In Experiment 1, the hypothesis tested was that the biostimulatory effect of bulls is associated with adrenal regulation and/or function in anestrous cows. The biostimulatory effect of bulls was associated with mean concentrations of cortisol in postpartum cows. Experiment 2 was designed to determine if acute (5-h daily) bull exposure alters characteristics of patterns of cortisol and luteinizing hormone (LH) concentrations in postpartum, anestrous cows. Cows exposed acutely to bulls exhibited fewer pulses of cortisol and more frequent pulses of LH than cows exposed to steers. However, it was not known if these changes were related to resumption of ovarian cycling activity in postpartum, anestrous cows. Experiment 3 was designed to test the hypothesis that patterns of cortisol concentrations are altered by continuous, 24-h daily, bull exposure, before and after resumption of ovarian cycling activity in postpartum, anestrous cows. Continuous bull exposure decreased cortisol pulse frequency before cows resumed ovarian cycling activity. Experiment 4 tested the hypothesis that the overall shape of patterns of cortisol and/or LH concentrations may differ between cows exposed acutely to bulls or steers in Experiment 2. Cows exposed acutely to bulls had more uneven patterns of LH concentrations than cows exposed to steers and as patterns of cortisol concentrations became smoother, patterns of LH become more uneven in cows exposed acutely to bulls. In Experiment 5, the hypothesis tested was that interval to resumption of ovarian cycling activity may depend upon duration of daily bull exposure. Cows resumed ovarian cycling activity sooner as duration of daily bull exposure increased. In conclusion, as duration of daily bull exposure increases, the biostimulatory effect of bulls alters activity of the HPA axis and this change may facilitate or support the function of the HPO axis and accelerate resumption of ovarian cycling activity in primiparous, postpartum, suckled, anestrous cows.

CHAPTER 1

INTRODUCTION

Reproductive efficiency in beef cattle production can be defined as the proportion of cows within a herd that become pregnant, give birth, and rear calves each year. Postpartum anestrus, 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 primiparous, 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 problems associated with postpartum anestrus; these include early weaning of calves, increasing feed intake of cows, synchronization of estrus in cows, etc. Unfortunately, these strategies can be costly, labor intensive, unsustainable, and socially unacceptable. There are many physiological, social, and environmental factors that influence interval from calving to resumption of ovarian cycling activity in postpartum, anestrous cows. One of these is the biostimulatory effect of bulls. This effect is defined as a reduction in the interval from calving to resumption of ovarian cycling activity in anestrous cows. The biostimulatory effect of bulls may be an effective management strategy to reduce postpartum anestrus that is cost effective, labor saving, sustainable, and more socially acceptable to both producers and consumers. However, to successfully implement the biostimulatory effect of bulls as a management strategy in practical situations we must first identify the conditions that are necessary to realize this effect, determine the

physiological mechanisms involved with this effect, and develop an understanding of how these physiological mechanisms accelerate resumption of ovarian cycling activity in postpartum, anestrous, suckled cows.

The physiological mechanism for the biostimulatory effect of bulls appears to involve a change in the hypothalamo-pituitary-ovarian (HPO) axis that results in an increase in the frequency of luteinizing hormone (LH) pulses (Fernandez et al., 1996; Roelofs et al., 2007), which in turn stimulates resumption of ovarian cycling activity. Recently, research from our laboratory indicates that the mechanism of this biostimulatory effect is mediated by pheromones that are produced and excreted by bulls (Berardinelli and Joshi, 2005a). In mammals, male pheromones are predominantly carried in and released from urine (for review, see, Izard 1983) and affect hypothalamic-pituitary-adrenal (HPA) activity in female rodents (Marchlewska-Koj and Zacharczuk-Kakietek, 1990; Mora and Sanchez-Criado, 2004) and *homo sapiens* (Wyart et al., 2007). However, it is not known if pheromones excreted by bulls affect the function and/or regulatory activities of the HPA axis in cows. In this dissertation, Experiments 1, 2, and 3 addressed the possibility that function and/or regulation of the HPA axis may be involved with the biostimulatory effect of bulls to accelerate resumption of ovarian cycling activity in postpartum, anestrous, suckled, beef cows. Then in Experiment 4, the mechanism whereby changes in HPA axis may be related to changes in HPO axis activity induced by bulls was explored using data from Experiment 2.

Included in this dissertation is an additional experiment to Experiments 1, 2, 3, and 4. This experiment is based on the observation that 24-h exposure of postpartum,

anestrous, suckled cows to bulls accelerated resumption of ovarian cycling activity (for review, see, Rekwot et al., 2000a). However, pheromones produced by bulls may not be perceived by cows every hour for 24-h daily even though cows are exposed continuously to the physical presence of bulls. It seems highly unlikely that continuous pheromonal stimulation would be necessary to induce a biostimulatory effect. In fact it may be that the sensory system for detection of pheromones would become refractive if stimulated by pheromones 24-h daily. The appropriate mode, by which bulls stimulate resumption of ovarian cycling activity may depend on the duration that cows perceive a pheromonal stimulus each day. Thus, the possibility that the duration of daily bull exposure may affect the time required for cows to respond to pheromones produced by bulls and accelerate resumption of ovarian cycling activity was investigated in Experiment 5.

This dissertation concerns the hypothesis related to physiological mechanisms whereby the biostimulatory effect of bulls alters the HPA and HPO axes to accelerate resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows. The first section of this dissertation is intended to give the reader the background necessary to evaluate the questions and hypotheses posed, the approaches used, and the results and conclusions of the experimentation derived by the author. This review includes: 1) a brief background of the physiological condition known as postpartum anestrus; 2) a perspective of the biostimulatory effect of bulls on the postpartum physiology of cows; 3) a review of the current knowledge of the mechanisms of the biostimulatory effect of bulls; 4) an overview of the literature related to pheromonal communication in mammals; 5) a discussion of pheromones in bovine reproductive

function and behavior; 6) identification of temporal and quantal aspects of the biostimulatory effect of bulls; 7) an acknowledgement of similarities between the biostimulatory effect of bulls and the male effect in sheep and goats; 8) an introduction to the effects of male pheromones on the HPA axis of female rodents and *homo sapiens*; 9) a synopsis of cortisol synthesis, secretion, and its general physiological effects; 10) notes related to the interaction between the HPA and HPO axes; and, 11) a perspective on how suckling and presence of offspring influence the HPA axis of lactating females.

CHAPTER 2

LITERATURE REVIEW

Postpartum Anestrus

Immediately after parturition most mammalian females, including the cow, begin to lactate and undergo a period of reproductive tract repair known as the puerperium. Uterine involution and endometrial remodeling occurs during the puerperium so as to sustain another pregnancy (Short et al., 1990). Additionally, ovaries recover their intrinsic ability to develop follicles in a wave-like pattern (Stagg et al., 1998). However, estrus and ovulation is constrained by lactation and the socio-psychological bond known as the cow-calf bond. This period of "constraint" is known as postpartum anestrus (Short et al., 1990). The interval of time that cows spend in this condition is measured by the time from calving to estrus, ovulation, resumption of ovarian cycling activity, or conception (Casida, 1968).

The principal factors that affect the length of postpartum anestrus are nutrition, suckling stimuli, and parity. Cows that are nutrient restricted, suckled frequently, and calve for the first time have longer intervals to resumption of ovarian cycling activity after calving than cows that are fed adequately, restricted from suckling calves, and have had more than one calf. Other factors that influence this interval are breed, environmental stress, dystocia, and social factors (for review, see, Short et al., 1990; Williams, 1990; Rhodes et al., 2003).

Regulation of postpartum anestrus is under control of the reproductive neuroendocrine system, which regulates hypothalamic release of gonadotropin releasing hormone (GnRH). The release pattern of GnRH in turn regulates the pulsatile secretion of luteinizing hormone (LH) from the anterior pituitary. During postpartum anestrus, activity of the hypothalamic GnRH pulse generator is modulated by the negative effects of serotonin, dopamine, endogenous opioids, (Williams, 1990) and estradiol-17β (Acosta et al., 1983). Together these molecules suppress the activity of the GnRH pulse generator and the result is the release of high amplitude, low frequency pulses of LH from the anterior pituitary, which signals the ovary to remain anovulatory (Acosta et al., 1983). This pattern of LH secretion is associated with high nutrient demands for lactation (Williams, 1990), low blood concentrations of leptin, insulin, and insulin-like growth factor 1 (IGF-1), and high concentrations of growth hormone (Chagas et al., 2006). As time postpartum progresses the maternal cow-calf bond wanes (Williams, 1990), nutrient demands for lactation decrease, and metabolic hormones such as leptin, insulin, and IGF-1 increase (Chagas et al., 2006) and growth hormone decreases (Chagas et al., 2006; Rhodes et al., 2003). These changes bring about changes in the pulsatile release of GnRH and LH and result in low amplitude, high frequency release of LH pulses 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., 1993; Wright et al., 1992; Rhodes et al., 2003; Chagas et al., 2006). This temporal release pattern of LH stimulates the final maturation of a dominant ovarian follicle, which synthesizes and secretes estradiol-17β (Fortune et al., 1991). As circulating concentrations of estradiol-17β increase, the

feedback effect of estradiol-17β switches from negative to positive feedback on GnRH pulse generation in the hypothalamus. High concentrations of estadiol-17β trigger behavioral estrus and the episodic or preovulatory surge release of LH that causes ovulation of the dominant follicle and formation and function of the corpus luteum, i.e., resumption of ovarian cycling activity (Short et al., 1990).

The Effect of Bull Exposure on the Postpartum Anestrous Cow

The effect of bull exposure on resumption of ovarian cycling activity in postpartum, anestrous cows was first documented in breeding experiments. In these experiments the occurrence of estrus during artificial insemination protocols was greater for groups of cows exposed to teaser bulls than cows not exposed to bulls (Nersesjan, 1962; Sipilov, 1966; Ebert et al., 1972). However, these data could have been biased because the presence of bulls may have enhanced the ability of observers to detect estrous behavior in groups of cows exposed to bulls (Foote, 1975). In the years following the report of these observations, numerous controlled studies indicated 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; Perez-Hernandez et al., 2002; Landaeta-Hernandez et al., 2004; 2006). The effect of bulls on acceleration of resumption of ovarian cycling activity, termed the “biostimulatory effect of bulls”, is defined as a reduction in the interval from

calving to resumption of ovarian cycling activity in anestrous *bos taurus* cows (Stumpf et al., 1992) and *bos indicus* cows (Rekwot et al., 2000a).

There are data to indicate that 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 resumed cycling activity before the breeding season than cows not exposed to bulls (Custer et al., 1990; Beradinelli and Joshi et al., 2005a). Berardinelli et al. (2007) reported that more cows exposed to the biostimulatory effect of bulls before and during a GnRH-based ES protocol became pregnant as a result of AI than cows not exposed to bulls. Furthermore, Tauck (2005) reported that more cows exposed continuously to bull urine displayed estrus in response to a progesterone-based ES protocol and became pregnant as a result of AI than cows exposed continuously to steer urine. Taken together, these data indicate that bull exposure may have a biostimulatory effect on both resumption of ovarian cycling activity and breeding performance of postpartum cows.

Although there are data from numerous studies that support the biostimulatory effect of bulls, there are a few studies that do not. Bonavera et al. (1990) reported that exposing cows to bulls did not shorten the interval from calving to the resumption of ovarian cycling activity in postpartum, anestrous cows. However, in this experiment a difference between cows exposed and not exposed bulls would have been difficult to detect, 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

fence-line contact with bulls had equal or longer intervals from calving to resumption of ovarian cycling activity than cows not exposed to fence-line contact with bulls. However, Fike et al. (1996) and Berardinelli and Tauck (2007) reported that fence-line contact of suckled cows with bulls had shorter intervals from calving to resumption of ovarian cycling activity than cows not exposed to fence-line contact with bulls. The difference between the results reported by Fike et al. (1996) and Berardinelli and Tauck (2007) and those of Shipka and Ellis (1998; 1999) may be explained by the fact that bulls in the latter experiments 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 bulls; whereas, cows in the former studies were allowed direct fence-line contact ("nose to nose") with bulls for 24-h daily.

It is interesting to note that the biostimulatory effect of bulls may be influenced by season. Fall-calving cows did not respond to the biostimulatory effect of bulls as readily as 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. One seasonal factor that may influence the ability of cows to respond to the biostimulatory effect of bulls could be temperature. Perhaps the physiological and sensory systems involved with the biostimulatory effect of bulls operate more efficiently and stimulate resumption of ovarian cycling activity in postpartum, anestrous cows to a greater extent as days become warmer and vice versa. Another important factor involved with seasonal changes is photoperiod. Photoperiod may interact with the biostimulatory effect of bulls on resumption of ovarian cycling activity in postpartum, anestrous cows.

Cows that calved in the summer displayed shorter intervals to resumption of ovarian cycling activity than cows that calved in the winter (Hauser, 1984) and the mechanism, by which photoperiod influences length of postpartum anestrus, appears to involve melatonin (Sharpe et al., 1986). Thus, melatonin secretion and/or photoperiod length may lengthen postpartum anestrus, counteracting the biostimulatory effect of bulls on resumption of ovarian cycling activity. However, additional studies are necessary to evaluate those seasonal factors that might be involved with the biostimulatory effect of bulls.

There is some evidence that indicates that the biostimulatory effect of bulls interacts with nutritive intake. Alberio et al. (1987) investigated the effect of bull exposure on cows fed to lose 0.9 kg of body weight each day. They reported that a greater proportion of cows not exposed to bulls had ovulations not associated with estrus and that interval to first ovulation was longer for cows exposed to bulls than cows not exposed to bulls. These data indicated that the presence of bulls may prolong postpartum anestrous in cows that are losing weight. Monje et al. (1992) further investigated the interaction between plane of nutrition and the biostimulatory effect of bulls. They reported that intervals from calving to resumption of ovarian cycling activity appeared shorter for cows exposed to bulls and fed a high plane of nutrition than cows not exposed to bulls and fed a high plane of nutrition. However, intervals from calving to resumption of ovarian cycling activity appeared longer for cows exposed to bulls and fed a low plane of nutrition than cows not exposed to bulls and fed a low plane of nutrition. Stumpf et al. (1992) interpreted data from Monje et al. (1992) as an indication that the biostimulatory

effect of bulls may be dependent on the nutritional plane of cows. They hypothesized that there would be a greater decrease in intervals from calving to resumption of ovarian cycling activity in cows fed a high plane of nutrition and exposed to bulls than cows fed a low plane of nutrition and exposed to bulls. However, they reported that intervals from calving to resumption of ovarian cycling activity for cows exposed to bulls and fed a low plane of nutrition were 14-d shorter than cows fed a low plane of nutrition and not exposed to bulls. Whereas, intervals from calving to resumption of ovarian cycling activity for cows exposed to bulls and fed a high plane of nutrition were only 6-d shorter than cows fed a high plane of nutrition and not exposed to bulls. These data indicate that the biostimulatory effect of bulls accelerates resumption of ovarian cycling activity to a greater extent in cows that are nutrient-restricted or fed at a low plane of nutrition.

Differences between results of Alberio et al. (1987) and Monje et al. (1992) and those of Stumpf et al. (1992) may be explained by the interpretation of data from Alberio et al. (1987) and Monje et al. (1992). Although Alberio et al. (1987) reported that intervals to first ovulation were longer for cows exposed to bulls than cows not exposed to bulls, they also reported that intervals from calving to estrus and calving to resumption of ovarian cycling activity did not differ between cows exposed and not exposed to bulls. Additionally, the proportion of cows that resumed ovarian cycling activity by 100 d after calving was greater for cows exposed to bulls than cows not exposed to bulls. These data indicate that more cows not exposed to bulls had ovulations not associated with estrus and that bull exposure accelerated resumption of ovarian cycling activity in cows that were losing body weight. Similarly, Stumpf et al. (1992) may have misinterpreted data

of Monje et al. (1992) who reported that intervals from calving to resumption of ovarian cycling activity appeared longer for cows exposed to bulls and fed a low plane of nutrition than cows not exposed to bulls and fed a low plane of nutrition. In the experiment of Monje et al. (1992), cows of each treatment were combined and managed as a single group on D 110 after calving. Furthermore, all cows in the treatments were exposed to bulls beginning on D 110 after calving. Mean intervals from calving to resumption of ovarian cycling activity for cows exposed and not exposed to bulls and fed a low plane of nutrition were greater than 110 d. It is difficult to formulate conclusions about experimental observations that occurred after treatments ended on D 110, and it may be more prudent to make conclusions based on the results of Stumpf et al. (1992), which indicated that the biostimulatory effect of bulls may accelerate resumption of ovarian cycling activity to a greater extent in cows that are nutrient-restricted. Therefore, the biostimulatory effect of bulls appears to interact with nutrient intake or energy metabolism to cause resumption of ovarian cycling activity in postpartum, anestrous cows. Perhaps the biostimulatory effect of bulls influences physiological mechanisms that may modulate production of metabolic hormones such as leptin, IGF-1, and growth hormone. If so, the effects of these hormones on generation of LH pulses (Chagas et al., 2006) and follicular growth and maturation (Rhodes et al., 2003) may facilitate changes in the HPO axis that are necessary for resumption of ovarian cycling activity in postpartum, anestrous cows.

Although the biostimulatory effect of bulls and plane of nutrition appear to interact to reduce the length of postpartum anestrus, there is no evidence yet that there is

an interaction between the biostimulatory effect of bulls and suckling stimuli. This may be because most experiments that investigated aspects of the biostimulatory effect of bulls were designed to equalize suckling stimuli over all treatments. There are few studies in which the interaction between the biostimulatory effect of bulls and suckling stimuli has been investigated. Suckling restricted to 2-h daily 8-h after milking did not interact with the biostimulatory effect of bulls on resumption of ovarian cycling activity in postpartum, anestrous, dairy cows (Perez-Hernandez et al., 2002). Additionally, Berardinelli and Joshi (2005b) reported that restricting suckling to twice daily in cows exposed to bulls starting 15, 35, and 55 days after calving had no effect on the percentage of cows that resumed ovarian cycling activity compared to cows exposed to bulls whose calves were allowed to suckle ad libitum. However, in this experiment calves were housed in pens adjacent to cows, thus, cows and calves could have maintained a social bond thereby negating the effect of restricted suckling through auditory and olfactory stimulation (Williams and Griffith, 1995; Lamb et al., 1997). Suckling is a major factor for determining the length of postpartum anestrus and future investigation is necessary to determine if there is an interaction between calf suckling stimuli and the biostimulatory effect of bulls.

The Mechanism of the Biostimulatory Effect of Bulls

Social Interaction Hypothesis

Social Interaction Hypothesis. Berardinelli et al. (2005) explored the hypothesis that a social bond formed between bulls and cows may cause changes in HPO axis necessary for cows to resume ovarian cycling activity sooner than cows not exposed to

bulls. This hypothesis was tested by exposing cows to bulls at calving for 30 d (familiar bulls), then switching half of these cows to exposure to unfamiliar bulls. As controls, they exposed cows to familiar ovariectomized (OVX) cows and then switched half of these cows to exposure to 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 of OVX cows or bulls did not affect intervals from calving to resumption of ovarian cycling activity in postpartum, anestrous cows. Thus it would appear that, social interactions and bonding between bulls and cows may not be an important factor that mediates the biostimulatory effect of bulls. However, the possibility exists that social bonding between bulls and cows occurs after peak lactation as the cow-calf bond begins to wane. Thus, switching cows from exposure to familiar bulls to exposure to unfamiliar bulls at 30 d after calving would not differentially affect the interval from calving to resumption of ovarian cycling activity. Future investigation is needed to address the possibility that social bonding between bulls and cows may be involved with the biostimulatory effect of bulls on resumption of ovarian cycling activity in postpartum, anestrous cows.

Effect of Bull Exposure on the Hypothalamic-Pituitary-Ovarian (HPO) Axis. The evidence presented thus far leads to the conclusion that the biostimulatory effect of bulls accelerates resumption of ovarian cycling activity in postpartum, anestrous cows and that there are factors that do or do not influence this response. The next question is, "How does the biostimulatory effect of bulls influence the reproductive neuroendocrine and endocrine regulation of the postpartum anestrous cow?" Previous reports have

established that postpartum, anestrous cows show an increase in LH pulse frequency directly before they resume ovarian cycling activity (Walters et al., 1982; Humphrey et al., 1983; Peters and Lamming, 1990). This led researchers to postulate that bull exposure could bring about resumption of ovarian cycling activity by stimulating the release of LH pulses from the pituitary at a greater frequency sooner after calving in cows exposed to bulls than in cows not exposed to bulls. Custer et al. (1990) were the first to investigate this possibility and reported that baseline and mean concentrations of LH and duration, amplitude, and frequency of LH pulses did not differ between cows exposed and not exposed to bulls soon after calving. In this study, intensive blood sampling was conducted weekly after the start of bull exposure 3 days after calving, therefore, any immediate or short-term changes in characteristics of LH concentration patterns that would mimic those observed by Walters et al. (1982), Humphrey et al. (1983), and Peters and Lamming (1990) would not have been detected. Consequently, Fernandez et al. (1996) addressed the possibility that bull exposure would have an immediate or short-term effect on characteristics of temporal patterns of LH concentrations by intensively collecting blood samples every 10 min for 4-h every three days from cows exposed continuously to bulls, intermittently exposed to bulls 2-h every 3 days, or not exposed to bulls. Cows exposed continuously to bulls and cows exposed to bulls intermittently had greater mean concentrations of LH and frequency of LH pulses than cows not exposed to bulls. These data indicated that the biostimulatory effect of bulls stimulated LH release from the pituitary soon after the start of bull exposure. Further support for the observation that systemic gonadotropin concentrations are altered within a short time

interval after bull exposure comes from an earlier study by Baruah and Kanchev (1993). They reported that mean LH and follicle stimulating hormone (FSH) concentrations increased within 80 min after oronasally administering bull urine to non-suckled dairy cows 9 d after calving. Recently, Roelofs et al. (2007) observed that frequency of LH pulses was greater in samples collected from non-suckled dairy cows during a single, 8-h bull exposure period than compared to the frequency of LH pulses obtained from the same cows during an 8-h sampling period 24-h earlier. Results from these studies lend strong support to the hypothesis that the mechanism whereby bull exposure accelerates resumption of ovarian cycling activity in postpartum, anestrous cows involves an alteration in the reproductive neruoendocrine-endocrine system to increase pulse release of hypothalamic GnRH. An increase in pulse release of GnRH is reflected as an increase in frequency of pulses of LH from the pituitary, which is required for final maturation of dominant follicles and eventually ovulation.

Pheromones

Biologically active substances or extroceptive chemosignals that affect reproductive behavior and or function of conspecifics were first termed “Pheromones” by Karlson and Luscher (1959). Pheromones are defined as airborne chemical substances released from the urine, feces, or cutaneous glands that are sensed by the olfactory or respiratory systems that cause behavioral and endocrine responses in conspecifics (for review, see, Rekwot et al., 2000a). Pheromones can be classified as signaling or priming types. Signaling pheromones cause immediate or short-term, stimulation and response;

which can be followed immediately by a period of non-stimulation and non-response. Priming pheromones stimulate a cascade of the neuroendocrine-endocrine events that generally have long-term, irreversible effects on reproductive function and behavior of conspecifics (Izard, 1983).

The observation made by Baruah and Kanchev (1993) mentioned in the previous section, is noteworthy in that it allows one to speculate that a urinary pheromone(s) may be involved with the biostimulatory effect of bulls. Thus, to develop the mechanism of the biostimulatory effect of bulls requires an understanding of the basic principles of pheromonal stimuli and how pheromones influence reproductive behavior and/or function in mammals. Pheromones were first described in mammals in the 1950's by van der Lee and Boot (1955) who reported that estrous behavior was suppressed or delayed in female mice that were maintained in groups or exposed to cages soiled with female-urine. This was closely followed by a report from Bruce (1959) who observed that pregnancy was blocked or terminated in female mice exposed to the presence of unfamiliar males or urine from unfamiliar males. The discoveries of the "Lee-Boot" and "Bruce" effects has led researchers to identify and characterize a vast array of pheromonal effects in mammals. The following is a discussion of pheromone sensory pathways, transport, and perception.

Pheromone Sensory Pathways

Odorant Receptor Neuron. Pheromones are sensed by odorant receptor neurons (ORN) located within the nasal cavity that are embedded within either the main olfactory

epithelium (MOE) or the epithelium of the vomeronasal organ (VNO). Odorant receptor neurons are characterized as having a single dendrite that arises at opposite ends of the cell body; they are termed bipolar neurons, and the dendrite projects MOE or VNO into the lumen of the nasal cavity. At the end of the dendrite is a "dendritic knob", which is differentiated into cilia or microvilli. The cilia have tapered tips and can range in diameter from 10 to 25 nm and length from 5 to 250 um. Some ORN are larger than others and can range in area from 70 to 3500 um^2. This range in size may explain how sensitivity to odorants varies within and between individuals; however, ORN are quite sensitive with a resting potential that ranges from -90 to -30 mV. Electro-chemical current through a single ion channel can generate an action potential, thus a single odorant molecule has the ability to produce a quantal-like current fluctuation in an ORN. Membrane receptors on ORN are classified as G-protein-coupled receptors. Receptor/ligand coupling leads to the propagation of either cyclic adenosine monophosphate (cAMP) or inositol 3-phosphate (IP3) second messenger pathways. In general, these second messenger pathways lead to action potentials by affecting K^+, Na^+, Ca^{++}, and Cl^- ion channels (Schild and Restrepo, 1998). In addition, gaseous messengers can modify the ability of an ORN to generate action potentials by inhibiting or opening ion channels, e.g., nitric oxide inhibits and carbon monoxide can either inhibit or open ion channels (Zufall et al., 2002).

Vomeronasal Organ. A specialized structure involved in olfaction that has been traditionally described as the pheromone sensory organ is known as the VNO. The VNO was first discovered by Jacobson in 1811 and has been described in many vertebrate

species including ruminants (Duvall et al., 1983). The VNO is an encapsulated, bilateral, sensory organ located in the medial-basal portion of the nasal cavity just below the nasal septum. The VNO epithelium consists of elongated glial-like support cells, basal stem cells, and specialized ORN called vomeronasal neurons (VN). Vomeronasal neurons make up the majority of the epithelium and extend dendritic knobs into the VNO lumen; however, unlike regular ORN, VN are devoid of cilia or microvilli (Zufall et al., 2002). Two types of receptors are found on VN within the VNO, V1R and V2R. V1R and V2R neurons are segregated in zones within the VNO; V1R are located in the apical layer while V2R are on the basal layer of the VNO epithelium. Both V1R and V2R receptors are 7–transmembrane receptors; however, N-terminal regions of V2R appear to interact so that two V2R receptors express one binding domain while each V1R receptor expresses one binding domain (Bigiani et al., 2002). Additionally, VN are characterized by long axons that coalesce and run laterally to and project through dorsal aspects of the nasal epithelium to synapse with neurons in the accessory olfactory bulb (Meisami and Bhatnagar, 1998).

Main Olfactory Epithelium. Traditionally, pheromone sensing has been understood to involve ORN within the VNO. Recent evidence indicates that both the VNO and MOE are involved in pheromone sensory pathways. Odorant receptor neurons within the MOE are similar to VN. The major difference is that neurons in the MOE use many different receptors to detect a wide variety of odorant types while the VNO detects only pheromones (Dulac and Torello, 2003). Additionally, axons of ORN from the MOE

bypass the accessory olfactory bulb and synapse with neurons of the main olfactory bulb (Meisami and Bhatnagar, 1998).

Accessory Olfactory Bulb (AOB) and Main Olfactory Bulb (MOB). All herbivores possess an AOB. The AOB is contiguous with but separated neurally from the MOB. The AOB is dorsal and medial to the MOB, which sits just below the frontal cortex of the brain. The AOB is made up of six layers, from outer to inner: the vomeronasal nerve layer, glomerular layer, external plexiform layer, layer of mitral/tufted (MT) cells, internal plexiform layer, and internal granular layer. This laminar organization is the same for the MOB. One major difference between the AOB and MOB is that the AOB is much smaller than the MOB. Also, MT cells are less organized in the AOB than MOB, which supports the idea that the MOB is adaptive or compatible with sensing many different odorant types while the AOB is sorted for instinctive odorant-guided behaviors (Meisami and Bhatnagar, 1998).

Neurons from the vomeronasal system and main olfactory system ultimately excite nerve fibers that follow divergent olfactory tracts within the brain. The vomeronasal system follows the accessory olfactory tract, which projects into the bed nucleus of the stria terminalis and medial aspects of the amygdala called the vomeronasal amygdala. The vomeronasal amygdala has projections into the supraoptic nucleus, medial preoptic nucleus, ventromedial nucleus, and premammarilary nucleus of the hypothalamus. The main olfactory system excites lateral olfactory tract fibers and nerve fibers that project to the anterior hippocampus. The lateral olfactory fibers connect with the primary olfactory cortex, entorhinal cortex, and cortical aspects of the amygdala.

Fibers from the cortical amygdala connect with and stimulate neurons within the premammilary nucleus of the hypothalamus. The main olfactory system connects with the primary olfactory cortex and entorhinal cortex and is thought to be involved with scent awareness and memory. The vomeronasal system does not connect with the cortex, but has connections to four nuclei within the hypothalamus. Due to this arrangement it is thought that the vomeronasal system conveys information that the animal is not aware of and does not remember (Meisami and Bhatnagar, 1998).

Pheromonal Transport

Pheromonal communication between individuals is a complex process that relies upon the transport of semi-volatile compounds from an individual into the environment. The mechanism by which transport occurs may be one of the most important keys to understanding the mechanism and control of pheromonal action. Pheromones can be transported from individuals to the environment through feces, urine, and cutaneous gland excretions (for review, see, Rekwot et al., 2000a). Evidence indicates that bovine pheromones are present in feces and urine (Baruah and Kanchev, 1993; Berardinelli and Joshi, 2005a), or in cervical mucus (Wright et al., 1994). Pheromonal transport has been studied extensively in mice and rats. A review of pheromonal transport in mice and rats may give us insight into pheromonal transport and communication in cattle.

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; Flower et al., 2000; Cavaggioni and Mucignat-Caretta, 2000; Achiraman and Archunan, 2002; Sharrow et al., 2002). Lipocalins are

approximately 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 et al., 2000). Some functions of lipocalins include retinol transport, cryptic coloration, olfaction, enzymatic synthesis of prostaglandins, and pheromonal 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 (Cavaggioni and Mucignat-Caretta, 2000). Two lipocalins have been identified as pheromonal transport proteins; major urinary protein (MUP) and α-2u globulin (Flower, 1996; 2000; Cavaggioni and Mucignat-Caretta, 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 for an extended period of time 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 the specificity of isoform production. The affinity of MUP for specific pheromones also varies with the isoform being produced. 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 (Cavaggioni and Mucignat-Caretta, 2000).

The other known pheromonal transport protein is α-2u globulin (A2U). Alpha-2u globulin is a close homologue 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 pheromonal transport that might occur in cattle. There has been very little study of pheromonal 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 pheromonal transport protein. This study utilized a polyclonal antibody 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, urine of the female elephant is at pH 8.4, similar to the pH of urine from domestic ruminants; whereas, the pH of the vomeronasal 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 intrinsically and extrinsically different from priming-type pheromone transport mechanisms.

Data reported by Izard (1983), Wright et al. (1992), and Berardinelli and Joshi (2005a) indicated that pheromones are involved in bovine reproductive processes related to the transition from anestrous to estrual states. Taken together with the aforementioned description and function of pheromones and transport vehicles found in other species, one

could hypothesize that any bovine pheromonal transport mechanism may involve a protein-pheromone interaction by the sex that produces the pheromone. The pheromone itself is probably a small semi-volatile hydrophobic compound produced by various tissues throughout the body, processed in the liver, transported by the blood and secreted into 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 a metabolite of or bear no relationship to androgenic steroids. For example, cells under the influence of androgens could produce a specific metabolite or product that 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 the 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 or feces could depend on androgens or vice versa.

Pheromones present in the urine or feces 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 the VNO or MOE, or 4) some other as yet undefined mechanism. Clearly, pheromonal transport is a very complex process involving the interaction of many distinct biochemical entities and processes.

Perception of Pheromones

The next point of discussion is how pheromones are perceived by conspecifics. In other words, "What is the mechanism involved with the 'release' of a pheromone(s) from the urine/feces/cutaneous glands into the environment to a site in the VNO or the MOE 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, 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; Boudjelal 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 cow (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) sequenced 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 vomeronasal 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 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, they found a specific membrane receptor for OBP in respiratory tissue, hela cells, cos cells and skin fibroblasts, but not in ciliated olfactory epithelium. These data indicate that the mechanism, by which OBP presents odorants to the olfactory epithelium, does not involve specific membrane receptors and may not be involved with perception of odorants. To date there have been no studies that have successfully demonstrated an interaction between OBP and ORN. However, Ko and Park (2008)

reported recently that OBP increased the sensitivity of ORN to odorants. Therefore, the function of OBP may be to present an odorant to ORN in such a way that it enhances the ability of ORN to bind and respond to odorants.

Summary of Hypothetical Pheromonal Perception and Sensory Pathways

The following is hypothetical summary of how the olfactory system may perceive and sense pheromones and is based on the previous discussion related to sensory pathways of pheromones and pheromonal perception. Odorant binding protein or a small protein that is similar to OBP binds pheromones in the nasal cavity. After binding, OBP must deliver the pheromone and activate MOE or VNO receptor neurons. The mechanisms of MOE and VNO activation are very sensitive (Schild and Restrepo, 1998) and could be stimulated 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 binding of some other small hydrophobic molecule that displaces the ligand(s) present on OBP binding sites. This displacement could then allow the interaction of a pheromone with its receptor neuron population. Odorant receptor neurons have the ability to encode stimulus strength (Keverne, 1999); therefore, it is logical to assume that a stimulation threshold must be reached for the entire subpopulation of neurons to reach action potential threshold and conduct a strong neural 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 either the VNO or MOE (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. Stimulating a primer-type pheromonal response.

Pheromones in Bovine Reproductive Behavior

There is some evidence for signaling-type pheromones related to reproductive processes in the bovine. Two specific compounds have been isolated in urine of estrual cows, di-n-propyl phthalate and 1-iodoundecane, that could possibly be estrus-specific compounds and act as a signal for the bull to detect estrus (Kumar et al., 2000). In this study, researchers compared gas chromatographic profiles from the urine of cows in estrus to urine profiles from cows not in estrus. The authors did not conduct a live animal experiment to confirm that specific compounds from estrual urine were biologically relevant. Additionally, Roelofs et al. (2008) made the observation that cows on farms where bulls were present and cows exposed to fence-line contact with bulls spent more time investigating for the presence of bulls than cows not exposed to fence-line contact with bulls. These observations support the existence of signaling-type pheromones related to reproductive behavior between bulls and cows.

In addition to signaling pheromones, there may be priming-type pheromones. Wright et al. (1994) showed that isolating postpartum, anestrous cows from female herd-mates lengthened the interval from calving to resumption of ovarian cycling activity. However, if isolated cows were exposed to vagino-cervical mucus from estrual cows then the effect of isolation was abolished. Supporting evidence for the existence of priming-

type pheromones in cows comes from a study by Berardinelli and Joshi (2005a). They reported that cows exposed to their own excretory products for 12-h daily had shorter intervals to resumption of ovarian cycling activity than cows not exposed to their own excretory products. These results beg the question as to whether or not bulls also produce priming-type pheromones. The first evidence for a primer-type pheromone produced by bulls comes from an experiment by Izard and Vandenberg (1982). They showed that weekly administration of bull urine to the nasal area of heifers increased the proportion of heifers showing estrus in a 7-week period compared to saline-treated heifers. Similarly, 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 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. Nevertheless, it indicates that something in bull urine influenced the reproductive neuroendocrine-endocrine system of postpartum, anestrous cows. Definitive evidence for bull to cow priming-type pheromones was reported by Berardinelli and Joshi (2005a). In this experiment, cows were exposed continuously to the physical presence of bulls, exposed to excretory products of bulls for 12 h daily, exposed to their own excretory products for 12 h daily, or not exposed to bulls, excretory products of bulls or cows beginning 35 d after calving. More cows exposed continuously to bulls, excretory products of bulls, or their own excreta resumed ovarian cycling activity during the experiment than cows not exposed to bulls, excretory products of bulls or excretory products of cows. Furthermore, cows exposed continuously to bulls or excretory

products of bulls had shorter intervals to resumption of ovarian cycling activity than cows exposed to their own excretory products or 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.

Certain priming-type pheromones in bovine could be “interchangeable” between sexes, that is, have the same function and molecular structure and may be excreted by both bulls and cows. This theory is supported by Burns and Spitzer (1992), who compared intervals from calving to resumption of ovarian cycling activity of postpartum, anestrous cows exposed continuously to the physical presence of bulls, exposed continuously to cows that were previously androgenized over a 30 d period with testosterone enanthate, or not exposed to bulls or androgenized cows. Intervals from calving to resumption of ovarian cycling activity did not differ between cows exposed to bulls and cows exposed to androgenized cows. However, intervals from calving to resumption of ovarian cycling activity were shorter for cows exposed to bulls and adrogenized cows than for cows not exposed to bulls. These data indicate that the biostimulatory effect of bulls could be mediated through an androgen-dependent pheromone(s). Testosterone is produced by the ovaries throughout the estrous cycle of cows. The probable source of testosterone in a cycling cow is the thecal cell layer of the lead or dominant follicle within a follicular wave. Kanchev and Dobson (1976) showed that 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 throughout the remainder of the estrous cycle. Also, Kesler et al. (1979) reported that a marked increase in

testosterone concentration occurred 7 d before behavioral estrus in cycling cows. Therefore, the effect of vagino-cervical mucus of cows reported by Wright et al. (1994), described above, could have been due to an increase in testosterone concentration 7 d before estrus in cycling cows, which may have caused the production of an androgen-dependent pheromone excreted into vagino-cervical mucus of estrual cows. Furthermore, Berardinelli and Joshi (2005a) reported that the interval to resumption of ovarian cycling activity of postpartum anestrous cows was reduced in cows exposed to their own excretory products. In this experiment, cows exposed to their own excretory products were housed in a small enclosed pen for 12 h daily while cows not exposed to excretory products were housed in larger open-shed, outdoor pens 24 h daily. In both treatments some cows began to cycle; however, cows exposed to their own excretory products were forced to be in close proximity to those cows that began showing estrus. These cows may have produced an androgen-dependent pheromone released through the discharge of their excretory products, including vagino-cervical mucus. When progressively more cows began to cycle, the pheromonal signal may have become strong enough to biostimulate anestrous pen-mates similar to that observed in cows exposed to bulls for 24 h daily and cows exposed to the excretory products of bulls. Cows housed in larger open-shed, outdoor pens were not forced to be in close proximity to the androgen-dependent pheromone produced by estrual cows, thus biostimulation of anestrous pen-mates did not occur. These data indicate the possibility that the biostimulatory effect of bulls is mediated by an androgen-dependent pheromone produced by bulls and cows. Furthermore, one might hypothesize that the pheromonal stimulus necessary to induce a

biostimulatory effect has a minimum effective threshold. This threshold must be met or exceeded to accelerate resumption of ovarian cycling activity in postpartum, anestrous cows.

Temporal and Quantal Aspects of the Biostimulatory Effect of Bulls

Based on results from Berardinelli and Joshi (2005a), Tauck et al. (2006) hypothesized that exposing cows to bull urine 24-h daily would mimic the biostimulatory effect of bulls to accelerate resumption of ovarian cycling activity in postpartum, anestrous, suckled cows. However, exposing cows to bull urine for 24-h daily did not cause a reduction in the interval from the start of the experiment to resumption of ovarian cycling activity or increase the proportion of cows that resumed ovarian cycling activity before the breeding season. They concluded that either bull urine was not the excretory product that carried pheromones or that the manner by which pheromones were presented to cows was not appropriate to cause resumption of ovarian cycling activity. This begs the question, "What is the appropriate manner to present pheromones produced by bulls to cows that will cause a biostimulatory effect?"

It is well known that bulls housed with cows for 24-h daily accelerate resumption of ovarian cycling activity in postpartum, anestrous beef cows (Custer et. al., 1990). However, pheromones produced by bulls may not be sensed or perceived by cows 24-h daily even though bulls are continuously housed with cows. Cows exposed to fence-line contact, nose to nose, with bulls resumed ovarian cycling activity sooner than cows not exposed to bulls (Fike et al., 1996), but later than cows housed in direct contact with bulls

(Berardinelli and Tauck, 2007). However, Shipka and Ellis (1998, 1999) reported that dairy cows exposed to bulls across a 6 to 8 m alley way did not accelerate resumption of ovarian cycling activity. Together, these observations indicate that proximity of cows to bulls is an important aspect to consider when evaluating the appropriate manner to expose cows to pheromones produced by bulls when bulls are not in the company of cows. Intuitively one would expect that the proximity of bulls to cows changes throughout the day, so that when bulls come into close contact with cows this is followed by a period wherein bulls and cows are not in close contact with each other and are separated by at least 4 to 8 m. Probably this cycle is repeated multiple times in a 24-h period so that pheromonal stimuli may be sensed and perceived by cows in recurring intervals throughout each day even if bulls are housed with cows for 24-h/d. The possibility exists that the biostimulatory effect of bulls requires a period of pheromonal stimulation, during which pheromones of bulls are in close proximity to cows. This is followed by a period of relaxation, during which pheromones of bulls are not sensed or perceived by cows. Furthermore, this pattern of stimulation and relaxation must be repeated throughout each day.

There is some evidence to indicate that duration of pheromonal stimuli, or number of stimulation and relaxation cycles that cows sense and perceive each day, influences the time required for cows to respond to the biostimulatory effect of bulls. Fernandez et al. (1996) reported that exposing cows to bulls for 2-h every third day for 18 d beginning 33 d after calving did not alter the interval from calving to resumption of ovarian cycling activity for cows compared to cows exposed continuously to bulls. However, postpartum

anestrus was attenuated in cows exposed to the excretory products of bulls 12-h daily (Berardinelli and Joshi, 2005a). These data indicate that the number of days required to accelerate resumption of ovarian cycling activity in anestrous cows decreases as cows perceive pheromones produced by bulls for either longer and longer daily intervals or perceive more and more pheromonal stimulation and relaxation cycles. Furthermore, the minimum duration of daily bull exposure required to accelerate resumption of ovarian cycling activity in postpartum, anestrous cows must be greater than 2-h every 3 d and less than 12-h per day.

The number of days of bull exposure required to cause a biostimulatory effect in postpartum, anestrous cows may change as time after calving increases. Based upon this premise, Berardinelli and Joshi et al. (2005b) evaluated the response of cows to bull exposure starting at 15, 35, and 55 d after calving. All cows exposed to bulls had shorter postpartum anestrous intervals from calving to resumption of ovarian cycling activity than cows not exposed to bulls. However, more cows exposed to bulls starting at D 55 after calving resumed ovarian cycling activity within 20 d of exposure compared to cows exposed to bulls starting on either 15 or 35 d after calving. These data indicate that postpartum, anestrous cows become more sensitive to the biostimulatory effect of bulls as time after calving increases.

In summary, pheromones produced by bulls may activate sensory pathways of cows during a period of pheromonal stimulation when cows are in close proximity to pheromones and sense threshold quantities of pheromones. This stimulation period may be followed by a period of relaxation, during which cows are greater than 4 to 8 m from

pheromones of bulls and sensory quantal thresholds are not met or exceeded. This pattern of pheromonal stimulation and relaxation cycles may be repeated throughout each day. When temporal, duration of daily cycles, and quantal, number of cycles each day, thresholds of stimulation and relaxation are met or exceeded, cows begin to undergo the appropriate neuroendocrine-endocrine changes that are necessary for resumption of ovarian cycling activity. Additionally, there appears to be a threshold number of days, during which temporal and quantal daily thresholds are met or exceeded. Furthermore as time after calving increases the threshold number of days required to cause resumption of ovarian cycling activity may decrease. Therefore, the biostimulatory effect of bulls appears to accelerate resumption of ovarian cycling activity through presentation of pheromones to cows in cycles of stimulation and relaxation throughout each day and over a number of days.

Similarities between the Biostimulatory Effect of Bulls and the Male Effect in Sheep and Goats

The ram effect or "male effect" in sheep (Schinkel, 1954) and goats (Shelton, 1960) is an example of a biostimulatory effect in small ruminants and may be comparable to the biostimulatory effect of bulls. One similarity between the male effect and the biostimulatory effect of bulls is that both involve activation of the HPO axis in females. The following is a synopsis of research in this area reviewed by Gelez and Fabre-Nys (2006). Introduction of males stimulates secretion of LH and ovulation in female sheep and goats during the transition from anestrus to estrus at the beginning of a breeding season. Females exhibit a preovulatory-like surge of LH within 6 to 54 h after the start of

male exposure. Another similarity between the effect of bulls and the male effect is that females in both species exhibit a period of time when they are less responsive to males. The proportion of cows that respond to the biostimulatory effect of bulls within the first 30 days after calving is very low. Similarly, female sheep and goats are not as responsive to the male effect in early seasonal anestrus as they are during mid- to late-seasonal anestrus. An additional similarity between the male effect and the biostimulatory effect of bulls is that both effects appear to be mediated by pheromones released in the excretory products of males. Exposure of ewes to ram urine or wool or does to buck urine can cause the same effect as exposure to the physical presence of males of these species; indicating that pheromones mediate the male effect in sheep and goats (for review, see, Gelez and Fabre-Nys, 2006). Finally, the duration and frequency of pheromonal stimuli may be an important factor to consider in both the biostimulatory effect of bulls and the male effect in sheep and goats. The minimum duration of daily bull exposure must be greater than 2-h every 3 d (Fernandez et al., 1996) and less than 12-h per day (Berardinelli and Joshi, 2005a). Similarly, Hawken et al. (2008) reported that synchrony of estrus was greater in ewes exposed to repeated fence-line contact or physical contact with rams than ewes not exposed to rams. Also, Hawken and Beard (2008) reported that ewes exposed continuously to rams had shorter intervals to estrus than ewes exposed to rams once for 24-h at the start of the experiment (D 0) or ewes exposed repeatedly to rams for 24-h on D 0, 17, and 34 of the experiment. These observations indicate that in these species, pheromonally mediated effects are dependent on quantal and temporal sensory and perceptive thresholds.

The male effect in sheep and goats appears to involve sensory stimulation of both the vomeronasal and main olfactory systems. Ablation of the main olfactory epithelium abolishes the male effect in ewes indicating that the male effect is dependent upon stimulation of the main olfactory system (Gelez and Fabre-Nys, 2006). The presence of rams and ram fleeces induced greater neural activity of GnRH neurons in the pre-optic area and organum vasculosum of the lamina terminalis of the hypothalamus in ewes (Gelez and Fabre-Nys, 2006). Additionally, the male effect appears to activate many neural pathways in brains of ewes. Ewes exposed to rams exhibit greater neural activity throughout the main olfactory and accessory olfactory systems than ewes exposed to ram or ewes fleeces (Gelez and Fabre-Nys, 2006). These data indicate that the presence of rams with ewes leads to activation of more neural pathways in the brain than the presence of ram odor alone. Furthermore the memory, learning, and novelty of male stimuli appear to be involved with the male effect in sheep. Stimulation of the main olfactory system leads to stimulation of learning and memory circuits in the brain; therefore, the male effect of sheep and goats can be enhanced if females have been previously exposed to males (for review, see, Gelez and Fabre-Nys, 2006). Additionally, Hawken and Beard (2008) reported that synchrony of estrus was greater for ewes that were exposed to novel rams every 17 d throughout the experiment than ewes exposed to the same rams throughout the experiment. These data support the conclusion that the male effect in sheep and goats involves learning and memory circuits in the female brain.

In contrast, there are no reports in the literature to indicat that the biostimulatory effect of bulls is influenced or enhanced by novel males or that learning and memory are

involved in this effect. Berardinelli et al. (2005) reported that interval to resumption of ovarian cycling activity and the proportion of cows that resumed ovarian cycling activity before the breeding season did not differ between cows exposed to familiar bulls or cows exposed to unfamiliar bulls. Although these aspects, learning and memory, of the male effect in sheep and goats do not appear to be consistent with the biostimulatory effect of bulls, similarities between the biostimulatory effect of bulls and the male effect in sheep and goats may yield insight into the pheromonal sensory and neural pathways involved with the biostimulatory effect of bulls.

Another similarity between the biostimulatory effect of bulls and the male effect in sheep is that exposing heifers to bulls or ewe lambs to rams accelerates the onset of puberty. In sheep this effect is physiologically similar to that observed in adult ewes. Introduction of rams increased LH pulsatility in prepubertal ewe lambs during the non-breeding season but did not cause ovulation (Ungerfeld et al., 2004). However, ram exposure shortly before the breeding season increased the number of prepubertal lambs that exhibited ovulation (Ungerfeld et al., 2004). Data from numerous studies indicate that exposure of heifers to bulls before the breeding season does not accelerate puberty (Berardinelli et al., 1978; Macmillan et al., 1979; Roberson et al., 1987; Wehrman et al., 1996). On the other hand there are two reports that indicate that under certain circumstances bull exposure accelerates onset of puberty in heifers. Roberson et al. (1991) evaluated the interaction between growth rate and bull exposure in prepubertal beef heifers. In their experiment they found that heifers fed at a high plane of nutrition and exposed to bulls attained puberty sooner than heifers fed a high plane of nutrition and

not exposed to bulls. However, age of puberty did not differ between heifers fed a medium plane of nutrition and exposed to bulls and heifers not exposed to bulls and fed a high or medium plane of nutrition (Roberson et al., 1991). Also, Rekwot et al. (2000b) reported that age of puberty was reduced in 15-month old *bos indicus* heifers exposed to bulls for 15 months compared to heifers not exposed to bulls. It appears that the biostimulatory effect of bulls and the ram effect accelerate onset of puberty in young female ruminants, but the effect appears to be dependent on growth rate and genetic background. The mechanism for the male effect in prepubertal lambs and the biostimulatory effect of bulls in prepubertal heifers is not known or well understood. The assumption is that these effects on acceleration of puberty are mediated pheromonally and involve a change in the HPO axis similar to that observed in the neuroendocrine-endocrine system of postpartum, anestrous cows or seasonally anestrous ewes exposed to males.

Hypothalamic-Pituitary-Adrenal (HPA) Axis and Male Pheromones

In rodents there appears to be a clear functional relationship between the HPA axis and pheromone-induced activation or inhibition of female reproductive events. Nichols and Chevins (1981) reported that female mice housed individually and exposed to male urine had greater basal concentrations of corticosterone than female mice housed individually and exposed to water. Furthermore, mean concentrations of corticosterone were greater in female mice 30 min after exposure to male urine than 30 min after exposure to water. Marchlewska-Koj and Zacharczuk-Kakietek (1990) reported that

concentrations of corticosterone increased sharply after female mice were transferred into cages soiled with male urine and feces. Subsequently, Ma et al. (1998) and Mora and Sanchez-Criado (2004) demonstrated that adrenalectomy abolished the effect of male urine to stimulate resumption of normal estrous cycles in aging females, while replacement therapy with corticosterone restored the responsiveness of aging females to male urine. More recently, Wyart et al. (2007) collected saliva samples before and after women were instructed to sniff a vial containing a putative male pheromone (androstadienone) 20 times over a 15-min period. They reported that salivary concentrations of cortisol were greater after than before sniffing androstadienone. These results indicate that olfactory (pheromone) activation of the HPA axis could be involved in triggering the events that initiate or re-enforce the activation of the hypothalamic-pituitary-ovarian axis to alter social and reproductive events in females.

Cortisol Synthesis, Secretion, and General Physiological Effects

The glucocorticoid, 11β, 17α, 21-trihydroxy-pregn-4-ene-3, 20-dione or cortisol, is a 21-carbon steroid molecule that is synthesized in and secreted from the zona fasiculata layer of the adrenal cortex. Low density lipoprotein (LDL) delivers cholesterol to zona fasiculata cells by binding to LDL receptors on these cells. Adrenocorticotropic hormone (ACTH) stimulates endocytosis of LDL to provide a pool of cholesterol within cells. Once inside the cell, cholesterol esterase acts on LDL to increase concentrations of free cholesterol within cells. Steroid transport activating receptor (STAR) protein, activated by ACTH binding, transports cholesterol to the inner-mitochondrial membrane.

Once cholesterol is inside the mitochondria, key enzymes known as the desmolase system (or cytochrome p450scc) convert it to pregnenolone. Pregnenolone is then transported to the cell cytoplasm, where it is converted to cortisol by a series of enzymatic conversions. 17α-hydroxylase converts pregnenolone to 17α-hydroxypregnenolone, which is converted to 17α-hydroxyprogesterone by 3 β-OH-dehydrogenase and Δ^5 Δ^4 isomerase. 21-Hydroxylase acts on 17α-hydroxyprogesterone to make 11-deoxycortisol, which is converted to cortisol by 11β-hydroxylase. Cortisol is then secreted from the cell into the blood where it binds to its transport molecule known as corticosteroid binding protein (CBP) or α-2 globulin produced in the liver. Only about 6% of cortisol is free or unbound in the blood at any one time. Cortisol in the unbound form is free to cause physiological effects or be metabolized by conjugation with glucuronic acids or sulfates and excreted (Hadley and Levine, 2006). In addition, many tissues have the ability to oxidize cortisol to its inert form, cortisone, by two isoforms of 11-β hydroxysteroid dehydrogenase (11-βHSD). Type 1 11-βHSD is an NADPH-dependent enzyme, which has the ability to convert cortisol to cortisone and cortisone to cortisol, while type 2 11-βHSD is an NAD^+ dependent enzyme and exclusively converts cortisol to cortisone (Thurston et al., 2003).

Circulating concentrations of cortisol are controlled primarily by the hypothalmo-pituitary-adrenal axis. Neuronal input from higher brain centers stimulates neurons in the paraventricular nucleus (PVN) of the hypothalamus to secrete corticotropin-releasing hormone (CRH), which stimulates ACTH secretion from corticotrophs of the anterior pituitary. ACTH stimulates the G-protein coupled receptor melanocortin 2 (MC2) in

zona fasiculata cells of the adrenal cortex to cause an increase in the activity of protein kinase A (PKA) and production of cyclic adenosine monophosphate (cAMP). Stimulation of cAMP production and activation of PKA appears to concurrently activate cholesterol ester hydrolase and STAR to increase both the production and secretion of cortisol. Additionally, an increase in cAMP production stimulates the accumulation of insulin-like growth factor II, which stimulates hypertrophy and hyperplasia of the adrenal cortex. Once cortisol is released into circulation it has a negative feedback effect to decrease ACTH secretion at the level of the pituitary, hypothalamus, and higher brain centers (Hadley and Levine, 2006).

In most mammals, concentrations of cortisol appear to follow a circadian rhythm that is a result of "imprinting" by maternal adrenal steroids, and in human beings appears to be set 3 months after birth and cannot be altered by changing light conditions. This circadian rhythm is regulated by neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus (Hadley and Levine, 2006). In cattle, daily cortisol concentrations follow a less pronounced circadian rhythm (Wagner and Oxenreider, 1972) and a more notable ultradian rhythm (Lefcourt et al., 1993). The circadian rhythm is characterized by cortisol concentrations reaching a zenith at 0530 h and nadir at 1800 h (Lefcourt et al., 1993). The ultradian rhythm is characterized by one cortisol pulse every 120 min (Lefcourt et al., 1993). In addition, Leining et al. (1980) reported that as photoperiod increased, concentrations of circulating glucocorticoids decreased in bulls. In agreement with these results are those of Berardinelli et al. (1992) who reported that mean, baseline, and amplitude of cortisol decreased from fall to spring as photoperiod decreased,

followed by an increase from spring to fall when photoperiod was increasing, in both *bos taurus* and *bos indicus* bulls. Therefore, in addition to daily rhythms, systemic patterns of cortisol appear to follow seasonal rhythms.

The effects of cortisol are mediated by both genomic and non-genomic activation of receptors in cells of target tissues. It is widely accepted that the genomic effects of cortisol are caused by cortisol binding to receptor complexes in the cytoplasm. After binding, these cortisol-receptor complexes are translocated to the nucleus where they bind specific recognition sites on DNA. However, in recent years there is progressively more evidence that cortisol has many non-genomic mechanisms of action. Non-genomic mechanisms are characterized by rapid response (< 5 min), and responses that are resistance to receptor blockade and independent of protein synthesis (Stahn et al., 2007).

Cortisol has many physiological roles. The first and probably most important physiological effect of cortisol is to sustain and permit the activity of catecholamines by stimulating re-uptake and blocking degradation in the synapses between neurons. Under stressful conditions vascular collapse and death will occur if glucocorticoids are not present to permit the actions of catecholamines. Secondly, cortisol acts to modulate intermediate metabolism by increasing gluconeogenesis in the liver, decreasing glucose uptake by adipose and muscle tissue, increasing proteolysis and lipolysis, and decreasing the affinity of insulin to cellular receptors. Also, cortisol has immuno-suppressant and anti-inflammatory roles that serve to shut down or reduce the inflammatory response. Additionally, cortisol is involved with maintaining the structure and integrity of the brain and may be involved with such functions as the establishment of learning and memory

circuits in the hippocampus and arousal circuits in the amygdala. Lastly, cortisol has important physiological roles in reproduction. Infusions of ACTH or dexamethasone can cause premature parturition, indicating that cortisol is a critical component of the timing of birth. Correlated with parturtion cortisol works concurrently with prolactin to stimulate lactogenesis in mammary glands (Hadley and Levine, 2006). Cortisol has been associated with suppressive effects on LH response to gonadotropin releasing hormone (GnRH) secretion from the hypothalamus. The molecular mechanisms by which cortisol accomplishes this effect are apparently mediated by glucocorticoid receptors in cells of the anterior pituitary. Cortisol binding to glucocorticoid receptors in the anterior pituitary does not appear to decrease GnRH receptor expression; rather, cortisol decreases the ability of gonadotrophs to release LH in response to GnRH (Breen et al., 2007; 2008).

Interaction between the HPA Axis and HPO Axis

Prolonged stress has long been identified as having a negative effect on reproduction (Dobson and Smith, 2000). As a result, any changes in hypothalmo-pituitary-adrenal (HPA) axis function or cortisol concentrations are almost always interpreted in a negative context because the effects on reproductive events are generally inhibitory. The following is a review of how changes in HPA axis function and/or regulation and cortisol concentrations affect the function of the hypothalamic-pituitary-ovarian (HPO) axis and factors that may influence how the HPO axis responds after stress related events: HPA activation by ACTH; and/or infusion of cortisol.

The hypothesis that activation of the HPA axis and an increase in circulating cortisol concentrations inhibits HPO axis function has been a subject of investigation since the early 1970's. Perhaps Short et al. (1973) were the first to test this hypothesis in beef cows. In this experiment, LH surges were induced in eighteen ovariectomized (OVX) cows by administering 10 mg of estradiol-17β (E_2) to each cow. Six cows received 100 mg of progesterone (P_4) and six received 100 mg of cortisol acetate injected four times at 12-h intervals starting 12-h before the E_2 injection. All eighteen cows had a preovulatory-like LH surge 16 to 24 h after E_2 injection. More cortisol-treated cows displayed estrus than progesterone-treated cows; however, the interval to estrus did not differ between cows in these treatments. These results indicated that an LH surge induced by E_2 is not suppressed by exogenous cortisol; however, this type of treatment does not exclude the possibility that activation of the HPA axis through ACTH could inhibit the function of the HPO axis. Matteri and Moberg (1981) tested this possibility by injecting cows with either ACTH or cortisol and measuring the LH surge after administration of GnRH. They found that exogenous cortisol did not inhibit the LH surge after GnRH injection, on the other hand, exogenous ACTH inhibited LH response to GnRH. These data indicated that activation of the HPA axis at the level of the pituitary is responsible for the negative effects of stress on HPO axis function. Activating the HPA axis through stress may have similar negative effects on HPO axis function. A subsequent study by the same laboratory investigated the possibility that stress during the follicular phase of the estrous cycle may inhibit LH secretion, thereby negatively affecting HPO axis function. Stoebel and Moberg (1982a) stressed seven

dairy heifers by shocking them with an electric hand-held prod for 1 sec every 2 min, for 15 min twice daily, 6 h apart, for four days, after heifers were treated with P_4 and E_2 to initiate estrus. Stressing heifers in this manner resulted in an increase in circulating cortisol from 9 ng/mL before stress to 55 ng/mL after stress. Stressing heifers did not affect baseline concentrations of LH, or amplitude and frequency of LH pulses in heifers that displayed estrus; however, two of the seven heifers did not display an LH surge. These data indicated that HPA activation through stress inhibited function of the HPO axis. This conclusion was further supported by Dobson et al. (1999) who transported ewes for 4 h and measured the ability of ewes to generate an LH surge during and after transportation. They found that transportation stress caused a delay in the onset of the LH surge. Together, these results indicate that activation of the HPA axis by exogenous ACTH and/or inducing a stress response inhibits HPO axis function. However, the end result of HPA axis activation, that is, increased circulating cortisol concentrations, does not necessarily inhibit function of the HPO axis.

In contrast, results from well-designed experiments indicate that cortisol alone can have very similar inhibitory effects on circulating concentrations of LH as activating of the HPA axis. Stoebel and Moberg (1982b) injected ACTH at 12-h intervals for 3.5 d and infused cortisol succinate into the jugular vein for 90-h during the follicular phase of the estrous cycle in Holstein heifers. Both ACTH and cortisol infusion delayed or inhibited estrous behavior, but cortisol infusion did not decrease basal concentrations of E_2 or LH. Li and Wagner (1983) reported that cortisol decreased the ability of cultured pituitary cells to release LH in response to GnRH and that inducing a hyperadrenal state

in heifers delayed the occurrence of an LH peak after GnRH administration. Pamanabhan et al. (1983) reported that cortisol administration decreased and ACTH did not affect LH secretion after GnRH was administered to the media of cultured pituitary cells. Daley et al. (1999) investigated the effect of infusing ewes with cortisol during the follicular phase of the estrous cycle. Cortisol infusion increased circulating cortisol concentrations to 64 ng/mL and suppressed both the E_2 rise before the LH surge and the onset of the LH surge. Subsequently, McFarlane et al. (2000) reported that cortisol infusion increased circulating cortisol concentrations to greater than 60 ng/mL and inhibited LH secretion in ewes during the luteal and follicular phases of the estrous cycle. More recently, Breen and Karsch (2006) investigated the effect of cortisol infusion on patterns of LH concentrations in OVX ewes at different times of the year. They reported that cortisol concentrations rose to 150 ng/mL after infusion of cortisol and suppressed LH pulse amplitude and frequency in OVX ewes during the breeding season, the transition from the breeding season to anestrus, and during anestrus. In summary, these results indicate that cortisol inhibits the release of LH at the level of the pituitary and appears to reduce the ability of the HPO axis to function properly.

Examination of the literature in this area yields divergent opinions as to the nature of the interaction between activation of the HPA axis and function of the HPO axis. One possible explanation for the inconsistent effects of cortisol on function of the HPO axis could be that the pituitary has the ability to release LH in response to GnRH when it is subjected to “low” intensity stressors that allow animals to habituate to stress. However, “high” intensity stressors stimulate a dramatic increase in cortisol concentrations via

CRH-stimulated ACTH release, which an animal cannot become inured to, and cause an irreversible chronic stressful state, during which luteotrophs are insensitive to GnRH and LH release is suppressed. That this may be the case is supported by Pitman et al. (1988) who reported that the ability of rats to habituate to stress depended on the intensity of the stressor. A chronic stressful state was induced in rats that were subjected to a high intensity stressor, restraint of all four legs, whereas, rats subjected to a low intensity stressor, restrained in buckets, habituated to this stressor within 3 d. Additionally, Ladewig and Smidt (1989) reported that bulls housed in deep straw and untethered had lower mean cortisol concentrations than bulls tethered and housed on open-slatted floors. However, when bulls were subjected to ACTH challenge they found that cortisol concentrations were greater in bulls housed in deep straw and untethered than in bulls tethered on open slatted floors. An interpretation of these results may be that bulls were more habituated to an ACTH-driven response to stressors if they had been previously exposed to low intensity stressors.

That this interpretation may be valid is supported by data reported by Echternkamp (1984). He showed that cows habituated to handling procedures had lower mean cortisol concentrations and greater mean concentrations of LH and frequency of LH pulses than cows that were not habituated to handling procedures before the intensive blood sampling period. Furthermore, temporal patterns of LH concentrations were not affected in cows that exhibited a rise in mean cortisol concentrations of 10 to 20 ng/mL. However, mean concentrations of LH and frequency of LH pulses were significantly decreased in cows that displayed a 10 to 20 fold increase in mean cortisol concentrations.

Thus, the HPO axis was not inhibited in cows that had habituated to the stress of intensive blood sampling. Additionally, Turner et al. (2005) reported that repeated acute stress does not adversely affect estrous behavior, ovulation rates, and pregnancy rates in female pigs. Furthermore, Thun et al. (1998) reported that subjecting cows to an acute low intensity stress, hoof restraints, increased mean cortisol concentrations from 4 ng/mL to 20 ng/mL but did not alter characteristics of temporal patterns of LH concentrations. Taken together, the results of Echternkamp (1984) and Thun et al. (1998) in cows and Turner et al. (2005) in pigs indicate that domestic females appear to habituate to stress and the function of the HPO axis is not inhibited if they are subjected to low intensity stressors applied over a short term. Finally, Breen et al. (2005) investigated the effect of infusing cortisol to ewes in the mid-follicular phase of the estrous cycle at one-half maximal or the maximal level induced by isolation-restraint (IR) stress. Cortisol infusion at the maximal level inhibited frequency of LH pulses, delayed the rise in E_2, and blocked or delayed the preovulatory FSH and LH surges. On the other hand, infusion of cortisol at half maximal levels did not inhibit these variables to the extent of maximal infusion. Again, these data indicate that the influence of cortisol concentrations or activation of the HPA axis on functions of the HPO axis depends on the intensity of the stressor and whether or not animals have been given the opportunity to habituate to that stressor.

Another possible explanation for the disparity associated with the effects of cortisol on functions of the HPO axis is that the hormone milieu in total at the time of stress may influence the effect of cortisol on function of the HPO axis. Daley et al. (2000) tested this possibility by measuring characteristics of LH concentration patterns in

wethers infused with either E_2 or cortisol separately, or concurrent infusion of E_2 and cortisol. As expected cortisol infusion increased mean concentrations from 22 ng/mL to 68 ng/mL; however, cortisol or E_2 infused separately did not inhibit LH concentration patterns. In contrast, cortisol infused in combination with E_2 decreased LH mean concentrations and LH pulse frequency. These data indicate that activation of the HPA axis and/or cortisol interacts with E_2 to enhance the negative feedback effect of E_2 on LH pulse frequency. Tilbrook et al. (1999) used a similar method to investigate the interaction of sex steroids with HPA activation on LH concentration patterns in gonadectomized and intact ewes and rams subjected to IR stress. Gonadectomized rams were pre-treated with testosterone and gonadectomized ewes were pre-treated with P_4 only, E_2 only, or P_4 in combination with E_2 before IR stress. Isolation-restraint stress increased cortisol in rams from 10.9 to 28.8 ng/mL and in ewes from 7.9 to 22.3 ng/mL and inhibited function of the HPO axis in both intact and gonadectomized ewes and rams. Luteinizing hormone amplitude but not frequency was lower after than before IR stress in rams pre-treated with testosterone. Control rams exhibited a decrease in frequency of LH pulses during IR stress, but amplitude of LH pulses was not affected. Additionally, IR stress decreased LH pulse frequency in control ewes; amplitude and frequency of LH pulses in ewes pre-treated with E_2; frequency of LH pulses in ewes pre-treated with P_4; and amplitude of LH pulses in ewes pre-treated with P_4 and E_2. These data indicate that cortisol may enhance the negative feedback effect of E_2 on the hypothalamus to decrease frequency and amplitude of LH pulses. Futhermore, Stackpole et al. (2006) investigated the effect of infusion of high and low doses of cortisol into gonadectomized ewes and

rams and low-dose cortisol infusion in intact ewes and rams. In gonadectomized ewes and rams infused with either high or low doses of cortisol, amplitude and frequency of LH pulses were decreased. However, cortisol did not affect characteristics of LH concentration patterns in intact rams and ewes. These data indicate that gonadectomized rams and ewes are more sensitive to the negative effects of cortisol on the HPO axis. The reasons for these observations may be that the steroid milieu differs between gonadectomized and intact sexes. The effects of cortisol on the HPO axis seem to be attenuated in the presence of gonads and their products.

The interaction between the physiological state of animals and HPA axis activation on function of the HPO axis was summarized in an editorial by Levine (2002). The salient points identified by the author were: 1) females exhibit changes in basal HPA activity and HPA activity in response to stressors during the estrous cycle, with the highest levels of cortisol in the pre-ovulatory period; 2) cortisol binding protein (CBP) is higher in females than males to insure that cortisol rapidly returns to baseline after stimulation by CRH-induced ACTH release; 3) CRH may inhibit GnRH release directly; 4) glucocorticoids inhibit reproductive hormone secretions at the level of the pituitary; 5) glucocorticoids inhibit gonadal steroid secretion and actions at target tissues; and, 6) testosterone in males inhibits HPA activity by decreasing CRH expression while E_2 in females has the opposite effect by stimulating CRH transcription through activation of E_2 receptors. The literature as a whole indicates that the stage of estrous cycle in females, sex of the animal, and the hormonal milieu at the time a stressor is applied influences the

effects that activation of the HPA axis or cortisol infusion have on function of the HPO axis.

Hypothalamic-pituitary-adrenal axis function and/or regulation and circulating concentrations of cortisol may have direct effects on ovarian function in addition to the effects on hypothalamic and pituitary aspects of the HPO axis. Glucocorticoids have an important role in the ovulatory process by inhibiting or attenuating the inflammatory response associated with ovulation. Cortisol concentrations are ten times greater in follicular fluid from ovulated follicles than in serum (Andersen, 2002). This increase could be a result of: 1) displacement of cortisol from CBP by 17α-OH-progesterone; 2) a decrease in the rate of conversion of cortisol to cortisone by type-2 11β- hydroxysteroid dehydrogenase; and, 3) activation of proteolytic enzymes necessary for ovulation may free cortisol from CBP (Andersen, 2002). Acosta et al. (2005) reported that the concentration of free cortisol is greater in the follicular fluid of preovulatory follicles than in anovulatory follicles in cows. These results indicate that "high" local cortisol concentrations may be required for final maturation and ovulation of dominant follicles in the cow. There is the possibility that activation of the HPA axis could support follicular uptake of cortisol in the ovary and be involved with final maturational steps of follicular development.

In summary, activation of the HPA axis has clear negative effects on the function of the HPO axis if animals are subjected to high intensity stressors or injected with ACTH. In contrast, if cortisol concentrations are relatively "low" and animals have the ability to adapt to low intensity stressors there is no apparent negative effect on function

of the HPO axis. However, the sex, physiological state, and hormonal milieu of the animal at the time of stress can dramatically influence the effect of HPA activation on function of the HPO axis. Additionally, cortisol appears to be involved with follicular development and ovulation. Therefore, altering adrenal function and/or regulation may support or inhibit ovarian function.

Effect of Suckling and Presence of Offspring on the HPA Axis

Suckling appears to influence regulation and/or function of the HPA axis. Smith and Vincent (1972) and Wagner and Oxenreider (1972) were the first to report that cortisol increases after suckling bouts in the cow. Subsequently, Ellicott et al. (1981) reported that cortisol increased within 10 min after the start of suckling and remained significantly higher than pre-suckling concentrations for 40 min, with peak cortisol concentrations occurring 20 min after the start of a suckling bout in young beef cows. Similarly, Dunlap et al. (1981a) reported that cortisol increased within 30 min after initiation of suckling and returned to basal concentrations within 45 min after initiation of suckling in postpartum beef cows. Additionally, Hoffman et al. (1996) reported that after postpartum, anestrous cows were reunited with calves, cortisol concentrations increased sharply and fell to basal levels after 30 min. These data indicate that lactation and the act of suckling stimulate activation of the HPA axis and increase circulating concentrations of cortisol in postpartum beef cows.

In contrast, Faltys et al. (1987) reported cortisol concentrations did not differ before and after cows were suckled. In addition, Tilbrook et al. (2006) reported that

cortisol concentrations and ACTH concentrations in response to IR stress were greatest in non-lactating ewes and lowest in lactating ewes that had lambs present and were allowed to be suckled. Additionally, cortisol and ACTH concentrations after IR stress were greater in lactating ewes if lambs were absent than in ewes with lambs present but unable to suck and cortisol and ACTH concentrations after IR stress was greater in lactating ewes with lambs present but unable to suck than lactating ewes with lambs present and able to suck. The interpretation of these results is that lactation, the presence of lambs with ewes, and the act of suckling interact to suppress activation of the HPA axis in response to stress.

At first glance, these results seem contradictory and confusing because suckling increased concentrations of cortisol in cows from data reported by Smith and Vincent (1972), Wagner and Oxenreider (1972), Ellicott et al. (1981), and Dunlap et al. (1981a). On the other hand, suckling had no effect on concentrations of cortisol in cows from data reported by Faltys et al. (1987) and suckling and the presence of lambs appeared to inhibit activation of the HPA axis in ewes from data reported by Tilbrook (2006). The conflicting observations of these experiments could be explained by the amount of time offspring were separated from dams before suckling bouts. Smith and Vincent (1972), Wagner and Oxenreider (1972), Ellicott et al. (1981), and Dunlap et al. (1981a) removed calves from cows for 12-h and reported that suckling increased cortisol concentrations in cows after calves were re-united with cows. However, Faltys et al. (1987) removed calves from cows for 6-h and reported that cortisol concentrations in cows did not differ

before and after re-uniting pairs. Therefore, activation of the HPA axis in response to suckling may depend upon how long calves are removed from cows.

The effect of suckling on function and/or regulation of the HPA axis may depend on how accustomed cows are to the presence of their calves. Stevenson et al. (1994) reported that cortisol concentrations were greater before calves were returned to cows after 12-h removal in mastectomized and udder-intact cows that had previous unrestricted contact with calves (CP) than in mastectomized cows that had restricted contact with calves (RP). Furthermore, cortisol concentrations decreased after calves were reunited with CP cows and did not change after calves were reunited with RP cows. These data indicate that cortisol concentrations decreased after cows were reunited with calves if cows were accustomed to the presence of their own calves. However, reuniting calves with cows did not effect cortisol concentrations if cows were not accustomed to the presence of their own calves.

Overall, these data indicate that suckling and reuniting offspring with dams influenced the HPA axis and increased circulating concentrations of cortisol if dams were separated from offspring for 12 hours. However, cortisol concentrations were not influenced; by the act of suckling if offspring were housed continuously with dams, by reuniting dams with offspring if separated for 6 hours, and if cows were not accustomed to the presence of their own calves.

CHAPTER 3

STATEMENT OF THE PROBLEM

As pointed out in the review of literature, the biostimulatory effect of bulls appears to be mediated by pheromones that influence reproductive behavior and function of cows. In mammals, male pheromones that affect female reproductive behavior or function are usually excreted in urine (for review, see; Izard, 1983). However, exposing cows to bull urine 24-h daily did not cause a reduction in the interval from the start of the experiment to resumption of ovarian cycling activity or increase the proportion of cows that resumed ovarian cycling activity before the breeding season (Tauck et al., 2006). One interpretation of this observation may be that bull urine is not the excretory product that carries pheromones; however, a more probable explanation may be that the manner, by which urinary pheromones were presented to cows, 24-h daily, was inappropriate to stimulate activity of the HPO axis and accelerate resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows.

Also from the review of literature, it appears that in rodents, the function and/or regulation of the HPA axis is an integral component of male pheromone stimulatory pathways that alter reproductive behavior and/or function in females. From these observations, it was proposed that pheromones produced by bulls may alter adrenal function and/or regulation of cows. When pheromones produced by bulls are presented to cows in an appropriate manner, activity of the HPA axis may support, facilitate, or stimulate HPO axis function and resumption of ovarian cycling activity in postpartum, anestrous, suckled cows.

The goals of this research were: 1) to determine if the HPA axis is involved with the physiological response of cows to pheromones produced by bulls, 2) if so, to define the role of the HPA axis in the biostimulatory effect of bulls on the function of the HPO axis and resumption of ovarian cycling activity, and 3) to determine if duration of daily bull exposure is a factor involved with the appropriate manner whereby bull-pheromonal stimuli accelerates resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows.

CHAPTER 4

EXPERIMENT 1: ADRENAL INVOLVEMENT IN THE BIOSTIMULATORY EFFECT OF BULLS

Introduction

Stress and stress hormones have long been associated with negative effects on reproductive activities. Stress associated release of cortisol and ACTH injections have been shown to cause a decrease in gonadotropin (LH and FSH) release in both cattle (Dunlap et al., 1981a) and sheep (Breen et al., 2005). However, Turner et al. (2005) reported that repeated acute stress does not adversely affect estrous behavior, ovulation rates, and pregnancy rates in female pigs. In contrast there is an indication that adrenal activation is involved in the response of females to male pheromones. Male urine sprayed into the nasal cavity of ovariectomized female rats stimulated an ACTH release causing the adrenal release of progesterone and corticosterone (Mora and Sanchez-Criado, 2004). Furthermore, adrenalectomy attenuated the resumption of normal cycling activity in female rats exposed to male pheromonal stimuli (Mora and Sanchez-Criado, 2004) indicating that adrenal activation may be involved in the physiological response of females to males pheromonal stimuli.

The objective of this study was to determine if systemic cortisol concentrations are associated with the biostimulatory effect of bulls on resumption of luteal function in primiparous, postpartum, anestrous, suckled, beef cows. In Trial 1, cows were exposed to either the continuous physical presence of bulls or not exposed to bulls. In Trial 2, cows were continuously exposed to either bull urine or steer urine.

Materials and Methods

Two trials were conducted at the Montana State University Bozeman Area Research and Teaching Facility. Animal care, handling, and protocols used in these experiments were approved by the Montana State University Agricultural Institutional Animal Care and Use Committee. Trials 1 and 2 were performed in 2003 and 2004, respectively.

Animals and Treatments

Trial 1. Twenty-eight spring-calving two-yr-old Angus X Hereford primiparous, postpartum, suckled, beef cows and four mature, epididymectomized Angus X Hereford bulls were used in this experiment. 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. Cows averaged 58 d postpartum at the start of the experiment (D -30), thirty days before the start of the breeding season (D 0). One week before the start of treatment cows were stratified by calving date, cow BW, calf birth weight, calf sex ratio, dystocia score, and BCS. Once cows were stratified they were assigned randomly to one of two treatments; exposure to mature bulls (BE; n = 13) or no bull exposure (NE; n =15).

Trial 2. Thirty-eight two-yr-old Angus X Hereford primiparous, postpartum, suckled, beef cows, four Angus X Hereford epididymectomized bulls, and four 1-yr-old Angus X Hereford steers were used in this experiment. 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 and at the start of the experiment, March 21, cows averaged 40 d postpartum. Two weeks before treatment started cows were stratified by calving date, cow BW, calf birth weight, calf sex ratio, dystocia score, and BCS and fitted with a controlled urine delivery device (CUDD; Tauck et al., 2006). Cows were then assigned randomly to either steer urine exposure (SUE; n = 19) or bull urine exposure (BUE; n = 19).

Animal Housing Areas (Trials 1 and 2)

At the start of each trial cows were moved from a common pasture area into two lots, designated north and south by their geographic location. Each lot contained four pens (41 m x 18 m) that were similar in east-west configuration, bunk space, aspect, slope, and connection to open-shed shelters. Cows exposed to bulls (BE; Trial 1) and cows exposed to bull urine (BUE; Trial 2) were housed in the north lot, cows not exposed to bulls (NE; Trial 1) and cows exposed to steer urine (SUE; Trial 2) were housed in the south lot. Cows were allowed to move between two pens within each lot. Lots were approximately 0.35 km apart. These lots and arrangements have proven to be effective in previous experiments involving bull-cow interactions (Berardinelli and Joshi, 2005a).

In Trial 1, bulls were housed with BE cows. In Trial 2, bulls and steers were housed away from cows in two separate pens approximately 80 m apart and in a separate lot area north of the lots that housed cows by approximately 0.4 km.

Bull Exposure (Trial 1)

Pens within the north lot were used for maintaining cows exposed to bulls (BE), while pens within the south lot were used for maintaining cows not exposed to bulls (NE). Cows assigned to either BE or NE treatments were placed into pens on D -30. Bull to cow ratio per pen was approximately 1:7.

Bull and Steer Urine Exposure (Trial 2)

Pens within the north lot were used for maintaining cows exposed continuously to bull urine (BUE), while pens within the south lot were used for maintaining cows exposed continuously to steer urine (SUE). Continuous exposure of cows to bull and steer urine was accomplished with a CUDD. Details of the components and construction of CUDDs, and urine collection stanchions, urine collection devices, handling of urine from bulls and steers, and filling of CUDDs are given in Tauck et al. (2006).

Nutrition (Trials 1 and 2)

Cows had free access to good quality, chopped mixed-grass alfalfa hay, and any pasture grasses that were available before the start of each experiment. Once cows and calves were moved into pens they were given free access to the same hay, 0.5 $kg \bullet hd^{-1} \bullet d^{-1}$ 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). In Trial 1, bulls had access to the same diet as cows. In Trial 2, bulls had ad libitum access to medium 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 (Trials 1 and 2)

To determine resumption of ovarian cycling activity, 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 in duplicate using solid-phase RIA kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) validated for bovine serum in our laboratory (Custer et al., 1990). Intra- and interassay CV for a serum pool that contained 0.42 ng/mL were < 10.0%, respectively; and for a pool that contained 3.1 ng/mL were < 7.0%, respectively, for blood samples from Trial 1. Intra- and interassay CV for a serum pool that contained 2.6 ng/mL of progesterone were 0.4 and 7.4%, respectively; and 3.4 and 11.0%, respectively, for a pool that contained 7.5 ng/mL in Trial 2. Progesterone concentration patterns were used to determine the occurrence of resumption of ovarian cycling activity and the intervals from the start of treatment to resumption of ovarian cycling activity. An increase of progesterone concentration, above the average progesterone baseline of individual cows in three consecutive samples that exceeded 1 ng/mL was used to determine the occurrence of resumption of ovarian cycling activity. Intervals from the start of treatment to resumption of ovarian cycling activity were determined by the number of days from the treatment to the lowest inflection point before a rise in three consecutive samples that exceeded 1 ng/mL. Cows that failed to exhibit a

rise in progesterone over three consecutive samples were assigned an interval from the start of treatment to the end of treatment.

Changes in overall mean concentrations of cortisol were determined by blood samples collected in equally spaced intervals throughout either bull or bull urine exposure periods in Trial 1 and 2. Blood samples obtained on Days 0, 8, 16, and 24 in Trial 1 and Days 0, 19, 38, and 57 in Trial 2 were assayed for cortisol concentrations using a solid-phase RIA kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) validated for bovine serum in our laboratory (Berardinelli et al., 1992). Intra- and interassay CV for a serum pool that contained 0.08 ng/mL were 4.8 and 11%, respectively; and for a pool that contained 7.0 ng/mL were 2.0 and 5.4%, respectively.

Statistical Analyses

Intervals from the start of treatment to resumption of ovarian cycling activity were analyzed by ANOVA for a completely randomized design using PROC GLM of SAS. Proportions of cows that resumed ovarian cycling activity by the end of the exposure period were analyzed by chi-square analyses using the PROC FREQ procedure of SAS.

Cortisol concentrations for each day of sampling of Trials 1 and 2 were analyzed by separate ANOVA for a completely randomized split-plot design using PROC GLM of SAS. The main plot included treatment (TRT) and Animal within Treatment (Anim(TRT)). The Anim(TRT) variance component was used to test the effect of treatment. The sub-plot included day (Day) and the interaction of TRT and Day. Means were separated by the PDIFF procedure of SAS.

Results

Trial 1

Stratification factors were similar between BE and NE cows (Table 1).

Table 1. Number of cows per treatment and least squares means for stratification factors, interval from exposure to resumption of ovarian cycling activity, and proportion of cows that resumed ovarian cycling activity for primiparous, anovular, suckled, beef cows exposed to bulls (BE) or not exposed to bulls (NE)

	Treatments			
Variable	BE	NE	SEM[a]	*P* value
n	13	15		
Calving date[b]	54.69	50.27	15.88	
Days postpartum at start of exposure	56.31	60.67	15.95	
Cow BW (kg)	520.66	519.28	44.24	
Cow BW change (kg)	-3.77	-12.91	14.03	
Calf sex ratio[c]	0.62	0.73		
Interval to resumption of ovarian cycling activity during the exposure period, d	3.76	18.2	11.56	< 0.05
% resuming luteal activity during the exposure period	100%	47%		< 0.05[d]

[a]SEM = Pooled standard error for means.

[b]Day of year.

[c]Calf Sex ratio = ratio of male to female calves, 1 = male and 0 = female.

[d]X^2 = 6.6, d.f. = 1.

More ($P < 0.05$) cows exposed to bulls (100%) resumed ovarian cycling activity by the end of the experiment than cows not exposed to bulls (47% ; Table 1). The interval from the start of treatment to resumption of ovarian cycling activity was shorter ($P < 0.05$) for cows exposed to bulls (3.76 d) than cows not exposed to bulls (18.2 d).

There was a TRT by Day interaction ($P < 0.01$) for systemic cortisol concentrations. This was due to a rapid increase ($P < 0.05$) in cortisol concentrations for cows exposed to bulls from D 0 to 8, while cows not exposed to bulls did not exhibit a rise in cortisol from D 0 to 8 (Figure 1).

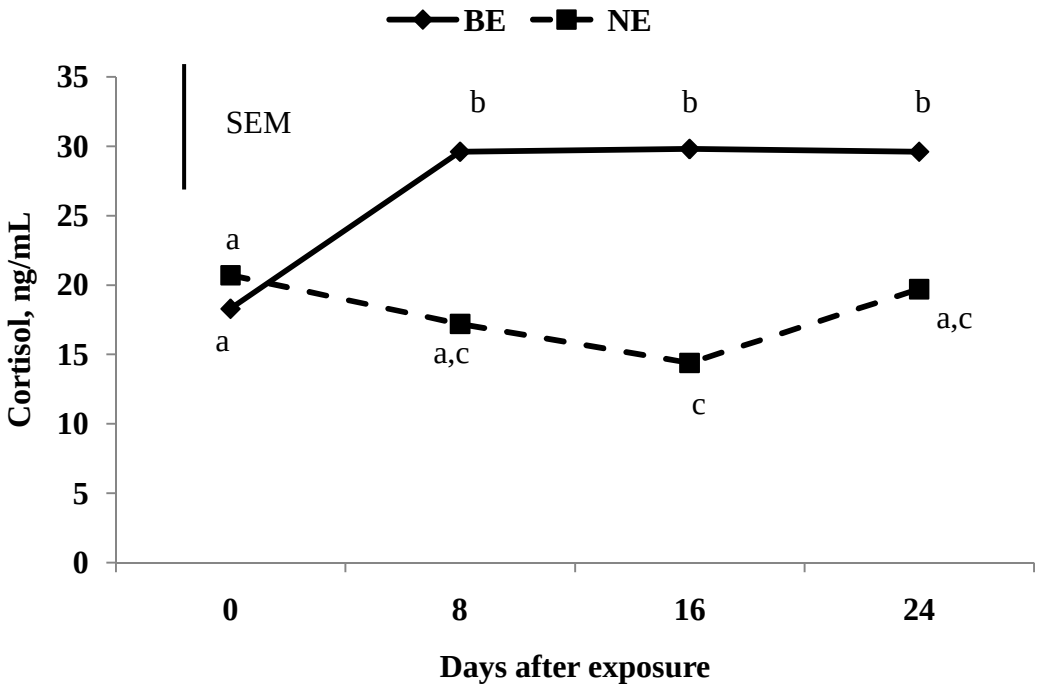

Figure 1. Cortisol concentrations in 8-d intervals during the 30 d bull-exposure period in primiparous, suckled, beef cows exposed (BE) or not exposed (NE) to bulls in Trial 1. Vertical line represent the pooled SEM (SEM = 8.3 ng/mL). Points that lack common letters differ ($P < 0.05$).

Thereafter, from D 8 to 16, cortisol concentrations in BE cows remained higher and more stable than cortisol concentrations in NE cows, which decreased from D 0 to D 16 (Figure 1).

Trial 2

Stratification factors were similar for cows exposed to bull urine (BUE) and steer urine (SUE; Table 2).

Table 2. Number of cows per treatment and least squares 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, interval from exposure to resumption of ovarian cycling activity (ROA), and proportion of cows that ROA for primiparous, suckled, beef cows exposed to bull urine (BUE) or exposed to steer urine (SUE)

	Treatment			
Variable	BUE	SUE	SEM	*P* value
n	19	19		
Calving date[a]	39	39	9.22	
Cow BW (kg)	557	550	40.90	
Cow BW change (kg)[b]	-37.20	-22.30	21.50	
Calf BW (kg)	35	39	9.10	
BCS	5.10	5.20	0.34	
BCS change[b]	-0.04	-0.04	0.35	
Calf sex ratio[c]	0.47	0.60		
Dystocia score[d]	1.00	1.05	0.16	
Interval to ROA, d	62.50	55.80	14.60	>0.10
% resuming ovarian cycling	15%	33%		>0.10[e]

[a]Day of year.
[b]Cow BW and BCS change are differences from the start of treatment to the end of treatment.
[c]Calf sex ratio = ratio of male to female calves, 1 = male and 0 = female.
[d]Dystocia Score: 0 = No assistance to 5 = Caesarean section.
[e]X^2 = 1.3, d.f. = 1.

There was no difference ($P > 0.10$) in the intervals from the start of the exposure period to the resumption of ovarian cycling activity between BUE and SUE cows, 62.5 and 55.8 d, respectively (Table 2). Likewise, proportions of cows that resume ovarian cycling

activity by the end of the exposure period did not differ ($P > 0.10$) between BUE and SUE cows, 15% and 33%, respectively (Table 2).

There was a TRT by Day interaction ($P < 0.05$) for patterns of cortisol concentrations (Figure 2).

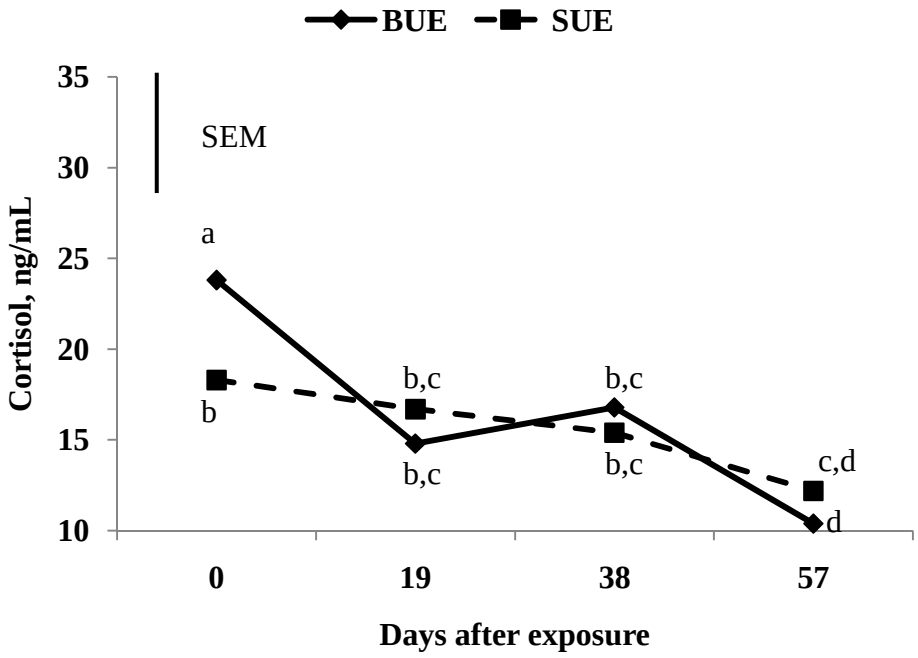

Figure 2. Cortisol concentrations in 19-d intervals during the 57 d urine-exposure period in primiparous, suckled, beef cows exposed to bull urine (BUE) or exposed to steer urine (SUE) in Trial 2. Vertical line represent the pooled SEM (SEM = 6.3 ng/mL). Points that lack common letters differ ($P < 0.05$).

This interaction appeared to be caused by the difference in cortisol concentrations between BUE and SUE on D 0 and the larger decrease ($P < 0.05$) in cortisol between D 0 and 19 in BUE than in SUE cows (Figure 2). Nevertheless, cortisol concentrations in both BUE and SUE cows decreased ($P < 0.05$) during the exposure period (Figure 2); and cortisol concentrations for any one day did not differ ($P > 0.10$) between BUE and SUE cows throughout the exposure period (Figure 2).

Discussion

To determine if the biostimulatory effect of bulls is associated with systemic cortisol concentrations, postpartum, anestrous, suckled, beef cows were exposed continuously to either the physical presence of bulls or bull urine. Cows in Trial 1, resumed ovarian cycling activity in response to the physical presence of bulls earlier than cows not exposed to bulls; however, in Trial 2, neither the proportion of cows that resumed ovarian cycling activity nor the interval to resumption of ovarian cycling activity differed between cows exposed to bull or steer urine. Thus, the physical presence of bulls caused a biostimulatory effect on resumption of ovarian cycling activity; whereas, continuous bull urine exposure did not stimulate resumption of ovarian cycling activity.

Cortisol concentrations in cows not exposed to bulls in Trial 1, ranged from 21 to 16 ng/mL. These data are consistent with previous reports for systemic circulating cortisol concentrations in cows 40 to 60 d postpartum, which ranged from 8 to 16 ng/mL (Wagner and Oxenreider, 1972; Dunlap et al., 1981a; Walters et al., 1982). However, in the present experiment cows exposed to the physical presence of bulls in Trial 1, exhibited a significant rise in systemic cortisol concentrations from 16 ng/mL on D 0, to 29 ng/mL throughout the remainder of the experiment. Wagner and Oxenreider (1972) reported that cortisol is released in a diurnal pattern with a change of approximately ± 1 ng/mL every h and peak cortisol release occurring at 4:00 am and 4:00 pm. The diurnal pattern of cortisol release cannot explain the results observed in Trial 1, because blood samples were collected within a 2-h period each sampling day between 9:00 am and 2:00 pm from cows in both treatments. It is possible that the increase in cortisol

concentrations observed in Trial 1, were due to an acute stress caused by the physical presence of bulls. Mean cortisol concentrations in cows that have been subjected to an acute physical stress, such as transportation, or cows that have been injected with adrenocorticotropic hormone (ACTH), to mimic a stress response, exhibit a rise in cortisol concentrations that meets or exceeds 60 ng/mL (Dunlap et al., 1981b; Dobson and Smith, 2000). Mean cortisol concentrations for cows in Trials 1 and 2 were well below this concentration, indicating that cows used in these experiments did not exhibit a typical ACTH-driven cortisol response and were not subjected to conditions that would cause a stress-like cortisol response.

In Trial 2, systemic cortisol concentrations decreased as time after calving increased for cows exposed to either bull or steer urine. This result is probably a photoperiodic effect and is consistent with the results of Leining et al. (1980) and Berardinelli et al. (1992) who reported that as photoperiod increased concentrations of circulating glucocorticoids decreased in bulls. Systemic cortisol concentrations did not differ between cows exposed continuously to mature bull urine or steer urine. This result might have been expected since the physical presence of bulls stimulated resumption of ovarian cycling activity in Trial 1, while continuous bull urine exposure did not stimulate resumption of ovarian cycling activity in Trial 2. Collectively, these results provide compelling evidence that elevated cortisol concentrations in postpartum, anovular cows are related to the biostimulatory effect of bulls. Thus, adrenal activation is a probable component of the pheromone-mediated biostimulatory effect of bulls on accelerating resumption of ovarian cycling activity in postpartum, anovular, suckled, beef cows.

In conclusion, bull urine exposure had no effect on circulating cortisol concentrations and this type of exposure did not reduce the postpartum anestrus interval from calving to resumption of ovarian cycling activity or increase the proportion of cows cycling by the end of the exposure period. However, the physical presence of bulls elevated systemic cortisol concentrations and this type of exposure shortened the postpartum anestrus interval to resumption of ovarian cycling activity in primiparous, postpartum, anovular, suckled cows. Therefore, it is possible that changes in cortisol concentrations is a critical component involved in the physiological mechanism of the biostimulatory effect of bulls that accelerates resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows.

CHAPTER 5

EXPERIMENT 2: CHARACTERISTICS OF TEMPORAL PATTERNS OF CORTISOL AND LUTEINIZING HORMONE IN PRIMIPAROUS, POSTPARTUM, ANESTROUS, SUCKLED, BEEF COWS EXPOSED ACUTELY TO BULLS

Introduction

The mechanism of the biostimulatory effect of bulls appears to involve the HPO axis to increase LH pulse frequency in response to acute (a single 8-h exposure period; Roelofs et al., 2007) bull exposure or chronic (24-h daily; Fernandez et al., 1996) bull exposure. Exposing cows to the excretory products of bulls accelerates resumption of ovarian cycling activity indicating that the mechanism for the biostimulatory effect of bulls is mediated by pheromones (Berardinelli and Joshi, 2005a). However, the effects of pheromones produced by bulls on neuroendocrine-endocrine events that precede resumption of ovarian cycling activity in cows are not well understood.

Observations from Experiment 1 indicated that postpartum, anestrous cows exposed to bulls resumed ovarian cycling activity sooner and had greater mean cortisol concentrations than cows not exposed to bulls. One interpretation of these results is that the HPA axis may be involved with the physiological pathway by which bull exposure accelerates resumption of ovulatory cycles in postpartum cows. The objectives of this experiment were to determine whether the acute physical presence of bulls affects mean concentrations and characteristics of temporal patterns of cortisol and LH concentrations.

Materials and Methods

Animals and Treatments

Sixteen 2-yr-old cows, two 4-yr-old bulls and two 1-yr-old steers were used in this experiment. All animals were crossbred Angus X Hereford. Cows and calves were maintained in a single pasture and had no contact with bulls, steers, or the excretory of bulls or steers from the previous breeding season until the start of the experiment at 67 ± 3.5 d (mean ± SD) after calving (D 0). Average calving date for these cows was Feb. 4, 2006. Before the start of the experiment, cycling status for each cow was rated by ultrasound examinations of each ovary for the presence or absence of a corpus luteum at 12 and 2 d before the start of the experiment. Cows that did not exhibit the presence of a corpus luteum on either ovary in both ultrasound examinations were used in this experiment.

Two days before the start of the experiment cows were stratified by body weight, BCS, calf birth weight, calving date, sex of calf, and dystocia score; no cows with a dystocia score above 3 on a scale of 1 to 5 were used in this experiment (1 = no assistance, 5 = Caesarean section). Cows were assigned randomly to be exposed to either the two mature bulls (EB; n = 8) or the two steers (ES; n = 8) for 5 h daily over a 9-d period (D 0 to 8).

Facilities

Cows were housed within pens in two separate lot areas. Pens within the north lot were used to maintain EB cows while pens within the south lot were used to maintain ES

cows. During each daily intensive blood sampling period and exposure period, cows were moved into individual stalls within open-air sheds adjacent to pens in which cows were housed. Sheds were similar in structure, area and light density. Light density within sheds was tested using a Minolta Autometer Pro Photometer (Konica Minolta Holdings Inc., USA) and tarps were used to manipulate the light density so that cows in each sampling area were exposed to the same amount of light each intensive sampling period. Cows were halter-restrained within side-by-side stalls during the daily sampling and exposure periods. Bulls or steers were contained within the immediate vicinity in front of the cows, unrestrained and allowed to eat, roam, and come into contact with the frontal aspect of each cow. Excretory products of bulls and steers were not cleaned from the exposure area during the experiment.

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 $kg \bullet hd^{-1} \bullet d^{-1}$ 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 15% (NRC, 1996). Bulls were fed 0.5 kg of cracked barley and good quality, chopped mixed-grass alfalfa hay. Steers were fed a finishing diet that consisted of 70% concentrate and 30% roughage throughout the experiment.

Intensive Blood Sampling Protocols

Two d before the start of the experiment each cow received an indwelling jugular catheter. Blood samples were collected daily from each cow at 15-min intervals for 6 h (1000 to 1600 h) beginning 1 h before the 5-h exposure period each day during the 9-d period (D 0 to 8). The maximum number of 6-h sampling periods that could be collected each day was 16 (1 sampling period for each cow in the experiment) and the total number of sampling periods over the 9-d period was 72 for each treatment. At the beginning of each collection day catheters were cleared of heparinized saline before blood was collected and flushed after collection of each blood sample using sterilized physiological saline solution (0.9%). Blood samples were refrigerated and centrifuged at 1285 x *g* for 30 min at 4°C the following day. Sera was harvested and stored at -20°C until assayed for cortisol and LH. Blood samples were collected by the same technician in each treatment throughout the experiment.

Resumption of Ovarian Cycling Activity

To confirm that cows remained anestrous throughout the experiment, resumption ovarian cycling activity was monitored by assay of daily progesterone concentrations in samples collected 1 h after start of the 6-h sampling period. Serum was assayed for progesterone concentration using the same assay as that used in Experiment 1. Intra-assay CV was less than 10% for serum pools. The criterion used to determine resumption of ovarian cycling activity was the same as that described in Experiment 1.

Cortisol and Luteinizing Hormone (LH) Assays

Cortisol concentrations in serum samples were assayed using the same assay as Experiment 1. Intra- and interassay CV were < 10% for pools of postpartum cow sera that contained 135 and 25 ng/mL, respectively, of cortisol. Concentrations of LH in serum samples were assayed in duplicate by liquid-phase double antibody RIA (Niswender et al., 1969). The primary antibody was NIDDK anti-oLH-1 AFP 192279Rb and bLH AFP11743B was used for iodination and standards. Both assay reagents were obtained from the National Hormone and Pituitary Program (NHPP) and Dr. A. Parlow (University of San Francisco, San Francisco, CA). Intra- and interassay CV for sera pools that contained 12.5 ng/mL were 10.9% and 15.4%, respectively, and for sera pools that contained 1 ng/mL were 7.4% and 17.7%, respectively.

Characteristics of Temporal Patterns
of Cortisol and LH Concentrations

Characteristics of temporal patterns of cortisol and LH concentrations were determined by the Pulsar algorithm (Merriam and Wachter, 1982) using PC PULSAR (PC-Pulsar, Gitzen and Ramirez, University of Illinois). This algorithm identified characteristics of daily temporal patterns and included mean of all samples in a sampling period (mean concentration), mean of samples that were identified as baseline concentrations (baseline concentration), frequency (pulse frequency), mean for apex sample concentrations over all pulses in a sampling period (pulse amplitude), and mean number for minutes that sample concentrations within pulses were above baseline concentrations for a sampling period (pulse duration).

Statistical Analyses

Characteristics of temporal patterns of cortisol and LH concentrations were examined by ANOVA for a completely randomized split-plot design using PROC GLM of SAS (SAS Inst. Inc., Cary, NC). The main plot included treatment (TRT) and Animal within Treatment (Anim(TRT)). The Anim(TRT) variance component was used to test the effect of TRT. The sub-plot included day (Day) and the interaction of TRT and Day. Means were separated by Bonferroni multiple comparison tests.

Relationships between temporal patterns of cortisol and LH concentrations were determined by regressing characteristics LH concentrations on respective characteristics cortisol concentrations within treatments using the PROC REG procedure of SAS. So that mean concentrations of LH were regressed on mean concentrations of cortisol and baseline concentrations of LH were regressed on baseline concentrations of cortisol, etc. The model was a standard regression model in which characteristics of temporal patterns of cortisol concentrations were used as the explanatory variable and characteristics of temporal patterns of LH concentrations were used as the dependent variable.

Results

None of the cows exposed to bulls or cows exposed to steers 5-h daily for 9 d resumed ovarian cycling activity during the experiment (0 of 8 and 0 of 8, respectively).

Adaptation to Handling and Intensive Blood Sampling Protocols

There were no ($P > 0.10$) TRT or TRT by Day interaction for mean cortisol concentrations for EB or ES cows from D 0 to D 2. Mean concentrations of cortisol

decreased ($P < 0.05$) from 10.2 ± 1.7 ng/mL (± SE) on D 0 to 2.8 ± 0.24 ng/mL on D 2 in both EB and ES cows (Figure 3).

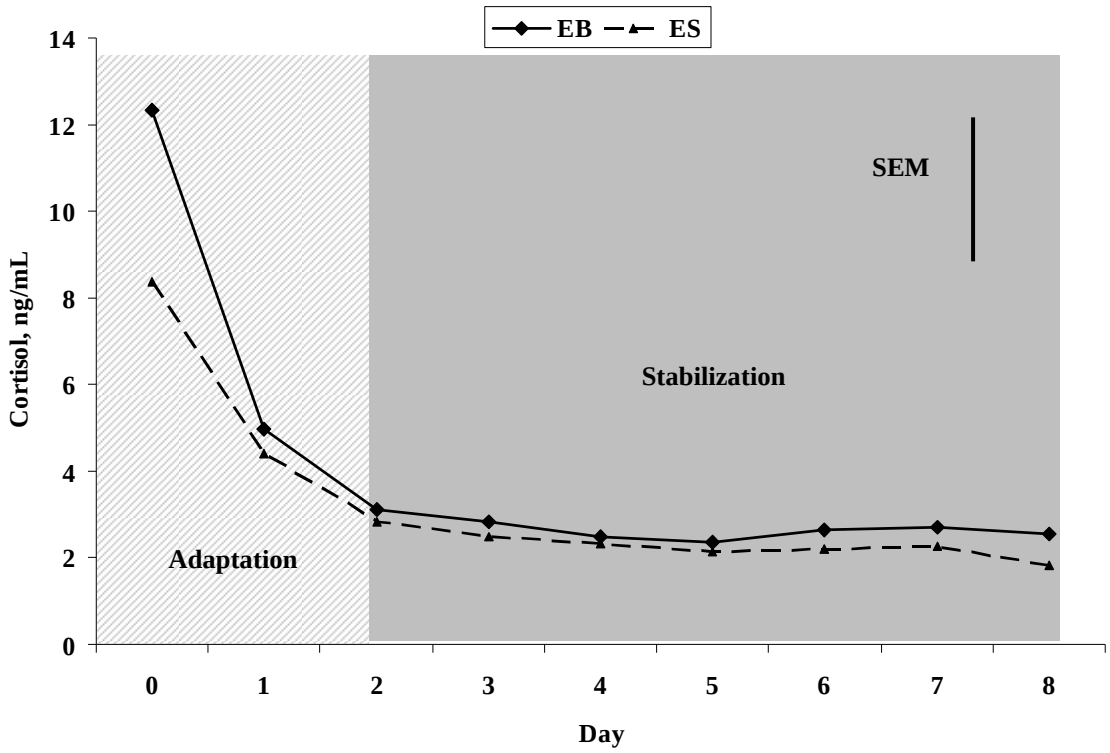

Figure 3. Least squares means for daily mean concentrations of temporal patterns of cortisol in postpartum, anestrous, suckled cows exposed to bulls (EB; n = 8) or steers (ES; n = 8) for 5 h daily over a 9-d intensive blood sampling period (D 0 = start of bull or steer exposure). The vertical bar represents the pooled standard error of the mean (SEM). The grey hashed box represents the period during which cows adapted to intensive blood sampling protocols (D 0 to D 2). The solid grey box represents the period of stabilization after the adaptation period (D 2 to D 8).

Thereafter, from D 2 through 8, daily mean concentrations of cortisol did not differ ($P > 0.10$) between Days or between TRTs (Figure 3); mean concentrations of cortisol for all cows from D 2 though 8 was 2.5 ± 0.10 ng/mL. The decrease in mean cortisol concentrations in EB and ES cows from D 0 to D 2 was attributed to cows adapting to stressors involved with the handling and intensive blood sampling protocols used in this

experiment. Consequently, analyses of treatment effects on characteristics of temporal patterns of cortisol and LH concentrations, and regression analyses focused on data derived from temporal patterns of cortisol and LH concentrations from D 2 through 8 of the experiment.

Characteristics of Temporal Patterns of Cortisol Concentrations, D 2 through D 8

There was no ($P > 0.10$) Day effect or TRT by Day interaction for characteristics of temporal patterns of cortisol concentrations from D 2 to D 8 of the experiment.

Table 3. Least squares means of characteristics of temporal patterns of cortisol concentrations (mean, baseline, pulse frequency, pulse amplitude, and pulse duration) in blood samples collected at 15-min intervals between 1000 and 1600 h for postpartum, anestrous, suckled cows exposed to bulls (EB) and cows exposed to steers (ES) from D 2 through D 8 of a 9-d exposure period[a]

	Treatment			
Variable	EB	ES	SEM[b]	*P* value
n	8	8		
Mean, ng/mL	2.7	2.4	2.4	>0.10
Baseline, ng/mL	1.2	1.0	1.6	>0.10
Pulse frequency/h	0.5	0.6	0.3	0.05
Pulse amplitude, ng/mL	6.1	5.6	5.3	>0.10
Pulse duration, min	58.2	50.4	22.4	0.09

[a]D 0 = Start of the experiment; cows were 67 ± 3.5 d postpartum.

[b]SEM = Standard error of the mean.

Baseline concentrations of cortisol, and amplitudes of cortisol pulses did not differ ($P > 0.10$) between EB and ES cows from D 2 through 8 (Table 3). However, cortisol pulse frequency (pulses/h) was lower ($P = 0.05$) and pulse duration tended ($P = 0.09$) to be longer in EB cows than in ES cows (Table 3).

Characteristics of Temporal Patterns
of LH Concentrations, D 2 through D 8

There was no ($P > 0.10$) Day or TRT by Day interaction for characteristics of temporal patterns of LH concentrations from D 2 to D 8 of the experiment. Mean, baseline, pulse amplitude, and pulse duration of temporal patterns of LH concentrations did not differ ($P > 0.10$) between EB and ES cows from D 2 through 8 (Table 4). However, frequency of LH pulses was greater ($P = 0.06$) in EB cows than ES cows over D 2 through 8 of the exposure period (Table 4).

Table 4. Least squares means of characteristics of temporal patterns of LH concentrations (mean, baseline, pulse frequency, pulse amplitude, and pulse duration) in blood samples collected at 15-min intervals between 1000 and 1600 h for postpartum, anestrous, suckled cows exposed to bulls (EB) and cows exposed to steers (ES) from D 2 through D 8 of a 9-d exposure period[a]

	Treatment			
Variable	EB	ES	SEM[b]	*P* value
n	8	8		
Mean, ng/mL	0.7	0.6	0.2	>0.10
Baseline, ng/mL	0.6	0.6	0.2	>0.10
Pulse frequency/h	0.3	0.2	0.36	0.06
Pulse amplitude, ng/mL	0.9	0.6	0.9	>0.10
Pulse duration, min	37.0	25.0	42.1	>0.10

[a]D 0 = Start of the experiment; cows were 67 ± 3.5 d postpartum.

[b]SEM = Standard error of the mean.

Relationships between Characteristics of
Temporal Patterns of Cortisol and LH Concentrations

Linear regression of characteristics of temporal patterns of LH concentrations on characteristics of temporal patterns of cortisol concentrations indicated that characteristics of cortisol concentrations were not related ($P > 0.10$) to characteristics of

LH concentrations for ES cows. However, regression analysis of pulse amplitudes of these hormones in EB cows indicated that as amplitudes of cortisol pulses increased, amplitudes of LH pulses decreased (b_1 = -0.04 [ng/mL]/[ng/mL]; $P < 0.05$; Table 5). Furthermore, as frequency of cortisol pulses increased, frequency of LH pulses tended to decrease (b_1 = -0.38 [pulses/h]/ [pulses/h]; $P = 0.06$; Table 5), and as baseline concentrations of cortisol increased, baseline concentrations of LH tended to increase (b_1 = 0.02 [ng/mL]/[ng/mL]; $P = 0.09$; Table 5). Mean concentrations and duration of cortisol pulses were not ($P > 0.10$) linearly related to mean concentrations or durations of LH pulses in EB cows (Table 5).

Table 5. Linear regressions of characteristics of LH concentration patterns on characteristics of cortisol concentration patterns (mean, baseline, pulse frequency, pulse amplitude, and pulse duration) in blood samples collected at 15-min intervals between 1000 and 1600 h for postpartum, anestrous, suckled cows exposed to bulls (EB) from D 2 through D 8 of a 9-d exposure period[a]

Variable	Y-Intercept	Slope[b]	R^2	*P* value
Mean, ng/mL	0.67	>-0.01	0.0	>0.10
Baseline, ng/mL	0.53	0.02	0.05	0.09
Pulse frequency/h	0.57	-0.38	0.06	0.06
Pulse amplitude, ng/mL	1.19	-0.04	0.08	<0.05
Pulse duration, min	36.5	>-0.01	0.0	>0.10

[a]D 0 = Start of the experiment; cows were 67 ± 3.5 d postpartum.
[b]Units for slopes are change in characteristic of LH per change in characteristic of cortisol.

Discussion

The focus of this experiment was to evaluate the adrenal response and HPO axis activity in primiparous, postpartum, anestrous, suckled cows during acute exposure to

bulls. In the present experiment, mean cortisol concentrations of cows were 10.2 ng/mL on D 0 and decreased to 2.5 ng/mL from D 2 through D 8. These values are close to and consistent with previous reports for systemic circulating cortisol concentrations in postpartum cows 40 to 60 d after calving, which ranged from 8 to 16 ng/mL (Wagner and Oxenreider, 1972; Dunlap et al., 1981b; Walters et al., 1982). Data for cortisol concentrations in the present study indicated that primiparous, postpartum, anestrous, suckled cows adapted to handling and sampling procedures within 2 d after the start of the experiment. Furthermore, characteristics of temporal patterns of cortisol concentrations, such as mean and baseline concentrations and amplitudes of pulses, did not differ between cows exposed to bulls or steers during the adaptation period. These results indicated that cows were not subjected to any undo stress throughout the experiment and that environment conditions were similar for cows exposed to bulls and steers. However, mean concentrations of cortisol during the "adaptation" period (D 0 and D 1) were significantly greater and more variable in cows of both treatments than during the "stabilization" period (D 2 through 8). Echternkamp (1984) reported that acute stress decreased mean concentrations of LH and frequency of LH pulses if cows showed a ten- to twenty-fold increase in cortisol concentrations. Thus, higher and more variable concentrations of cortisol during the "adaptation" period may have masked or artificially inflated potential differences between treatments. Therefore, data for cortisol and LH concentrations during the adaptation period were excluded from the analyses of characteristics of temporal patterns of hormone concentrations to better assess the effect of acute bull exposure.

Mean concentrations of cortisol in postpartum, anestrous cows did not differ between cows exposed to bulls and steers 5 h daily for 9 d. This result is inconsistent with those of Experiment 1, in which anovular cows exposed to bulls for 30 d starting 58 d after calving resumed ovarian cycling activity sooner and had greater mean cortisol concentrations 9 d after the start of bull exposure than cows not exposed to bulls. However, the increase in cortisol concentrations for cows responding to the biostimulatory effect of bulls observed in Experiment 1 could have been due to an increase in cortisol concentrations associated with the expression of estrus. Humphrey et al. (1983) reported that cortisol concentrations increased from less than 10 ng/mL during a 10-d period before estrus to greater than 40 ng/mL during a 3-d period after estrus in postpartum cows during the transition for anestrus to estrual states. Therefore, the increase in mean cortisol concentrations observed in cows responding to the biostimulatory effect of bulls in Experiment 1 could have been influenced by ovulation, formation of corpus hemorrhagicum, and expression of estrus and not associated with the presentation of bull-pheromonal stimuli to cows. In light of this observation and the results of the present experiment, it appears that pheromonal stimuli presented by bulls may not induce an increase in mean concentrations of cortisol in postpartum, anestrous cows exposed to bulls.

The most significant results of this study were that duration of cortisol pulses tended to be longer and frequency of pulses were reduced in cows exposed to bulls than in cows exposed to steers 5 h daily for 9 d. One interpretation of these results is that bulls produce pheromones that immediately alter adrenal function and/or regulation over the

short-term. This interpretation is much like the effect of male pheromones on physiological responses in females and is consistent with data from Mora and Sanchez-Criado (2004) who reported that male urine sprayed into the nasal cavity of ovariectomized female rats stimulates ACTH release from the pituitary, which causes adrenal release of progesterone and corticosterone. Thus, pheromones produced by bulls and presented to cows acutely (5 h daily) appear to alter adrenal function and/or regulation of primiparous, postpartum, anestrous, suckled cows.

The increase in duration and decrease in frequency of cortisol pulses observed in postpartum, anestrous cows exposed to bulls may not have been due exclusively to cows sensing pheromones and responding to the biostimulatory effect of bulls, but may have been influenced by cows transitioning from anestrus to ovarian cycling activity. Berardinelli and Joshi (2005b) reported that the mean interval to resumption of ovarian cycling activity for cows exposed to bulls 55 d after calving was 16 d. Based on this result it is logical to assume that some cows in the present experiment that were exposed to bulls at 67 d after calving started to transition from anestrus to ovarian cycling activity during the 9-d experimental period. Humphrey et al. (1983) reported that concentrations of cortisol during the postpartum anestrous period are greatest for two weeks before anestrous cows resume ovarian cycling activity. Although no EB or ES cows resumed ovarian cycling activity during the experimental period, some cows may have begun transitioning from anestrus to ovarian cycling activity during this 9-d period. Therefore, the transition period from anestrus to ovarian cycling activity may have influenced

characteristics of cortisol concentration patterns in primiparous, postpartum, anestrous, suckled cows exposed to bulls.

The second objective of this experiment was to evaluate characteristics of temporal patterns of LH concentrations in cows exposed to bulls or steers 5 h daily for 9 d. The results indicated that LH mean and baseline concentrations, pulse amplitude, and pulse duration were not affected in cows exposed to bulls. However, cows exposed to bulls had greater LH pulse frequency than cows exposed to steers under the conditions of this experiment. This observation is consistent with Fernandez et al. (1996) who reported that LH pulse frequency was greater in cows exposed to bulls for 2 h every 3 d for 18 d than in cows not exposed to bulls. Furthermore, Roelofs et al. (2007) reported that LH pulse frequency was greater in dairy cows exposed to bulls for a single 8-h exposure period than an 8-h sampling period the previous day. Thus, it appears that bull exposure has an immediate effect on the hypothalamic-pituitary-ovarian (HPO) axis to cause increased LH pulse frequency in primiparous, postpartum, anestrous, suckled cows.

The final and perhaps most important objective of this experiment was to determine if characteristics of temporal patterns of cortisol concentrations are related to characteristics of temporal patterns of LH concentrations. The results indicated that there was no relationship between characteristics of temporal patterns of cortisol and LH concentrations for cows exposed to steers over the 9-d experimental period. However in cows exposed to bulls, as amplitudes and frequency of cortisol pulses decreased, amplitudes of LH pulses increased and frequency of LH pulses tended to increase. These results are consistent with McFarlane et al. (2000) and Breen and Karsh (2006) who

reported that frequency and amplitude of LH pulses were decreased by infusing cortisol into intact and ovariectomized ewes, and indicate that cortisol has an inhibitory effect on LH secretion. In light of these results and the results of the present experiment that frequency of cortisol pulses was lower and duration of cortisol pulses tended to be longer in cows exposed to bulls suggest that changes HPA axis caused by the biostimulatory effect of bulls may facilitate changes in the HPO axis and accelerate resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows.

In conclusion, bull exposure for 5 h daily over a 9-d period did not appear to alter mean concentrations of cortisol or LH; however, bull exposure altered temporal patterns of cortisol and LH concentrations by decreasing frequency of cortisol pulses, tending to increase duration of cortisol pulses, and increasing frequency of LH pulses. Even though no biostimulatory effect, i.e., resumption of ovarian cycling activity, was observed in cows within the 9-d experiment, these findings indicated that the physical presence of bulls for 5 h daily over a 9-d period stimulated the HPO axis and influenced adrenal function and/or regulation. Therefore, bulls may release a pheromone(s) that has an immediate effect on activity of the HPO axis and adrenal function and/or regulation of primiparous, postpartum, anestrous, suckled cows. This effect may facilitate or support the physiological mechanism whereby the biostimulatory effect of bulls accelerates resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows.

CHAPTER 6

EXPERIMENT 3: THE BIOSTIMULATORY EFFECT OF BULLS ON CHARACTERISTICS OF TEMPORAL CORTISOL CONCENTRATION PATTERNS IN PRIMIPAROUS, POSTPARTUM, ANESTROUS, SUCKLED, BEEF COWS DURING THE TRANSITION FROM ANESTRUS TO OVARIAN CYCLING ACTIVITY

Introduction

The results from Experiment 2 indicated that cows exposed acutely to bulls (5 h daily) had fewer cortisol pulses and more LH pulses than cows exposed acutely to steers over a 9-d exposure period. One interpretation of these results may be that one or more pheromones released by bulls concurrently stimulates the HPO axis and alters the HPA axis in postpartum, anestrous, suckled, beef cows. However, it was not evident that the changes observed in Experiment 2 related to changes in the activity of the HPA axis were associated with the transition period from a physiological state of anestrus to ovarian cycling activity. Thus, the objectives of this experiment were to determine if; 1) resumption of ovarian cycling activity is accelerated, 2) characteristics of temporal patterns of cortisol concentrations are altered, and 3) changes in characteristics of temporal patterns of cortisol concentrations are associated with resumption of ovarian cycling activity, in cows exposed continuously to bulls.

Materials and Methods

Animals and Treatments

Thirteen spring-calving two-yr-old Angus X Hereford primiparous, postpartum, anestrous, suckled, beef cows and two mature epididymectomized Angus X Hereford bulls were used in this experiment. Cows and calves were maintained in a single pasture and had no contact with bulls or their excretory products from the previous breeding season until the start of the experiment (D 0). Average calving date for these cows was Feb. 5, 2007. Before the start of the experiment cycling status for each cow was rated by two ultrasonic examinations of each ovary for the presence or absence of a corpus luteum. The first and second ultrasonic examinations were conducted 15 and 5 d, respectively, before the start of the experiment. Cows that did not exhibit the presence of a corpus luteum on either ovary in both ultrasound examinations were used in this experiment.

The interval from calving to the start of the experiment averaged 72.5 ± 3.6 d. Two d before the start of the experiment cows were stratified by body weight, BCS, calf birth weight, calving date, sex of calf, and dystocia score and assigned randomly to be exposed (BE, n = 8) or not exposed (NE, n = 5) to bulls from D 0 to the end of the exposure period (D 44). Blood samples were collected at 15-minute intervals from cows in each treatment during a 10-d period (D 7 through D 16) for evaluation of characteristics of temporal patterns of cortisol concentrations.

Facilities

Cows were housed within pens in separate lot areas. Pens within the north lot were used to maintain BE cows while pens within the south lot were used to maintain NE cows. During the 10-d intensive blood sampling period cows were moved into open-air sheds adjacent to the assigned pen for each treatment and were halter-restrained within side-by-side stalls. Sheds were the same in structure, area and light density. Calves for NE cows and calves and bulls for BE cows were able to come within 2 m of cows during the daily intensive blood sampling period.

Nutrition

Cows had free access to good quality, chopped mixed-grass alfalfa hay, and any pasture grasses that were available before start of the experiment. Once cows and calves were moved into pens, they were given free access to the same hay, 0.5 $kg \bullet hd^{-1} \bullet d^{-1}$ cracked corn, water, and a trace mineral-salt supplement. Average body weight of cows was 464 ± 40.4 kg. The TDN of the diet was 97% of the recommended energy requirement for lactating beef cows with a mature weight of 533 kg (NRC, 1996). Bulls were fed the same diet as cows.

Intensive Blood Sampling Protocols

Two d before the start of the experiment (D 0), each cow received an indwelling jugular catheter. To habituate cows to handling and sampling protocols, cows were moved into individual stalls and subjected to intensive blood sampling protocols over a 4-h period on D 5 and 6. From D 7 to D 16 of the experiment, blood samples were

collected from each cow at 15-min intervals from 1000 to 1400 h. At the beginning of each daily intensive sampling period, catheters were cleared of heparinized saline before blood was collected and flushed after collection of each blood sample using sterilized physiological saline solution (0.9%). Blood samples were refrigerated and centrifuged at 1285 x *g* for 30 min at 4°C the following day. Sera was harvested and stored at -20°C until assayed for cortisol. Blood samples were collected by the same technician in each treatment throughout the experiment.

Cortisol Assay

Cortisol concentrations in serum samples were assayed using the same assay as that in Experiment 1. Intra- and interassay CV were < 10% for pools of postpartum cow sera that contained 31 ng/mL and 3.5 ng/mL, respectively, of cortisol.

Characteristics of Temporal Patterns of Cortisol Concentrations

Characteristics of patterns of cortisol concentrations were determined using the same method as given in Experiment 2.

Resumption of Ovarian Cycling Activity

To determine resumption of ovarian cycling activity, blood samples were collected from each cow by jugular venepuncture at 3-d intervals from the start (D 0) to the end of the experiment (D 44) except during the intensive blood sampling period (D 7 to 16). During the intensive blood sampling period, progesterone was assayed daily in samples collected 1 h after start of the 4-h sampling period. Serum was assayed for progesterone concentration using the same assay as that used in Experiment 1. Intra- and

interassay CV for a serum pool that contained 2.5 ng/mL of progesterone were 0.4 and 18%, respectively; and less than 10% for both intra- and interassay CV for a pool that contained 5.5 ng/mL. The criterion used to determine resumption of ovarian cycling activity was the same as that described in Experiment 1.

Statistical Analyses

Intervals to resumption of ovarian cycling activity were evaluated by ANOVA for a completely randomized design using PROC GLM of SAS (SAS, Cary, NC). The model included treatment (TRT). Proportions of cows that resumed ovarian cycling activity before, during, and before and during the intensive blood sampling period, and during the experiment were analyzed by chi-square using the PROC FREQ procedure of SAS. Characteristics of temporal patterns of cortisol concentrations were evaluated by ANOVA for a completely randomized split-plot design using PROC GLM of SAS. The main plot included TRT and Animal within TRT (Anim(TRT)). The Anim(TRT) variance component was used to test the effect of TRT. The sub-plot included day (Day) and the interaction of TRT and Day. Means were separated by Bonferroni multiple comparison tests.

Characteristics of cortisol concentrations before and after cows resumed ovarian cycling activity were used to elucidate the effect of resumption of ovarian cycling activity on characteristics of temporal patterns of cortisol concentrations within TRTs using PROC ANOVA of SAS. Data for this analysis were classified as daily sampling periods for each cow before and after resumption of ovarian cycling activity. These sampling periods were used as experimental units for this analysis. The maximum number of daily

sampling periods for any individual cow was 10. For example, if a cow resumed ovarian cycling activity on D 5 of the experiment she would have 5 sampling periods represented before and 5 sampling periods represented after resumption of ovarian cycling activity. The model included cycling status (Not cycling or Cycling) and animal within cycling status (Anim(Cycling Status)). Anim(Cycling Status) was used as the error term for cycling status.

Results

Resumption of Ovarian Cycling Activity

The proportion of cows that resumed ovarian cycling activity before the intensive blood sampling period did not differ ($P > 0.10$) between BE and NE cows (Table 6). Additionally, the proportion of cows that resumed ovarian cycling activity during the intensive sampling period did not differ ($P > 0.10$) between BE and NE cows (Table 6). However, more ($P = 0.05$) BE cows resumed ovarian cycling activity before and during the intensive sampling period than NE cows (Table 6). All cows resumed ovarian cycling activity before the end of the experiment (Table 6). Interval from the start of the experiment (D 0) to resumption of ovarian cycling activity was shorter ($P < 0.05$) for BE than NE cows (Table 6).

Table 6. Proportions of cows that resumed ovarian cycling activity (ROA) before the intensive blood sampling period (D 7 through 16), during the intensive blood sampling period, before and during intensive blood sampling, and during the experiment, and interval from the start of the experiment (D 0) to resumption of ovarian cycling activity for primiparous, postpartum, anestrous, suckled cows exposed continuously to bulls (BE) or not exposed (NE) to bulls for 45 d[a]

Variable	Treatment[b]		χ^2	*P* value
	BE	NE		
n	8	5		
Proportion of cows that ROA before intensive sampling, %	37.5 (3)	20.0 (1)	0.44	> 0.10
Proportion of cows that ROA during intensive sampling, %	37.5 (3)	0.0 (0)	2.44	> 0.10
Proportion of cows that ROA before and during intensive sampling, %	75.0 (6)	20.0 (1)	3.75	0.05
Proportion of cows that ROA during the experiment, %	100.0	100.0	NA	NA
Interval to ROA, d	11.4	21.0	7.6[c]	< 0.05

[a]Cows were 72.5 ± 3.6 d postpartum at the start of the experiment.
[b]Numbers within parentheses indicate number of cows of the total number of cows in each treatment.
[c]SEM = Standard error of the mean.

Characteristics of Temporal Patterns of Cortisol Concentrations Throughout the Intensive Blood Sampling Period, D 7 through 16

One BE cow that resumed ovarian cycling activity before the intensive blood sampling period was excluded from analyses of characteristics of temporal patterns of cortisol concentrations because of the inability to successfully re-catheterize this cow

after the first day of the intensive blood sampling period. Day or the TRT by Day interaction did not affect ($P > 0.10$) characteristics of temporal patterns of cortisol concentrations. Characteristics of temporal patterns of cortisol concentrations did not differ ($P > 0.10$) between BE and NE cows throughout the intensive blood sampling period from D 7 to 16 (Table 7).

Table 7. Least squares means for overall mean and baseline concentrations, pulse frequency, pulse amplitude, and pulse duration of cortisol in postpartum, suckled, beef cows exposed continuously to bulls (BE) and not exposed to bulls (NE) throughout the intensive blood sampling period (D 7 to D 16)[a]

	Treatment			
Variable	BE	NE	SEM[b]	*P* value
n	7[c]	5		
Mean cortisol, ng/mL	5.9	5.1	4.1	> 0.10
Baseline cortisol, ng/mL	3.6	3.1	2.9	> 0.10
Pulse frequency/h	0.4	0.4	0.2	> 0.10
Pulse amplitude, ng/mL	11.3	9.3	8.9	> 0.10
Pulse duration, min	63.4	60.6	49.2	> 0.10

[a]D 0 = Start of bull exposure; cows were 72.5 ± 3.6 d postpartum.

[b]SEM = Standard error of the mean.

[c]One BE cow was excluded from these analyses.

Characteristics of Temporal Patterns of Cortisol Concentrations Before and After Cows Resumed Ovarian Cycling Activity

Mean cortisol, pulse amplitude, and pulse duration did not differ ($P > 0.10$) between BE and NE cows before resumption of ovarian cycling activity (Table 8). However, cortisol pulse frequency was lower ($P < 0.05$) and cortisol baseline concentration tended to be greater ($P = 0.07$) in BE cows than NE cows before cows resumed ovarian cycling activity (Table 8).

Table 8. Least squares means for overall mean and baseline concentration, pulse frequency, pulse amplitude, and pulse duration of cortisol in postpartum, anestrous, suckled, beef cows exposed continuously to bulls (BE) and not exposed to bulls (NE) before cows resumed ovarian cycling activity during the intensive blood sampling period (D 7 to D16)[a]

	Treatment			
Variable	BE	NE	SEM[b]	*P* value
n	5	4		
Mean cortisol, ng/mL	7.1	5.2	3.1	> 0.10
Baseline cortisol, ng/mL	4.5	3.1	2.1	0.07
Pulse frequency/h	0.4	0.5	0.2	< 0.05
Pulse amplitude, ng/mL	13.0	9.8	9.7	> 0.10
Pulse duration, min	70.3	65.2	48.8	> 0.10

[a]D 0 = Start of bull exposure; cows were 72.5 ± 3.6 d postpartum.

[b]SEM = Standard error of the mean.

Only one NE cow resumed ovarian cycling activity before the intensive blood sampling period; thus, it was not possible to test the effect of bull exposure on characteristics of cortisol concentration patterns after cows resumed ovarian cycling activity. Nevertheless, changes in characteristics of temporal patterns of cortisol concentrations in BE cows before and after resumption of ovarian cycling activity may yield insight into the physiological mechanism involved with alteration of the HPA axis and the biostimulatory effect of bulls. Overall mean and baseline concentrations of cortisol were greater ($P < 0.05$), and pulse frequency was lower ($P = 0.05$) for sampling periods of BE cows before resumption of ovarian cycling activity than after resumption of ovarian cycling activity (Table 9). However, amplitudes and durations of cortisol pulses in BE cows after resumption of ovarian cycling activity did not differ from those observed before BE cows resumed ovarian cycling activity (Table 9).

Table 9. Least squares means for overall mean and baseline concentration, pulse frequency, pulse amplitude, and pulse duration of cortisol in postpartum, suckled, beef cows exposed continuously to bulls (BE) before (Not Cycling) and after (Cycling) cows resumed ovulatory activity during the intensive blood sampling period (D 7 through D 16)[a]

	Treatment			
Variable	Not Cycling	Cycling	SEM[b]	*P* value
Number of sampling periods	35	34		
Mean cortisol, ng/mL	6.6	4.7	2.5	< 0.05
Baseline cortisol, ng/mL	4.4	2.8	1.1	< 0.05
Pulse frequency/h	0.35	0.48	0.2	0.05
Pulse amplitude, ng/mL	10.9	9.0	9.8	> 0.10
Pulse duration, min	66.2	60.9	43.9	> 0.10

[a]D 0 = Start of bull exposure; cows were 72.5 ± 3.6 d postpartum.

[b]SEM = Standard error of the mean.

Discussion

In Experiment 2, cows were exposed 67 ± 3.5 d after calving to bulls or steers for 5-h daily for 9 d. Frequencies of cortisol pulses were decreased and duration of cortisol pulses tended to be increased by acute bull exposure (5 h daily). This result could have been influenced by cows transitioning from anestrus to ovarian cycling activity. The aim of the present experiment was to determine if the biostimulatory effect of bulls alters characteristics of temporal patterns of cortisol concentrations before and after the transition from anestrus to ovarian cycling activity. Berardinelli and Joshi (2005b) reported that the mean interval from the start of bull exposure to resumption of ovarian cycling activity was 16 d for postpartum anestrous cows exposed continuously to bulls starting 55 d after calving. Based on this observation we postulated that cows exposed

continuously to bulls starting 65 to 75 d after calving would respond to the biostimulatory effect of bulls and resume ovarian cycling activity 10 to 15 d after the start of bull exposure. The present experiment was designed in the anticipation that characteristics of temporal patterns of cortisol concentrations of cows in each treatment could be monitored before and after cows resumed ovarian cycling activity. Thus, the experiment started 72 d after cows calved, cows were exposed continuously to bulls, and blood samples were collected intensively from cows beginning 7 d after the start of the experiment.

Continuous exposure of cows to bulls did not appear to alter characteristics of cortisol concentration patterns during the intensive blood sampling period (D 7 to 16) in this experiment. It would appear that these results are inconsistent with those of Experiment 2. Differences of results between these experiments could be explained by the duration that cows were exposed to bulls each day. In the present experiment, cows were exposed to bulls initially on D 0 and continuously to bulls 24 h daily throughout the experiment. On the other hand, cows in Experiment 2 were exposed initially to bulls on D 0 for 5 h and had no contact with bulls or pheromones of bulls for a 19-h period. Thereafter, the duration and frequency of exposure was 5 h daily with and 19 hours without the presence of bulls or bull pheromones. There is a possibility that changes in frequency and duration of cortisol pulses are dependent on the frequency and duration of bull exposure. Cows exposed over a short-term (5 h daily) or acutely to bulls in Experiment 2 may have experienced what could be considered a novel stimulus each day. Whereas, cows exposed to bulls continuously (24 h daily) in the present experiment may have adapted to the physical presence of bulls by the seventh day of exposure or the start

of the intensive blood sampling period. Changes in duration and frequency of cortisol pulses of cows exposed continuously to bulls may be detectable only for a short period of time after the start of exposure. These results are much like those reported by Ladewig and Smidt (1989) who investigated the effect of tethering and floor-type on characteristics of cortisol concentration patterns in bulls over a 38-d period. They reported that on D 2 to 5 of the experiment that frequency of cortisol pulses was reduced and duration of cortisol pulses were lengthened in bulls that were tethered on slatted floors than in bulls not tethered on straw-bedded floors. However, on D 32 to 38 there were no differences in characteristics of cortisol concentration patterns between bulls in each treatment. These results indicated that subjecting bulls to a mild stressor, in this case slatted floors, caused a reduction in frequency and increase in duration of cortisol pulses. However, after a time, bulls were able to adapt to this stressor and frequency and duration of cortisol pulses returned to values observed in non-stressed bulls. One could conclude that when cows are exposed acutely to bulls the HPA axis responds to the presence of bulls in each repeated exposure the same as the first exposure. However, if cows are exposed continuously to bulls the ability of the HPA axis to respond to pheromonal stimuli of bulls diminishes over time, in other words, cows habituate or adapt to pheromonal stimuli of bulls.

In contrast, a more probable explanation of the differences in observations of characteristics of temporal patterns of cortisol concentrations between Experiment 2 and the present experiment could be that physiological changes in the HPA axis induced by bull-pheromones changed once cows resumed ovarian cycling activity. In Experiment 2

none of the cows resumed ovarian cycling activity during the 9-d experimental period. However, in the present experiment three cows exposed to bulls and one cow not exposed to bulls resumed ovarian cycling activity before the intensive blood sampling period (≤ D 7) and three cows exposed to bulls resumed ovarian cycling activity during the intensive sampling period. Thus, the occurrence of resumption of ovarian cycling activity in cows that resumed ovarian cycling activity before or during the intensive sampling period may have affected characteristics of temporal patterns of cortisol concentrations in cows exposed continuously to bulls. When data for characteristics of temporal patterns of cortisol concentrations were analyzed before cows resumed ovarian cycling activity, cortisol pulse frequency was lower and baseline concentrations tended to be greater in cows exposed continuously to bulls than in cows not exposed to bulls. These data are consistent with those of Experiment 2, in that, cortisol pulse frequency was lower and baseline concentrations were numerically greater for cows exposed acutely to bulls than cows exposed acutely to steers.

Subsequently, an evaluation of the effect of resumption of ovarian cycling activity on HPA axis function and/or regulation was conducted by analyzing characteristics of cortisol concentrations in sampling periods before and after cows exposed to bulls resumed ovarian cycling activity. The analyses showed that mean and baseline concentrations of cortisol were greater and pulses of cortisol occurred less frequently before than after resumption of ovarian cycling activity in cows exposed to bulls. Unfortunately, this type of analysis was not possible for cows not exposed to bulls because only one cow resumed ovarian cycling activity before or during the intensive

blood sampling period. Nevertheless, these data indicate that bull exposure altered adrenal function and/or regulation before cows resumed ovarian cycling activity.

In conclusion, the continuous physical presence of bulls accelerated resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows and decreased cortisol pulse frequency before cows resumed ovarian cycling activity. These findings indicate that the biostimulatory effect of bulls altered the activity and/or function of the HPA axis before cows resumed ovarian cycling activity. Therefore, the effect of bulls on resumption of ovarian cycling activity may be related to the release of one or more pheromones that alter the HPA axis and thereby change adrenal function before cows resume ovarian cycling activity. This change in adrenal function and/or regulation may facilitate changes in the HPO axis that bring about resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows.

CHAPTER 7

EXPERIMENT 4: THE RELATIONSHIP BETWEEN SHAPES OF TEMPORAL PATTERNS OF CORTISOL AND LH CONCENTRATIONS IN PRIMIPAROUS, POSTPARTUM, ANESTROUS, SUCKLED, COWS EXPOSED TO BULLS OR STEERS

Introduction

The mechanism by which cortisol influences secretion of LH appears to be mediated through glucocorticoid receptors in luteotrophs of the anterior pituitary. Cortisol binding to glucocorticoid receptors in the anterior pituitary does not appear to decrease GnRH receptor expression but decreases the ability of gonadotrophs to release LH in response to GnRH (Breen et al., 2007; 2008). This indicates that cortisol acts to "decouple" the stimulus-secretion pathway induced by GnRH binding to its receptor on luteotrophs. Thus, fluctuating concentrations of cortisol may influence the pulsatile release of LH by acting as a physiological "barrier" to LH release from luteotrophs in response to GnRH. In other words, the response of luteotrophs to GnRH and the subsequent release of LH would be inhibited when cortisol concentrations are relatively "high". If one assumes that a pulse of cortisol inhibits LH secretion when concentrations are high, then the size of the readily releasable pool of LH in luteotrophs may increase. Subsequently, as cortisol concentrations fall the ability of luteotrophs to secrete LH in response to GnRH would increase; thereby, increasing the probability that a pulse of LH will occur.

As pulses of cortisol become longer and less frequent the overall shape of concentration patterns of cortisol appears smoother or less uneven. This change in shape may influence the ability of luteotrophs to secrete LH in response to GnRH. One could hypothesize that LH secretion from the anterior pituitary in response to GnRH may be synchronized as shapes of temporal patterns of cortisol become smoother and less uneven. The objectives of this experiment were to determine if the unevenness of patterns of cortisol or LH concentrations differ between cows exposed to bulls or exposed to steers, and determine if unevenness of patterns of cortisol concentrations are related linearly to the unevenness of patterns of LH concentrations in cows exposed to bulls and cows exposed to steers.

Materials and Methods

Animals, treatments, facilities, nutrition, blood sampling, and cortisol and LH assays were those of Experiment 2. The temporal patterns of cortisol and LH concentrations were those from Experiment 2. The following narrative describes the rationale and derivation for the variable termed the Mean-Baseline coefficient related to "smoothness" of patterns of cortisol and LH concentrations for evaluating the relationship between patterns of cortisol and LH concentrations.

Mean-Baseline Coefficient

The pulsar algorithm uses amplitude and duration of hormone concentration trends to identify pulses during a sampling period. If a trend in concentrations is to be classified as a pulse it must meet thresholds of amplitude and duration that are based on

an estimate of the noise or variation within each temporal concentration pattern. Sample concentrations that are included in the noise make up the estimate of the baseline concentration. Thresholds for trends in duration decrease as the amplitude of that trend increases and vice versa. However, some trends in temporal patterns of hormone concentrations are not included in estimates of the baseline or pulses. Therefore, some data that may contribute to treatment differences are lost, albeit very little.

To address this problem, it was determined that hormone concentration patterns could be evaluated as shapes that have surface features that can be described as even, i.e., flat and smooth, or uneven, i.e., rough and jagged. The characteristics of temporal patterns of hormone concentrations that contribute to the unevenness or smoothness of lines are pulse frequency, pulse amplitude, and pulse duration. The assumption was that one could coalesce these variables into a single, unitary variable termed “mean-baseline coefficient”. Mean-baseline coefficients were calculated by subtracting the baseline concentration derived from PULSAR analyses from the mean concentration of a sampling period. The result of this calculation gives an estimate of the degree of unevenness or evenness of the line. As baseline concentration nears mean concentration the line becomes smoother and the mean-baseline coefficient decreases. However, if a line has more peaks and valleys the estimate of the mean-baseline coefficient increases. The mean-baseline coefficient depends on the estimate of the noise within the sampling period or baseline; however it is not dependent upon thresholds for trend amplitudes or durations. Any data that are not incorporated into estimates of the baseline or part of a pulse are included in the estimate of the mean-baseline coefficient. The resulting

reduction in the amount of data lost may contribute to understanding how concentration patterns of different hormones are related to each other. A graphical representation used for deriving the mean-baseline coefficient is given in Appendix A.

Statistical Analyses

The effect of bull exposure on mean-baseline coefficients was examined by ANOVA for a completely randomized split-plot design using PROC GLM of SAS. The main plot included treatment (TRT) and Animal within TRT (Anim(TRT)). The Anim(TRT) variance component was used to test the effect of TRT. The sub-plot included day (Day) and the interaction of TRT and Day. Means were separated by Bonferroni multiple comparison tests.

Relationships between shapes of patterns of cortisol and LH concentrations were determined by regressing mean-baseline coefficients of LH on mean-baseline coefficients of cortisol within TRTs using the PROC REG procedure of SAS. The model was a standard regression model, in which mean-baseline coefficients of cortisol were used as the explanatory variable and mean-baseline coefficients of LH were used as the dependent variable.

Results

Mean-baseline coefficients for “shapes” of temporal patterns of cortisol concentrations did not differ ($P > 0.10$; SEM = 1.1 ng/mL) between EB and ES cows (1.46 ng/mL and 1.42 ng/mL, respectively) from D 2 through 8 in Experiment 2. Mean-baseline coefficients were greater ($P < 0.05$; SEM = 0.12) for temporal patterns of LH

concentrations in EB than ES cows (0.12 ng/mL and 0.06 ng/mL, respectively). Mean-baseline coefficients for temporal patterns of LH concentrations were linearly related ($P < 0.05$) to mean-baseline coefficients of temporal patterns of cortisol concentrations in EB cows (b_1 = -0.04 [ng/mL]/[ng/mL]; R^2 = 0.11; Figure 4).

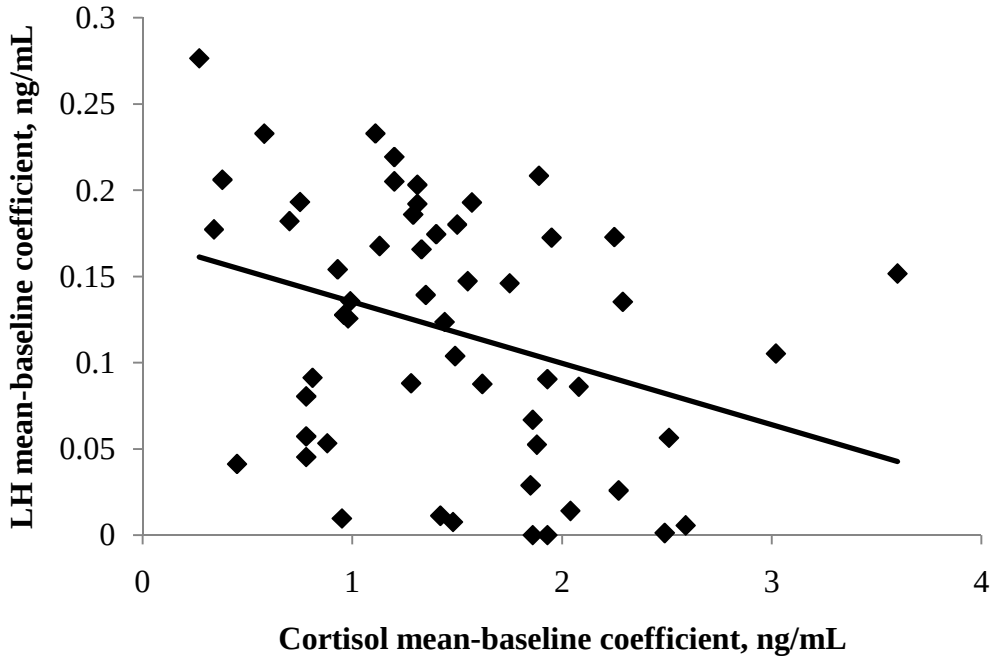

Figure 4. Linear regression of mean-baseline coefficients for temporal patterns of LH concentrations on mean-baseline coefficients for temporal patterns of cortisol concentrations in cows exposed to bulls (EB) for 5 h daily from D 2 through D 8 of a 9-d experimental period initiated 67 ± 3.5 d after calving in Experiment 2. Y-intercept (b_0) = 0.17 ng/mL; slope (b_1) = -0.04 [ng/mL]/[ng/mL]; ($P < 0.05$); R^2 = 0.11.

However, there was no ($P > 0.10$) linear relationship between mean-baseline coefficients for temporal patterns of cortisol and LH concentrations in ES cows (b_1 = -0.02; R^2 = 0.03).

Discussion

In light of the results obtained for mean concentrations of cortisol for bull-exposed cows in Experiment 1, perhaps the most surprising result of Experiments 2 and 3 was that mean cortisol concentrations were not influenced by either acute or continuous bull exposure. Based on results of Experiment 1, an increase in overall mean concentrations of cortisol was expected in cows exposed to bulls. When this was not observed in Experiment 2, it was hypothesized that mean cortisol concentrations may increase before or after cows resume ovarian cycling activity; however this was not the case. One possible explanation of these disparate results is that variation associated with mean cortisol concentrations between animals could have masked treatment effects on mean cortisol concentrations. This conclusion may be valid if one considers that a low number of animals were used in both Experiments 2 and 3. However, the only consistent result that we observed in Experiments 2 and 3 was that cortisol pulses were less frequent and cortisol pulse duration tended to be longer in cows that were exposed to bulls before cows resumed ovarian cycling activity.

Although unexpected, these results could explain how the biostimulatory effect of bulls accelerates resumption of ovarian cycling activity and yield valuable insight into the general mechanism of resumption of ovarian cycling activity in postpartum cows. Based on the results reported by Fernandez et al. (1996), Roelofs et al. (2007) and the results of Experiment 2, it is reasonable to conclude that exposing cows to bulls stimulates an increase in the frequency of LH pulses. The first question that arises after examining the

results of Experiments 2 and 3 is; “What are the physiological ramifications of decreased frequency of cortisol pulses on the function of the HPO axis?”

The mechanism by which cortisol influences LH secretion appears to be mediated through glucocorticoid receptors present in luteotrophs of the anterior pituitary. Cortisol binding to glucocorticoid receptors in the anterior pituitary does not appear to decrease GnRH receptor expression but decreases the ability of gonadotrophs to release LH in response to GnRH (Breen et al., 2007; 2008). Based on this mechanism, one could hypothesize that a decrease in frequency of LH pulses would be observed when cortisol concentrations are high. Thus, fewer cortisol pulses would result in an increase in frequency of LH pulses. That this may be the case is supported by results of Experiment 2, which indicated that as frequency of cortisol pulses decreased frequency of LH pulses increased. However, we also observed that duration of cortisol pulses tended to be longer and amplitude of cortisol pulses was numerically greater in cows exposed to bulls than in cows exposed to steers. One would expect that frequency of LH pulses would decrease as cortisol concentrations remain elevated. Therefore, longer cortisol pulses with greater pulse amplitudes, in cows exposed to bulls, would have an inhibitory effect on the HPO axis and decrease LH pulse frequency. However, frequencies of LH pulses were greater in cows exposed to bulls than in cows exposed to steers. The dichotomy in these results seems contradictory, confusing, and hard to interpret.

Analysis of a variable that incorporates pulse frequency, duration, and amplitude into one response variable may add to the understanding and interpretation of results from Experiment 2. As frequency and duration of hormone pulses decrease, the overall

shapes of patterns of hormone concentrations appear smoother. The smoothness of temporal patterns of cortisol and LH concentrations was measured using the response variable termed the “mean-baseline coefficient”. Mean-baseline coefficients are calculated by subtracting baseline concentrations from the overall mean of a concentration pattern. This measurement was based on the idea that fluctuating concentrations of cortisol may influence the pulsatile release of LH. When GnRH is released by the hypothalamus it binds to receptors on gonadotrophs of the anterior pituitary. However, cortisol may act as a molecular “dam” or barrier to LH release from luteotrophs in response to GnRH. If the cortisol-induced barrier is applied and removed in a synchronous manner this may increase the readily releasable pool of LH in luteotrophs of the anterior pituitary. An increase in the readily releasable pool of LH in luteotrophs may result in greater LH secretion from luteotrophs in response to GnRH; thereby, increasing the probability that an LH pulse will occur, increasing frequency of LH pulses. Thus, patterns of LH concentrations should become less smooth and more uneven as patterns of cortisol concentrations become smoother and less uneven.

The first objective of Experiment 4 was to determine if mean-baseline coefficients of temporal patterns of cortisol and LH differ between cows exposed acutely each day to bulls or steers. It was expected that mean-baseline coefficients of cortisol would be lower in cows exposed to bulls than in cows exposed to steers and that mean-baseline coefficients of LH would be greater in cows exposed to bulls than in cows exposed to steers. These expectations were based on the observation that frequency of cortisol pulses was lower in cows exposed to bulls than in cows exposed to steers and frequency

of LH pulses was greater in cows exposed to bulls than cows exposed to steers. Mean-baseline coefficients of patterns of LH concentrations were greater in cows exposed to bulls than in cows exposed to steers; however, mean-baseline coefficients of cortisol concentrations did not differ between cows exposed acutely to bulls or steers. This result is not entirely unexpected because mean-baseline coefficients incorporate amplitudes, duration, and frequency of pulses. Cortisol pulse amplitudes were numerically higher and durations of cortisol pulses tended to be longer in cows exposed to bulls than in cows exposed to steers. Therefore, this result could reflect increased durations and amplitudes of cortisol pulses.

The most profound result of this experiment was that mean-baseline coefficients of patterns of LH concentrations were negatively related to mean-baseline coefficients of patterns of cortisol concentrations in cows exposed acutely to bulls each day. This relationship was not observed in cows exposed acutely to steers each day. These data may indicate that the relationship between unevenness of LH and cortisol concentration patterns may depend upon the degree of change in unevenness of patterns of cortisol and not the absolute value of mean-baseline coefficients. Although mean values of mean-baseline coefficients for temporal patterns of cortisol concentrations did not differ between cows exposed to bulls and cows exposed to steers, mean-baseline coefficients for cows exposed to bulls may have decreased in cows exposed acutely to bulls each day. Whereas, mean-baseline coefficients of cortisol concentrations for cows exposed to steers may have remained static throughout each day. Thus, cows exposed to steers may not have exhibited an increase in frequency of LH pulses because shapes of temporal patterns

of cortisol did not change. Nevertheless, the relationship between mean-baseline coefficients of patterns of cortisol and LH concentrations in cows exposed to bulls indicates that as the overall shape of patterns of cortisol concentrations changed from uneven to smooth, the overall shape of patterns of LH concentrations changed from smooth to uneven. This observation is consistent with the hypothesis that cortisol may "dam" or inhibit LH release in response to GnRH and increase the ability of luteotrophs to secrete LH in response to subsequent GnRH stimulation. Thus, as temporal patterns of cortisol concentrations changed from uneven to smooth, this change resulted in more LH being secreted from luteotrophs in response to GnRH after concentrations of cortisol decreased to basal levels and increased in frequency of LH pulses in primiparous, postpartum, anestrous , suckled cows exposed to bulls.

In conclusion, mean-baseline coefficients of temporal patterns of cortisol concentrations were not altered by exposing primiparous, postpartum, anestrous, suckled cows to bulls 5 h daily for 9 d. However, mean-baseline coefficients for temporal patterns of LH concentrations were greater for cows exposed to bulls than cows exposed to steers. Furthermore, mean-baseline coefficients of LH concentrations were negatively related to mean-baseline coefficients of cortisol concentrations in cows exposed acutely to bulls. These results indicate that the overall shape of temporal patterns of cortisol concentrations may dictate shapes of patterns of LH concentrations and lend further support to the hypothesis that the biostimulatory effect of bulls alters temporal patterns of cortisol concentrations, and these changes support or facilitate the function of the HPO axis to accelerate resumption of ovarian cycling activity in primiparous, postpartum,

anestrous, suckled, beef cows. Further investigation is needed to establish whether or not this effect is unique to the biostimulatory effect of bulls on resumption of ovarian cycling activity or is a general mechanism involved with resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows.

CHAPTER 8

EXPERIMENT 5: THE EFFECT OF DURATION OF DAILY BULL EXPOSURE ON RESUMPTION OF OVARIAN CYCLING ACTIVITY IN PRIMPAROUS, POSTPARTUM, ANESTROUS, SUCKLED, BEEF COWS

Introduction

Based on the review of literature the mechanism of the biostimulatory effect of bulls on resumption of ovarian cycling activity in cows appears to be dependent upon temporal and quantal thresholds of bull-pheromonal stimuli. Previous data have given some insight that the duration of bull-pheromonal stimuli that is needed to cause resumption of ovarian cycling activity in primiparous, anestrous, suckled cows is greater than 2 h every 3 d (Fernandez et al., 1996) and lesser than 12 h/d (Berardinelli and Joshi, 2005a). However, it is not well understood if the mechanism of the biostimulatory effect of bulls is dependent on some threshold for duration of daily stimulus or if altering the duration of bull-pheromonal stimuli that cows perceive each day influences the time required for anestrous cows respond to the biostimulatory effect of bulls and resume ovarian cycling activity. The objectives of this experiment were to determine if resumption of ovarian cycling activity is accelerated in primiparous, anestrous, suckled cows exposed to bulls for 0, 6, or 12 h daily and if so, are the intervals from calving or the start of bull exposure to resumption of ovarian cycling activity related linearly to the duration of daily bull exposure.

Materials and Methods

Animals and Treatments

Thirty-nine, spring-calving, two-yr-old Angus X Hereford primiparous, suckled beef cows and four mature epididymectomized Angus X Hereford bulls were used in this experiment. Cows and calves were maintained in a single pasture and had no contact with bulls or their excretory products from the previous breeding season until the start of the experiment (D 0). Average calving date for these cows was Feb. 18, 2008. Before the start of the experiment, cycling status of each cow was rated by two ultrasound examinations of each ovary for the presence or absence of a corpus luteum. The first and second ultrasonic examinations were conducted 10 and 2 d, respectively, before the start of the experiment. Cows that did not exhibit the presence of a corpus luteum on either ovary in both ultrasound examinations were used in this experiment.

The interval from calving to the start of the experiment averaged 51.5 ± 2.3 d. Two d before the start of the experiment cows were stratified by calving date, calf birth weight, dystocia score, cow body weight, cow BCS, and sex of calf and assigned randomly to be exposed to bulls for 12 h daily (BE12; n = 15), 6 h daily (BE6; n =14) or not exposed to bulls (NE; n = 10) for 45 d.

Facilities and Daily Bull Exposure

Cows were housed within pens in separate lot areas. Pens within the south lot were used to maintain BE12 and BE6 cows while pens within the north lot were used to maintain NE cows. A common holding pen, approximately 0.35 km from the lot that

housed NE cows and 30 m from the lot that housed BE12 and BE6 cows, was used to house bulls before and after daily exposure periods. During daily exposure periods, two bulls were moved from the common holding pen into the pen that housed BE12 cows and two bulls were moved into the pen that housed BE6 cows. Cows in each treatment were exposed to bulls at 0700 h each day for 45 d (D 0 to 44). At 1900 and1300 h bulls were removed from pens that housed BE12 and BE6 cows, respectively, and housed in the common holding pen until the following day. Cows in each treatment could not see or smell bulls before or after daily exposure periods.

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 $kg \bullet hd^{-1} \bullet d^{-1}$ cracked corn, water, and a trace mineral-salt supplement. Average body weight of cows was 467.5 ± 37.7 kg. The TDN of the diet was 110% of the recommended energy requirement for lactating beef cows with a mature weight of 533 kg (NRC, 1996). Bulls were fed the same diet as cows.

Blood Sampling, Progesterone Concentrations, and Resumption of Ovarian Cycling Activity

Blood samples were collected from each cow by jugular venepuncture every other day from the start (D 0) to the end of the experiment (D 44). Serum was assayed for progesterone concentration using the same assay as in Experiments 1 and 3. Intra- and interassay CV for a serum pool that contained 2.2 ng/mL of progesterone were 10.2 and

15.4%, respectively, and 8.9 and 11.8%, respectively, for a pool that contained 5.75 ng/mL. Progesterone concentrations in these samples were used to determine the interval from calving to resumption of ovarian cycling activity, interval from the start of the experiment to resumption of ovarian cycling activity, and the proportion of cows that resumed ovarian cycling activity during the experiment. The same criteria were used to assess resumption of ovarian cycling activity as described in Experiments 1 and 3. Progesterone concentrations and resumption of ovarian cycling activity were confirmed by transrectal ultrasonographic examination of ovaries of each cow using a Titan ultrasound with a 7.5 to 10 MHz rectal transducer every other day throughout the 45-d exposure period used in this experiment (SonoSite Inc., Bothell, WA, USA). The presence of a corpus luteum in the same anatomical position of an ovary in 4 successive scans was used as evidence to confirm resumption of ovarian cycling activity. Cows that failed to exhibit a rise in progesterone over three consecutive samples and did not have a corpus luteum in their ovaries were assigned an interval from the start of treatment to the end of the experiment.

Statistical Analyses

Intervals from calving and the start of the experiment to resumption of ovarian cycling activity were evaluated by ANOVA for a completely randomized design using PROC GLM of SAS (SAS, Cary, NC). The model included treatment (TRT) and means were separated using Bonferroni Multiple Comparison tests. Linear regression analyses were used to determine the relationship between intensity of bull exposure (hours/d) and intervals (days) from calving and the start of the experiment to resumption of ovarian

cycling activity using the PROC REG procedure of SAS. Data for intervals from calving and from the start of the experiment to resumption of ovarian cycling activity showed heterogeneous variances among treatments using Bartlett's Box *F*-test. Therefore, data from calving and the start of the experiment to resumption of ovarian cycling activity that were used in ANOVA were transformed by raising intervals to the power of 6 and 10.3, respectively. Least squares means and standard errors of means for intervals from calving and the start of the experiment to resumption of ovarian cycling activity, reported herein, were transformed to original values after analysis. Linear regression analyses were conducted by raising intervals from calving and the start of the experiment to resumption of ovarian cycling activity and hours of daily bull exposure to the power of 6 and 10.3, respectively. The sixth and tenth-third root of slopes and Y-intercepts for regression lines for interval from calving and the start of the experiment to resumption of ovarian cycling activity, respectively, were used to estimate days for Y-intercepts and d/h for slopes. Differences in proportions of cows that resumed ovarian cycling activity during the experiment were analyzed by chi-square using the PROC FREQ procedure of SAS.

Results

Mean interval from calving and the start of the experiment to resumption of ovarian cycling activity was shorter ($P < 0.05$) for BE12 cows than for NE cows (Table 10). Additionally, mean interval from calving to resumption of ovarian cycling activity for BE6 cows was shorter ($P < 0.05$) than for NE cows, but did not differ ($P > 0.10$) from

BE12 cows (Table 10). Interval from the start of the experiment to resumption of ovarian cycling activity did not differ ($P > 0.10$) between BE6 cows and either BE12 or NE cows (Table 10). More ($P < 0.05$) BE12 cows resumed ovarian cycling activity during the experiment than NE cows (Table 10). The proportion of cows that resumed ovarian cycling activity did not differ ($P > 0.10$) between BE6 cows and BE12 cows; however, the proportion of cows that resumed ovarian cycling activity during the experiment tended ($P = 0.08$) to be greater for BE6 cows than for NE cows (Table 10).

Table 10. Number of cows per treatment and least squares means for intervals from calving and from the start of the experiment (D 0) to resumption of ovarian cycling activity (ROA) and proportions of cows that resumed ovarian cycling activity for primiparous, anestrous, suckled, beef cows exposed to bulls for 12 h daily (BE12), 6 h daily (BE6) or not exposed to bulls (NE) for 45 d starting 51.5 ± 2.3 d (± SE) after calving

	Treatment[a]				
Variable	BE12	BE6	NE	SEM	*P* value
n	15	14	10		
Interval from calving to ROA, d[b]	85.5[x]	91.3[x,y]	101.9[z]	13.6	<0.05
Interval from D0 to ROA, d	37.4[x]	41.4[x,y]	44.4[y]	7.0	0.06
Proportion that resumed ovarian cycling activity during the experiment, %	60.0[x]	42.9[x,y]	10.0[y]	6.23[c]	<0.05

[a]Means and proportions that lack common superscripts differ ($P < 0.05$).
[b]Cows that failed to exhibit a rise in progesterone over three consecutive samples were assigned an interval from calving or from the start of experiment (D 0) to the end of the experiment (D 44).
[c]X^2 value.

There was a linear relationship (b_1 = -7.64 d/h; $P < 0.05$) between interval from calving to resumption of ovarian cycling activity and hours per day that cows were exposed to bulls (Figure 5).

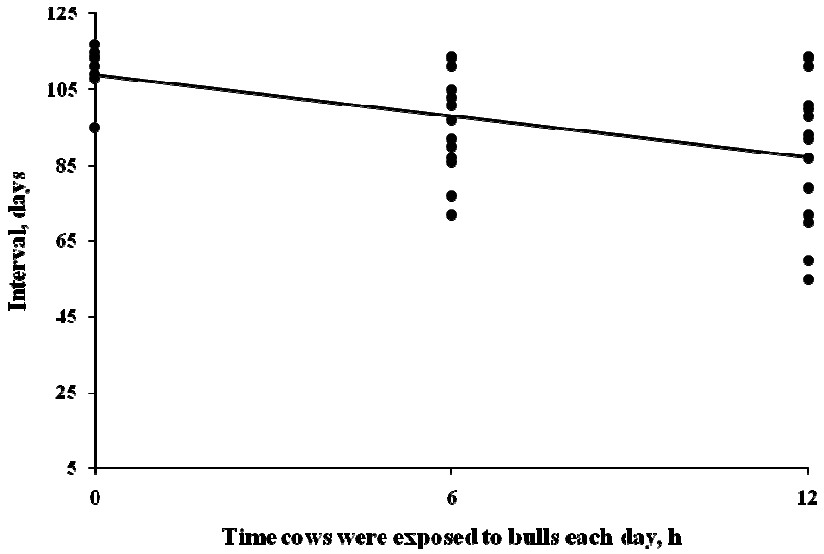

Figure 5. Linear regression of interval from calving to resumption of ovarian cycling activity on hours of daily exposure for postpartum, anestrous, suckled cows exposed to bulls for 0, 6, or 12 h. Y-intercept (b_0) = 105.3 d; slope (b_1) = -7.64 d/h ($P < 0.05$); R^2 = 0.13.

Likewise, there was a linear relationship (b_1 = -3.3 d/h; $P < 0.05$) between interval from the start of the experiment to resumption of ovarian cycling activity and hours of daily bull exposure (Figure 6).

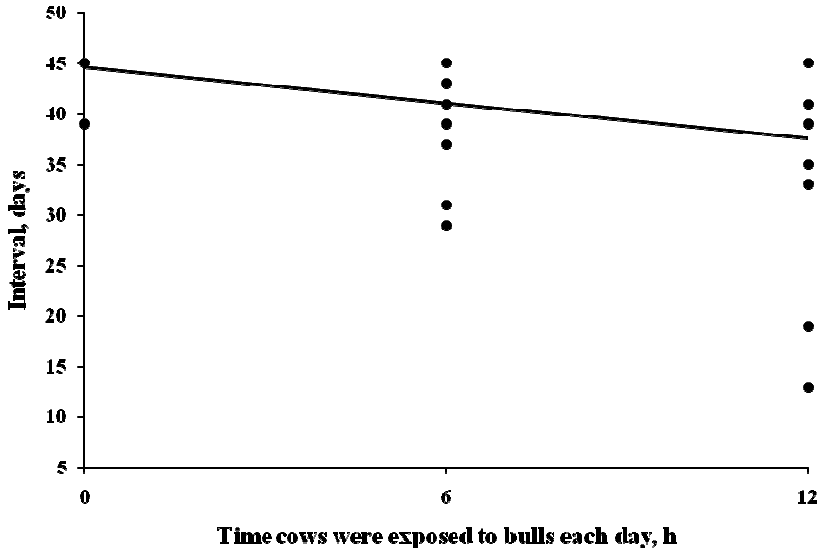

Figure 6. Linear regression of interval from the start of bull exposure to resumption of ovarian cycling activity on hours of daily exposure for postpartum, anestrous, suckled cows exposed to bulls for 0, 6, or 12 h. Y-intercept (b_0) = 43.9 d; slope (b_1) = -3.3 d/h ($P < 0.05$); $R^2 = 0.12$.

Discussion

An interpretation of the literature regarding temporal and quantal factors associated with the biostimulatory effect of bulls on acceleration of resumption of ovarian cycling activity is that pheromonal stimuli need to be presented to anestrous cows in stimulation and relaxation cycles throughout each day over a number of days. There is some evidence in the literature to indicate that duration of daily pheromonal stimulus, i.e., the number of stimulation and relaxation cycles cows perceive each day, influences the time required for cows to respond to the biostimulatory effect of bulls and resume ovarian cycling activity. Fernandez et al. (1996) reported that exposing cows to bulls for 2 h every third day for 18 d beginning on D 33 after calving did not alter the interval from calving to resumption of ovarian cycling activity compared with cows exposed

continuously to bulls. Additionally, resumption of ovarian cycling activity was attenuated in cows exposed to the excretory products of bulls for 12 h daily (Berardinelli and Joshi, 2005a). Based on these data, it was hypothesized that the number of days of bull exposure required to cause resumption of ovarian cycling activity in anestrous cows decreases as cows perceive pheromones produced by bulls for longer intervals each day or perceive more pheromonal stimulation and relaxation cycles each day. This means that the duration of daily bull exposure, or pheromonal stimuli, must be greater than 2 h every 3 d and less than 12 h daily to accelerate resumption of ovarian cycling activity in anestrous cows.

The aim of the current study was to determine whether or not the interval to resumption of ovarian cycling activity in postpartum, anestrous, suckled cows exposed to pheromonal stimuli produced by bulls depends upon the daily duration of pheromonal stimulus or number of pheromonal stimulation and relaxation cycles that cows perceive each day. In the present experiment, intervals to resumption of ovarian cycling activity were shorter and the proportions of cows that resumed ovarian cycling activity by the end of the experiment was greater for cows exposed to bulls for 12 h daily than for cows not exposed to bulls. These data are consistent with those of Berardinelli and Joshi (2005a) who reported that a greater proportion cows exposed to excretory products of bulls for 12 h daily resumed ovarian cycling activity sooner than cows not exposed to bulls or the excretory products of cows. Taken together, these results indicate that exposing postpartum, anestrous cows to presence of bulls or their excretory products for 12 h daily accelerated resumption of ovarian cycling activity.

Interestingly, intervals from calving and start of the experiment to resumption of ovarian cycling activity did not differ between cows exposed to bulls for 6 h daily and cows exposed to bulls for 12 h daily. However, interval from calving to resumption of ovarian cycling activity was shorter for cows exposed to bulls for 6 h daily than for cows not exposed to bulls and the proportion of cows that resumed ovarian cycling activity during the experiment tended to be greater for cows exposed to bulls for 6 h daily than for cows not exposed to bulls. These data indicate that the biostimulatory response of cows exposed to bulls for 6 h daily was intermediate between that of cows exposed to bulls for 12 h daily and for cows not exposed to bulls. Therefore, the biostimulatory effect of bulls on resumption of ovarian cycling activity appears to rely on duration of daily bull exposure in a dose-dependent manner.

An important finding that supports the aforementioned conclusion was the significant linear relationship between duration of daily bull exposure and interval to resumption of ovarian cycling activity. Using the data presented herein, one could expect a decrease in the interval from calving to resumption of ovarian cycling activity of approximately 7.64 d for every hour increase that cows are exposed to bulls each day and a decrease in the interval from the start of bull exposure to resumption of ovarian cycling activity of 3.3 d for every hour increase that cows are exposed to bulls each day. These results support the hypothesis that the number of pheromonal stimulation and relaxation cycles perceived by cows each day influences the number of days required for cows to respond to the biostimulatory effect of bulls and resume ovarian cycling activity.

Results of the present experiment indicate that cows responded to pheromonal stimulus from bulls in a dose-dependent manner. Cows that did not perceive the minimum daily dose or threshold of pheromone stimulation and relaxation cycles would not receive the appropriate biostimulatory signal and would not resume ovarian cycling activity. However, as duration of daily bull exposure increased, the opportunity for cows to perceive daily pheromone stimulation and relaxation cycles also increased. When postpartum anestrous cows perceived a threshold dose or number of pheromone stimulation and relaxation cycles each day they responded to the biostimulatory effect of bulls by resuming ovarian cycling activity. The dose-dependent manner by which pheromones produced by bulls accelerated resumption of ovarian cycling activity in postpartum anestrous cows may explain disparate reports in the literature concerning fence-line contact and intermittent exposure of cows to bulls. Shipka and Ellis (1998; 1999) reported that fence-line contact of cows with bulls separated by 6 to 8 m did not accelerate resumption of ovarian cycling activity; whereas, Fike et al. (1996) and Berardinelli and Tauck (2007) reported that nose-to-nose fence-line contact of cows with bulls accelerated resumption of ovarian cycling activity in postpartum, anestrous cows. Using the result of the present experiment with those of Shipka and Ellis (1998; 1999), Fike et al. (1996) and Berardinelli and Tauck (2007), one could conclude that cows separated from bulls by 6 to 8 m may not sense sufficient doses of bull-pheromonal stimuli each day to cause a biostimulatory effect. On the other hand cows in close, nose-to-nose, contact with bulls may sense thresholds of bull-pheromonal stimuli each day that activate the pheromonal-biostimulatory pathway and accelerate resumption of ovarian

cycling activity. Additionally, the concept that cows sense and respond to pheromones produced by bulls in a dose-dependent manner may explain the results of Fernandez et al. (1996) who reported that cows exposed to bulls for 2 h every 3 d for 18 d did not resume ovarian cycling activity sooner than cows not exposed to bulls. Simply put, exposing cows to bulls for 2 h every 3 d for 18 d may not deliver sufficient doses of daily bull-pheromonal stimulus that are required to accelerate resumption of ovarian cycling activity in postpartum anestrous cows. Thus, the concept that cows respond to bull-pheromonal stimulation and relaxation cycles in a dose-dependent manner may be a critical component of the pheromonal mechanisms by which the biostimulatory effect of bulls accelerates resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows.

In conclusion, exposing primiparous, postpartum, anestrous, suckled cows to bulls for 12 h daily reduced the intervals from calving and the start of bull exposure to resumption of ovarian cycling activity and increased the proportion of cows that resumed ovarian cycling activity within 45 d after the start of bull exposure. Furthermore, intervals from calving and the start of exposure to resumption of ovarian cycling activity were linearly related in a dose-dependent manner to the duration daily of bull exposure. Therefore, the duration of bull-pheromonal stimuli that cows perceived each day may be an integral component of the biostimulatory effect of bulls on resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled, beef cows. This further supports the hypothesis that the biostimulatory effect of bulls is dependent on temporal

and quantal thresholds of pheromonal stimulation to cause a response in the HPO axis that causes resumption of ovarian cycling activity.

CHAPTER 9

GENERAL DISCUSSION

The results of Experiments 1 through 4 leads to the conclusion that the HPA axis is involved in the response of cows to pheromones produced by bulls. It appears as though bull-pheromonal stimuli(us) modulates adrenal activity of postpartum, anestrous cows and influences temporal patterns of cortisol concentrations by decreasing frequency and increasing durations or cortisol pulses. Additionally, characteristics of temporal patterns of LH concentrations appear to be related to characteristics of temporal patterns of cortisol concentrations: as frequency and amplitude of cortisol pulses decrease, frequency and amplitude of LH pulses increase. Interestingly and unique to this dissertation is the finding that shapes of temporal patterns of LH concentrations appear to be related to shapes of temporal patterns of cortisol concentrations in cows exposed to bulls. Mean-baseline coefficients indicated that as shapes of cortisol patterns become smoother, shapes of LH patterns become more uneven. As frequency of cortisol pulses decrease and shapes of cortisol patterns become smoother these changes may act as a physiological “dam” (Breen et al., 2007, 2008) that synchronizes secretion of LH from the anterior pituitary and increases frequency of LH pulses. Thus, bull-pheromonal stimulus altered characteristics and shapes of temporal patterns of cortisol concentrations and these changes may support the function of the HPO axis in postpartum, anestrous, suckled cows.

As shown in Figure 7 factors such as nutrition, lactation, suckling, etc. may result in a temporal pattern of cortisol concentrations that is characterized as being uneven and having frequent and short-duration pulses. This pattern of cortisol concentrations may inhibit function of the HPO axis causing infrequent pulses of LH. However, when pheromones produced by bulls are perceived by cows in the appropriate manner, frequency of pulses of cortisol decrease, duration of pulses of cortisol increase, and the overall shape of patterns of cortisol concentrations become smoother.

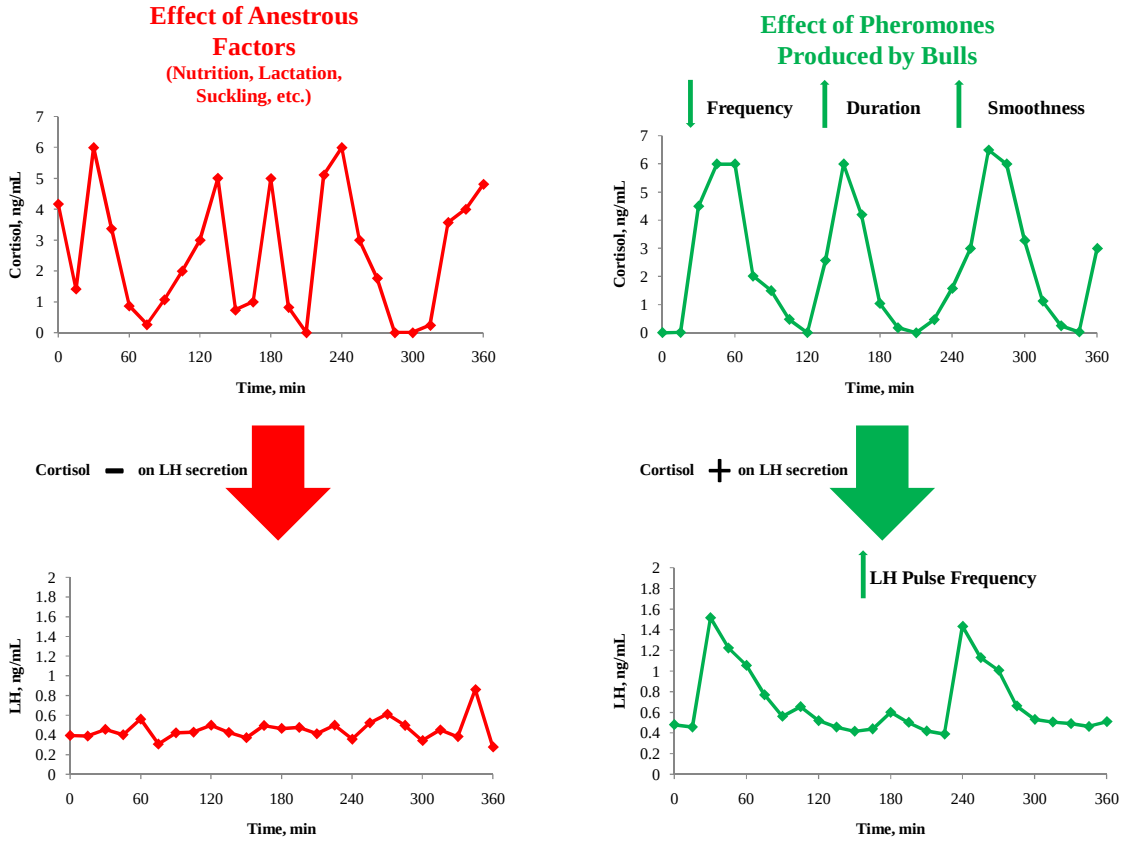

Figure 7. Graphical representation of the effect of factors that influence length of postpartum anestrus and the biostimulatory effect of bulls on the hypothalamic-pituitary-adrenal axis based on results from Experiments 1 through 4 of this dissertation.

These changes in temporal patterns of cortisol concentrations cause an increase in frequency of LH pulses. An increase in the frequency of LH pulses stimulates growth and maturation of the dominant follicle, which leads to the preovulatory increase in secretion of E_2 from the dominant follicle and surge of LH. The LH surge leads to ovulation of the dominant follicle and formation of a functional corpus luteum, i.e. resumption of ovarian cycling activity.

Hypothetical Paradigm for the Mechanism Involved with the Biostimulatory Effect of Bulls

In this section, a model is introduced for the physiological mechanism involved in the biostimulatory effect of bulls to accelerate resumption of ovarian cycling activity in postpartum, anestrous, suckled cows. This model, presented in Figure 8, incorporates the results and interpretations of the experiments presented in this dissertation and results and interpretations of experimentation summarized in the review of literature. In the left portion of Figure 8, pheromonal production, transport, and sensory pathways based on the review of literature are presented. In the right half of Figure 8, a summary of results from previous research at Montana State University and from Experiment 5 of the dissertation is presented. In this representation the shaded box at the top of the figure labeled “What we know” contains one or two asterisks: if a single asterisk appear within a box this indicates that there is limited data to support the information contained therein; if there are two asterisks within a box then there is substantial evidence to support the information within the box; if there are no asterisks or a question mark then these are speculative or hypothetical ideas based on information from other species.

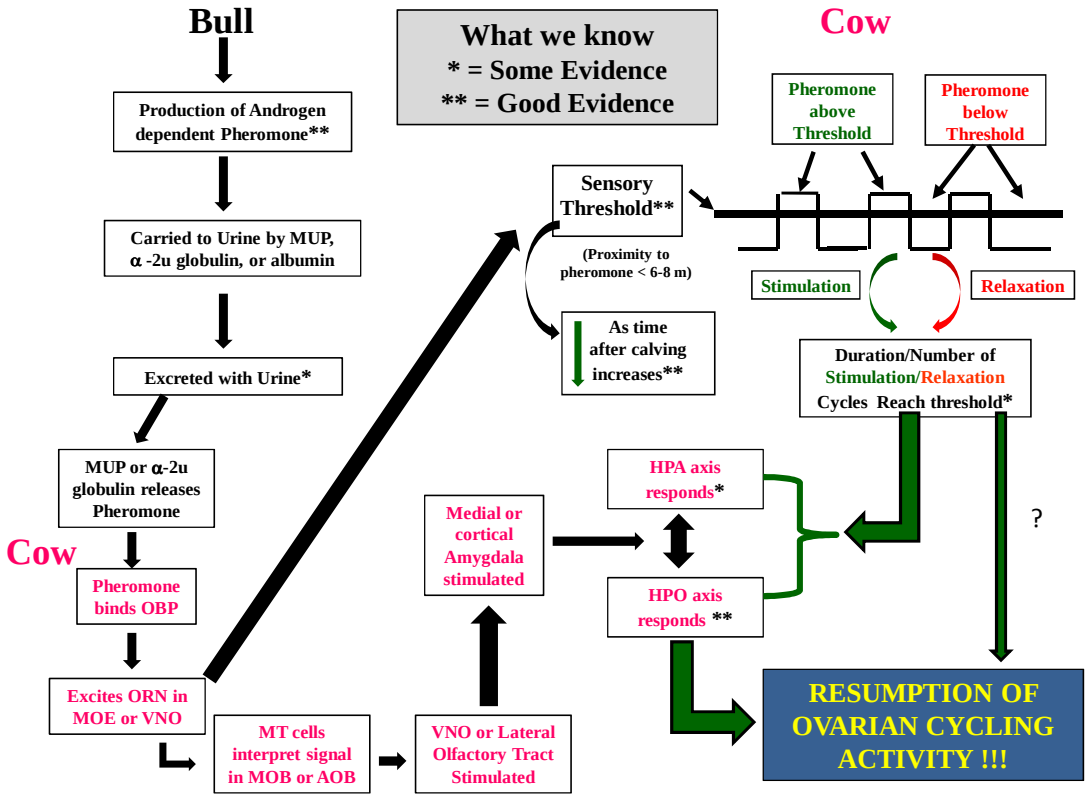

Figure 8. An illustration of the hypothetical mechanism of the biostimulatory effect of bulls to accelerates resumption of ovarian cycling activity in postpartum, anestrous, suckled cows.

The following is a summary of the hypothetical physiological model by which bulls accelerate resumption of ovarian cycling activity in primiparous, postpartum, anestrous, suckled cows presented in Figure 8. Bulls produce an androgen-dependent pheromone (Burns and Spitzer, 1992) that is carried from the blood to the urine by major urinary protein (MUP), α-2u globulin, albumin, or a combination thereof (Cavaggoini and Mucignat-Carretta, 2000). This pheromone is excreted in urine, feces, cutaneous secretions, or a combination of these excretory products (Izard, 1983; Berardinelli and Joshi, 2005a). Alpha-2u globulin, MUP, or albumin release the androgen-dependent pheromone into the environment after it has been excreted (Flower, 1996). This

pheromone(s) is then sensed by the cow by binding to odorant binding protein (OBP) in the nasal mucosa (Pelosi, 2001). The pheromone-OBP complex excites odorant receptor neurons (ORN) in the main olfactory epithelium (MOE; Gelez and Fabre-Nys, 2006) or vomeronasal organ (VNO; Schild and Restrepo, 1998), which stimulates mitral tufted (MT) cells in either the main olfactory bulb (MOB) or accessory olfactory bulb (AOB; Meisami and Bhatnagar, 1998). Depending on which bulb is stimulated, the signal is transmitted through the vomeronasal or lateral olfactory tracts, which stimulate the medial or cortical amygdala, respectively. It is within these central nervous system structures that perception occurs by interacting with hypothalamic, hippocampal, and cortical centers that interpret the strength, duration, etc. of the pheromonal signal. Perception of the pheromone stimulates hypothalamic-pituitary-ovarian (HPO) activity by influencing hypothalamic-pituitary-adrenal (HPA) function (Experiments 1 - 4). Cows perceive pheromones when they are within less than 6 to 8 m of bulls (Berardinelli and Tauck, 2007) or excretory products of bulls (Berardinelli and Joshi, 2005a). Perception of pheromones leads to stimulation of pheromonal sensory systems of cows above some as yet unknown threshold. As time after calving increases the threshold for perception and sensory pathways decrease (Berardinelli and Joshi, 2005b). Each stimulation period is followed by a period of relaxation (Tauck et al., 2006) and cows respond to this stimulation sooner as the duration of daily stimulation and relaxation cycles increase (Experiment 5). The end result is an increase in the frequency of LH pulses that is facilitated by activation of the HPA axis. This change in frequency of LH pulses stimulates growth and maturation of a dominant follicle, which leads to the

preovulatory increase in secretion of E_2 from the dominant follicle and the preovulatory release of LH. The LH surge causes ovulation of the dominant follicle and formation of a functional corpus luteum, i.e., resumption of ovarian cycling activity.

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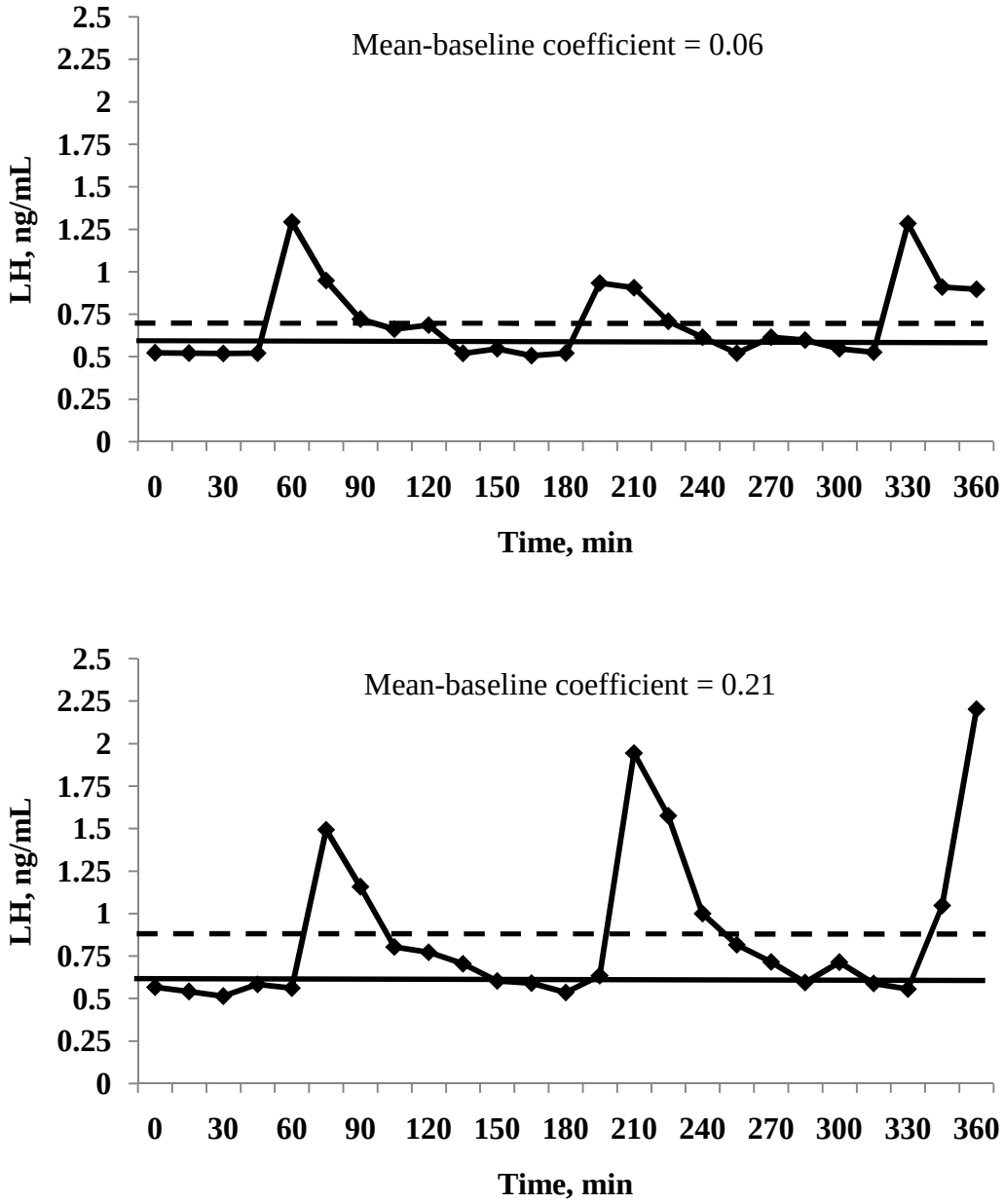
APPENDIX A:

CALCULATION OF MEAN-BASELINE COEFFICIENT



Graphical representation of the relationship between shapes patterns of luteinizing hormone (LH) concentrations and calculated mean-baseline coefficients. The top graph represents a concentration pattern that is indicative of a smooth or less uneven line. The bottom graph represents an uneven or jagged line. Solid lines represent baselines derived from PULSAR algorithm. Dashed lines represent the mean concentration of all samples during the sampling period. Mean-baseline coefficients are calculated as the difference between mean and baseline concentrations. As mean-baseline coefficients increase, concentration patterns become more uneven and display greater divergence between zeniths and nadirs.