EFFECT OF RAMS ON TEMPORAL HORMONE CONCENTRATIONS IN TARGHEE EWES DURING THE TRANSITION INTO THE BREEDING SEASON

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Richard Bryan McCosh

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APPROVAL

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Richard Bryan McCosh

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Dr. James G. Berardinelli

Approved for the Department of Animal and Range Sciences

Dr. Glenn C. Duff

Approved for The Graduate School

Dr. Carl A. Fox

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Richard Bryan McCosh

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ABSTRACT

The biostimulatory effect in sheep is known to cause a relatively rapid increase in LH pulse frequency in ewes that accelerates resumption of ovulatory activity during the transition into the breeding season. In addition there is the possibility that the biostimulatory effect involves changes in the metabolic status of ewes. Experiment 1, the hypotheses were that exposing seasonally anovular ewes to rams would not alter patterns of cortisol concentrations and that these changes are not associated with changes in temporal characteristics of LH concentrations. Cortisol pulse duration was longer in ewes exposed to rams (RE) than in ewes exposed to wethers (NE). The number of LH pulses, and LH pulse frequency was greater in RE ewes than in NE ewes. In RE ewes, as the pulse frequency and number of cortisol pulses increased there was a linear decrease in LH pulse frequency and number of LH pulses. The hypotheses in experiment 2 were that were that resumption of luteal activity and temporal patterns of cortisol, leptin, prolactin, IGF-1, T3 and T4 do not differ among virgin ewes exposed to rams during the transition into the breeding season. Resumption of luteal activity began earlier ($P < 0.05$) in RE than in NE ewes. Concentrations of T4 in RE ewes decreased less rapidly and over a longer interval before increasing by the end of the sampling period than those in NE ewes. Concentrations of PRL were greater in RE than in NE ewes 4 d after exposure but decreased over the next 12 d; whereas, PRL decreased in NE ewes during the first 6 d then increased over the next 14 d. In conclusion, ram exposure during the transition into the breeding season alters hypothalamic-pituitary-adrenal axis activity, which is related to an increase in LH pulse frequency, that hastens luteal activity. In addition, the onset of luteal activity is associated with alterations in metabolic status in ewes exposed to rams during the transition in to the breeding season.

CHAPTER 1

INTRODCTION

Reproduction requires considerable energetic investment for ewes, as such reproduction in general is restricted to favorable environmental conditions and specifically limited to periods of sufficient nutritional intake (for review, see, Scaramuzzi et al., 2006). Though this makes good evolutionary sense, these processes provide an obstacle for maximum sheep production. Ewes are a seasonally polyestrous species with a natural breeding season in autumn and early winter, and an approximately 5 mo long gestation. Productivity and therefore profitability of a ewe can be quantified by mass of lamb brought to market per year. Breeding ewes earlier in the year will result in earlier lambing and heavier lambs at weaning. The biostimulatory effect of rams, also known as "the ram effect," is a method of hastening resumption of estrus and ovulation in ewes. The biostimulatory effect of rams is a good candidate production technique because it is relatively inexpensive, labor saving, sustainable and socially acceptable (Martin and Kadokawa, 2006). Though this technique is in practice in many sheep production facilities around the world, certain components of the physiological mechanism of the biostimulatory effect of rams are not well understood.

The biostimulatory effect of rams involves olfactory, visual and tactile interactions of rams and ewes to induce ovulation in these females (for review, see, Delgadillo et al., 2009; Martin et al., 1986; Schinckel, 1954). Generally, exposing ewes to rams during the transition into the breeding season results in a relatively rapid increase in

pulsatile secretion of the pituitary gonadotropin known as luteinizing hormone (LH; Martin et al., 1980; Poindron et al., 1980). This increase in LH pulse frequency enhances follicular growth and maturation and ultimately ovulation, usually within 2 to 3 d, with normal estrous cyclicity thereafter (Atkinson and Williamson, 1985). It is known that postpartum, anovular cows exposed acutely to bulls exhibit changes in cortisol concentration patterns that coincide with changes in LH concentration patterns (Tauck et al., 2010). Furthermore, cows exposed to bulls tended to have higher leptin concentrations and lower non-esterified fatty acid (NEFA) concentrations (Olsen, 2009).

There is the possibility that the biostimulatory effect of rams also involves acute changes in cortisol concentration patterns as well as alterations of metabolic hormones concentrations in ewes during the transition into the breeding season. Experiment 1 of this thesis focuses on the temporal concentrations of cortisol and LH in ewes during the first 6 h of exposure to rams. Experiment 2 of this thesis was designed to determine the effect of the presence of a ram on systemic concentrations of cortisol, thyroxine (T4), tri-iodothyronine (T3), leptin, prolactin, IGF-1 in ewes over a 22-d period during the transition into the breeding season.

This review of literature encompasses: 1) an overview of the endocrinology of reproduction in ewes; 2) a review of regulation of metabolic processes; 3) an overview of the effect of stress on reproduction; and, 4) a review of the biostimulatory effect or rams.

CHAPTER 2

LITERATURE REVIEW

Reproductive Endocrinology of the Ewe

The reproductive endocrine system includes three organs, the hypothalamus, adenohypophysis and ovaries. The hypothalamus is central to integrating a variety of stimuli and orchestrating signals to the adenohypophysis. The adenohypophysis is the site of synthesis and secretion of gonadotropins. The ovaries are the source of female gametes and synthesize and secrete steroid and protein hormones that, among other functions, modulate the reproductive endocrine system.

Hypothalamus

The hypothalamus of the brain is primarily composed of neurons and is a relatively small structure located in the medio-basal aspect of the brain. The hypothalamus is the most basal portion of the diencephalon in proximity to the third ventricle. It is anatomically defined anteriorly by the optic chiasm dorsally by the thalamus and posteriorly by the mammilary bodies (Hafez and Hafez, 2000). Regions within the hypothalamus are termed “nuclei.” These are roughly defined as clusters of neurons with similar function and ultrastructure.

The hypothalamus is a portion of the brain that is responsible for regulating a variety of autonomic systems including reproduction, heart rate, appetite, water and salt balance, and metabolic activity. Many of these functions are controlled by neurosecretory neurons that integrate intrinsic and extrinsic signals, in response to these stimuli, peptide

neurohormones are synthesized and secreted. Neurosecretory neurons are classified as either part of the parvocellular system or magnocellular system. Neurosecretory cells of the parvocellular system project axons to the median eminence region of the hypothalamus that end on capillaries of the hypothalamic-pituitary portal system. Neurohormones from these cells influence anterior pituitary function, as such, these neurohormones are known as hypophysiotropic factors. The magnocellular system is composed of relatively large neurons located in the paraventricular and suprachiasmatic nuclei of the hypothalamus. Axons of these neurons project on capillaries of the posterior lobe of the pituitary. These neurons synthesize oxytocin and vasopressin in their soma and transport them within the axon for storage and release into the circulation from the neurohypophysis.

Gonadotropin releasing hormone (GnRH). Gonadotropin releasing hormone is a decapeptide synthesized and secreted from a subset of neurosecretory neurons scattered throughout the hypothalamus. The majority of GnRH-immunoreactive cells have been localized to the preoptic hypothalamic area (POA), the diagonal band of broca, the anterior hypothalamus and the mediobasal hypothalamus in sheep (Caldani et al., 1988) and rats (Merchenthaler et al., 1989). Each of these GnRH neuron-containing nuclei have extensions to the external zone of the median eminence in sheep (Jansen et al., 1997; Lehman et al., 1986; Silverman et al., 1987) and rats (Merchenthaler et al., 1989), and to a lesser degree, the organum vasculosum of the lamina terminalis in sheep (Caldani et al., 1988; Jansen et al., 1997; Lehman et al., 1986). GnRH neurons in sheep typically have a

bipolar or multipolar morphology, with few synaptic inputs, and many have a relatively long primary dendrite in excess of 500μm (Lehman et al., 1986).

Gonadotropin releasing hormone neurons secrete GnRH in a pulsatile manner (Moenter et al., 1992); each pulse stimulates the release of hypophyseal gonadotropins (Schally et al., 1971). The temporal pattern of pulsatile release can be described as either tonic- or surge type release. Tonic release refers to small, somewhat random episodes of hormone secretion followed by periods of variable length quiescence before the next episode. Surge release refers to hormone release with a larger magnitude, and higher frequency that leads to temporal summation of hormone concentrations. It is thought that GnRH is secreted in the tonic mode by a random pulse generating system that can be modified or otherwise functionally adjusted by a variety of factors including steroid hormone milieu (Karsch et al., 1987), photoperiod (Karsch et al., 1993), disease state (for review, see, Karsch and Battaglia, 2002), stress (Oakley et al., 2009), metabolic status (for review, see, Wade and Schneider, 1992) and other environmental stimuli. During a GnRH surge however, the secretion pattern of GnRH changes quite dramatically, from low amplitude and frequency to a sustained elevation of GnRH concentrations (Moenter et al., 1992).

Kisspeptin. Kisspeptin (formerly known as metastin) has been shown to play an important role in the regulation of reproduction since 2003 when a mutation in GPR54 was identified as the cause of hypogonadotropic hypogonadism in humans (de Roux et al., 2003). GPR54 was an orphan G- protein coupled receptor, and since then, kisspeptin has been identified as its ligand (de Roux et al., 2003). Since then, kisspeptin has been

associated with the initiation of puberty in primates (Plant, 2006) and rodents (Seminara et al., 2003), control of the estrous cycle in rodents (Maeda et al., 2007) and seasonality in sheep (for review, see,Clarke et al., 2009). Specifically, kisspeptin administration can cause an LH surge and ovulation in anestrus ewes (Caraty et al., 2007).

Pulsatile secretion of GnRH is a tightly regulated phenomenon that requires considerable modulation to reflect the multitude of stimuli that females experiences. It is known that regulation of GnRH secretion does not appear to occur at the level of GnRH secretory cells, because GnRH secretory cells do not possess receptors for ovarian steroid hormones (for review, see, Herbison, 1998). Instead, a relatively recently discovered population of kisspeptin expressing cells have been identified as regulators of GnRH. Kisspeptin, is a peptide composed of either 54, 14 or 10 amino acid analogues. It is the product of the KiSS1 gene that targets G protein coupled receptors (GPR54) present on GnRH neurosecretory neurons and cells of other tissues (Kotani et al., 2001). Kisspeptin has been localized in the arcute (ARC) nucleus and the preoptic area of the hypothalamus in sheep. In rodents, populations of kiss-immunoreactive cells have been found in the ARC and in the anteroventral-periventricular nucleus (for review, see, Lehman et al., 2010c).

The positive feedback effects of estradiol on GnRH are associated with actions of kisspeptin as evidenced by findings that KiSS1 expression in the preoptic area and caudal arcute nucleus are greater during the late follicular phase than during the luteal phase in ewes (Smith et al., 2009). Furthermore, a greater number of cells colocalize kisspeptin and Fos (an indicator of transcription) in ovariectomized ewes administered a surge

inducing dose of estradiol than ovariectomized control ewes (Smith et al., 2009). These findings indicate that surge- like concentrations of estradiol increase kisspeptin signals, which in turn increase GnRH secretion.

Evidence that the negative feedback effect of estrogen on GnRH secretion is associated with kisspeptin signaling comes from findings that ovariectomized mice supplemented with estradiol to mimic luteal phase concentrations reduced Fos expression in kisspeptin cells (Clarkson et al., 2008). Furthermore, ovariectomy increased kisspeptin immunoreactivity in ewes (Pompolo et al., 2006), suggesting that low concentrations of estradiol inhibits kisspeptin production and in turn reduces GnRH secretion.

Neurokinin B. It has been known for some time that the 10 amino acid peptide neurokinin B (NKB) plays a role in GnRH regulation in a number of species. In sheep, the stimulation of the NKB receptor (NK3R) has a stimulatory effect on LH secretion, presumably through stimulation of GnRH secretion (Billings et al., 2010). GnRH neurons do not express NK3R but Kisspeptin-Neurokinin B- Dynorphin A (KNDy) neurons do, indicating the NKB operates at the level of KNDy neurons (Amstalden et al., 2009). Furthermore, mutations in TAC3 or TACR3 genes which code for NKB and NK3R, respectively, have been associated with hypogonadotropic hypogonadism (Topaloglu et al., 2009). Taken together these results provide evidence that NKB acts as a stimulator of GnRH activity via KiSS in sheep and humans. However, this may not be the case all species, as NK3R agonists have been found to elicit an inhibitory effect on gonadotropin concentrations in rodents (Navarro et al., 2009; Sandoval-Guzman and Rance, 2004).

Dynorphin A. Dynorphin A is a 13 amino acid endogenous opioid ligand of the κ opioid receptor that has been implicated in mediating the effect of progesterone of GnRH secretion. Evidence that endogenous opioid peptides are important in negative feedback effect of GnRH and LH originate from findings that ovariectomized ewes treated with progesterone and luteal phase ewes experience an increased GnRH (Horton et al., 1987) and LH (Whisnant and Goodman, 1988; Yang et al., 1988) pulse amplitude when administered EOP agonists. However, ovariectomized ewes treated with estradiol do not experience a change in LH pulse frequency (Whisnant and Goodman, 1988). Together, these results suggest that dynorphin is an important modulator of the effect of progesterone and that estradiol feedback effect is mediated by an alternative system.

Kisspeptin- Neuroknin B- Dynorphin A cells (KNDy). Kisspeptin-Neurokinin B-Dynorphin A neurons have been found to coexpress kisspeptin, neurokinin B and dynorphin A in the arcute nucleus of sheep (Goodman et al., 2007). Kisspeptin-Neurokinin B- Dynorphin A cells have been shown to colocalize ovarian steroid hormone recpeptors for estrogen (Franceschini et al., 2006; Goubillon et al., 2000) and progesterone (Foradori et al., 2002) in sheep, this provides a link from ovarian steroid hormones to GnRH that was previously missing. Additionally, receptors for leptin (Smith et al., 2006) and glucocorticoids (for review, see, Lehman et al., 2010a) have also been identified in KNDy cells, indicating that inputs from a variety of stimuli may affect GnRH secretion through these neurons. Also, it is important to note that KNDy cells in the arcute nucleus have been found to receive input from other KNDy cells, meaning that these neurons may be able to act as a single unit (Foradori et al., 2002).

Relationship Between KNDy Neurons and the GnRH Random Pulse Generator. It has been hypothesized that KNDy cells localized in the arcuate nucleus (ARC) are the source of the GnRH pulse generator in sheep (for review, see, Lehman et al., 2010a) and goats (for review, see, Maeda et al., 2010). Though kisspeptin does present a plausible mechanism for mediating the biphasic effects of estradiol, it alone does not account for the spontaneous release patterns of GnRH that underlie reproductive processes. The model for GnRH pulse generator proposed by Lehmen et al. (2010a) and Meadea et al. (2010) involves the following interactions of NKB and Dyn. It is thought that NKB is initially released from some KNDy neurons and that a paracrine and autocrine positive feedback system amplifies the NKB signal, leading to Kisspeptin release from KNDy neurons. NKB also causes the release of Dynorphin from KNDy cells, which inhibits kisspeptin secretion, ending the signal. In addition, the release of dynorphin initiates a negative feedback system to end the release of dynorphin, thereby clearing the neuron and providing an opportunity for another pulse.

Hypophysis

The hypophysis or pituitary gland is a small endocrine gland situated within the sella turcica, a depression within the sphenoid bone, at the base of the brain, near the optic chiasm. The hypophysis is made up of two distinct tissue types. The adenohypophysis or anterior lobe of the pituitary gland consists largely of specialized epithelial cells that arise embroyologically from Rathke's pouch, an invagination of the buccal ectoderm. The neurohypophysis or posterior lobe of the pituitary gland develops

from the neural ectoderm of the brain and as such consists mostly of nerve endings (axonal bouttons) and blood vessels (for review, see, Hafez and Hafez, 2000).

The adenohypophysis synthesizes and secretes seven hormones into general circulation under the direction of parvocellular system of the hypothalamus. Hypophysiotropic factors synthesized by neurosecretory neurons in the hypothalamus are released into the neurohemal spaces of the primary capillary plexus of the hypothalamic-hypophysial portal vascular system to the pars distalis of the adenohypophysis. The hypophysial portal system consists of primary capillary bed located in the median eminence and infundibular stem that collects hypophysiotropic factors, and a connected secondary capillary bed that vascularizes the pars distalis and distributes hypophysiotropic factors with the pars distalis. Hormone- specific secretory cells in the pars distalis secrete a particular hormone in response to a specific hypophysiotropic factor. The adenohypophysis is arranged so that secretory cells that secrete each hormone are clustered together, giving rise to zones of origin for each pituitary hormone (for review, see, Hafez and Hafez, 2000).

Gonadotropins. Gonadotropins are a class of glycosylated protein hormones that are secreted from gonadotropes of the adenohypophysis. Gonadotropins include luteinizing hormone (LH), which regulates follicular maturation, ovulation and luteinization in females, and regulates testosterone synthesis and secretion by Leydig cells in males. The other major pituitary gonadotropin is follicle stimulating hormone (FSH), which regulates follicular development in females and regulates sperm production in males (for review, see, Hafez and Hafez, 2000). There is evidence from

immunohistolgoical studies to suggest that each of the gonadotropins are secreted from individual gonadotrope cells in rats, whereas in humans vacuoles containing LH and vacuoles containing FSH were both observed in the same cells (for review, see, Moriarty, 1973).

Gonadotropins are dimeric glycoproteins that share a common α-subunit and differ in the β-subunit. Biologic effect of the hormone is dependent on the β-subunit; however, the β-subunit alone confers no biological activity. The molecular weight of the gonadotropins is approximately 32,000 daltons, with each subunit weighing the same, approximately 16,000 daltons (for review, see, Bernard et al., 2010).

Throughout the estrous cycle, LH and FSH are produced in different proportions to support ovarian activity. Gene expression for either the LH or FSH β-subunit is directed by a number of factors including GnRH pulse frequency, GnRH receptor concentration, gonadal steroids and non-steroidal substances such as inhibin, activin, and follistatin among other factors (for review, see, Bernard et al., 2010). Gonadotropins are released from gonadotropes under influence of pulsatile fluctuations in GnRH secretion from the hypothalamus. Gonadotropin release occurs in small bursts that correlate with vacuole exocytosis from the axon terminals of GnRH neurons. Gonadotropin release from gonadotropes is initiated by GnRH binding to GnRH receptors initiating a G protein coupled reaction leading to spikes of intracellular calcium and hormone release.

Ovaries

The primary functions of the ovaries are to develop follicles for the production of fertilizable ova and to produce steroid hormones. Ovaries in most large monovulating and biovulating mammals are small, ovoid shaped, paired glands that are typically located in the lumber area of abdomen in close proximity to the kidneys. Ovaries are suspended from the anterior portion of the broad ligament known as the mesovarium. The mesovarium also supports ovarian arteries and veins as well as lymphatic vessels and nerves that supply ovaries (for review, see, Hafez and Hafez, 2000).

Estrogens. The dominant ovarian estrogen, estradiol 17- β, is synthesized and secreted primarily by granulosa cells of a developing follicle. It should be noted that only granulosa cells contain the aromatase enzymes necessary for final stages of the biosynthetic pathway of estrodiol synthesis from cholesterol. Estrodiol synthesis by granulosa cells is dependent on a source of 19-carbon androgens from thecal cells (for review, see, Hillier et al., 1994). This finding gave way to the current "two gonadotropin, two cell" mechanism for estrodiol synthesis because theca cells produce androgens under influence of LH; whereas, aromatase activity in granulosa cells is stimulated by FSH (Whitelaw et al., 1992).

Estrodiol exerts its effects on numerous tissues throughout the body including the hypothalamus, hypophysis, mammary tissue, vagina, cervix, uterus, oviducts, skin, muscle, and liver. The effects of estrodiol are mediated by steroid binding to cytosolic estrogen receptors that exerts their action in the nucleus of cells. There are two distinct isoforms of the nuclear receptor, ERα and ERβ, both of which work through binding to

estrogen response elements located near target genes of target cells (for review, see, Hafez and Hafez, 2000). Additionally, a membrane bound form of the estrogen receptor has been found in a variety of cell types, and is believed to capable of mediating quicker actions than the nuclear receptors (Kelly and Levin, 2001).

Progesterone. Progesterone is secreted primarily from luteal cells of the corpus luteum, though the adrenal gland and placenta are also sources of this hormone (for review, see, Hafez and Hafez, 2000). Progesterone elicits a variety of responses in the female including preparing the endometrium and myometrium for implantation of the blastocyst , maintenance of pregnancy, stimulation of the alveoli of the mammary glands for lactation, and acts in concert with estradiol to regulate the estrous and menstrual cycles and reproductive behavior. Progesterone plays an important role in the regulation of the estrous cycle because it has a negative feedback effect on GnRH pulsatility that results in lower LH pulsatility (Goodman and Karsch, 1980). Despite its typically dampening role in reproductive processes, progesterone also acts as a priming agent that is necessary to induce the positive feedback effect of estradiol on GnRH secretion and estrous behavior in species that have seasonal estrous cycles (Caraty and Skinner, 1999).

Metabolic Regulation and Reproduction

Cortisol

Cortisol is the dominant adrenal glucocorticoid in sheep. Its structure, 11β, 17α, 21- trihydroxy-pregn-4ene-3, 20-dione, confers a variety of metabolic actions including protein, carbohydrate and lipid metabolism, and is most notably associated with stress.

The hypophysiotropic factor, cortisol releasing hormone (CRF), ultimately regulates cortisol secretion by stimulating the secretion of adrenocorticotropin (ACTH) from the pars distalis of the adenohypophysis. Adrenocorticotropin enhances the production of cortisol from the zona fasciculate of the adrenal gland through up regulation of a number of enzymes responsible for steroid biosynthesis. Cortisol is released into the blood and binds to the specific transport protein known as corticosteroid binding protein (for review, see, Hadley and Levine, 2006). “Unbound” cortisol can then exert effects on a variety of target tissues or be metabolized to an inactive form and/or excreted in urine or feces. Cortisol regulates cellular activities through either cytosolic receptor-genomic mechanisms that requires transcriptional events in the cell nucleus, or non- genomic, more rapid actions independent of protein synthesis (Stahn et al., 2007).

Cortisol plays an important role in intermediary metabolism, or the processes necessary for cells to utilize energy sources. Cortisol acts in an anabolic manner in hepatocytes of the liver by stimulating the synthesis of enzymes necessary for gluconeogenesis. This results in increased glucose production which can be secreted into the blood or stored as glycogen. Cortisol also acts in a catabolic manner in skeletal muscle and adipose tissue by inhibiting glucose uptake and stimulating proteolysis and

lipolysis. Free fatty acids and amino acids from these tissues are secreted into the blood in response to cortisol which can then be used as a substrates for gluconeogenesis in the liver (for review, see, Hadley and Levine, 2006).

Cortisol and Stress. A stress is any stimuli that disrupts homeostasis in an organism, and can originate from a variety of sources including physiological, psychological or physical (Dobson and Smith, 1995). It is known that prolonged stressful stimuli have a negative impact on reproduction (for review, see, Dobson and Smith, 2000). Experiments in which exogenous cortisol agonists were administered provide evidence that stress of this type can reduce the response of LH to GnRH. Results such as these provide a mechanism whereby reproduction can be inhibited by stress through the actions of cortisol (Breen et al., 2008). It is important to note that stress induced by an injection of a single hormone does not entirely mimic activation of the hypothalamic-pituitary-adrenal (HPA) axis; other endocrine responses such as the release of opioid peptides (Owens and Smith, 1987) and biological amines (Willems et al., 1999) have been associated with nociception. Interestingly, short term stressful stimuli, such as restraint, have not caused negative impacts on LH and gonadal steroid secretion in cows (Thun et al., 1998) or pigs (Turner et al., 2005), despite transiently elevating cortisol concentrations. Perhaps more importantly, are Echternkamp's (1984) findings that the influence of stress on gonadotropin secretion is dependent on the magnitude of adrenal output, but that this can be overcome by adaptation to the stressful stimuli. From these results it is clear that the effect of stress on reproduction is a complicated matter riddled

with exceptions, no doubt emanating from an incomplete understanding of the interacting mechanisms involved with stress and response to stress.

Thyroid Hormones

Thyroid hormones, triiodothyronine (T3) and thyroxine (T4) mediate a variety of cellular functions including lipid, carbohydrate and protein metabolism as well as oxygen consumption which among other factors defines an organisms' basal metabolic rate. Normal growth and development are also dependent on thyroid hormones (Norman and Litwack, 1997). Similar to other endocrine systems, thyroid hormones are controlled primarily through hypothalamic regulation and the secretions of a hypophysiotropic factor known as thyrotropin releasing hormone (TRH) secreted from neurons of the paraventricular nucleus and preoptic area into the hypophysial portal system. Thyroid stimulating hormone (TSH) is a heterodimeric glycoprotein that is released from the pars distalis of the adenohypophysis. In response to TSH, follicular cells of the thyroid exhibit an enhanced production and release of (T4), and its transporter protein, thyroglobulin. Thyroxine undergoes mono-deiodination to produce T3, the biologically active hormone, that binds to thyroid receptors in the nucleus of target cells and exerts its effects by altering transcription (for review, see, Hadley and Levine, 2006). By stimulating transcription, thyroid hormones can cause a diverse set of effects in many different target tissues.

It is known that thyroid hormones are necessary for a number of reproductive processes, by being permissive of the effects of other hormones, including glucocorticoids and gonadotropins (for review, see, Hadley and Levine, 2006). In

addition, the initiation of the transition from the breeding season to anestrus is dependent on thyroid hormones in ewes (Moenter et al., 1991) and rams (Parkinson and Follett, 1994).

Leptin

Energy balance is defined as the difference between food intake and energy expenditure and is a tightly controlled phenomenon (Casanueva and Dieguez, 1999). The mechanism(s) by which energy balance is regulated did not begin to become apparent until the discovery of the "ob" gene product, leptin (Zhang et al., 1994). This 167 amino acid peptide has since been identified as an important signaling molecule for energy homeostasis, feeding behavior and reproductive function (Zieba et al., 2008). Leptin is secreted proportionally to the amount of fat mass in that organism (for review, see, Chan and Mantzoros, 2001). Although leptin is primarily secreted from adipose tissue, the expression of the ob gene has been observed in many other sites including skeletal muscle (Wang et al., 1998), bone marrow (Hoggard et al., 1998), fetal cartilage (Hoggard et al., 1998), placenta (Masuzaki et al., 1997) and mammary tissue (Smith-Kirwin et al., 1998). In addition, both leptin and the receptor for leptin (LR) are expressed in the adenohypophysis of humans where they were colocalized with cells that synthesize and secrete ACTH, growth hormone, LH, and FSH (Jin et al., 1999).

Five isoforms of leptin receptor have been identified to date; these isoforms can be categorized as either „long' or „short.' The short variety is released from cells and acts as a binding protein in general circulation. The long variety is the signaling form. Once leptin binds to this type of receptor, the receptor homodimerizes and activates

JAC2/STAT and MAPK pathways. Effects of leptin may also be modulated by the number of leptin receptors as well. Dryer et al. (1997) reported that ewes on a restricted diet express long form leptin receptors at higher levels than well fed ewes, thereby heightening the potential to receive a signal.

Shortly after the discovery of the importance of leptin in ob/ob mice, it was noticed that these mice exhibited a plethora of reproductive dysfunctions. Since then, leptin has been considered to play a permissive role in puberty and in the maintenance of secretion of GnRH in the hypothalamus (Chan and Mantzoros, 2001). However, GnRH secretory neurons do not possess leptin receptors in rats (Hakansson et al., 1998) or monkeys (Finn et al., 1998). Instead, leptin receptors have been identified on kisspeptin secretory neurons in mice (Smith et al., 2006). It should be noted that the majority of leptin receptors identified in the mouse hypothalamus are located on neurons that have direct contact with KiSS neurons (Louis et al., 2011). Intracerebral infusion of leptin was shown to increase KiSS1 mRNA expression in male rats (Castellano et al., 2006), and fasted ewes (Backholer et al., 2010), thereby providing evidence that leptin concentrations in the hypothalamus effect the KiSS system.

In addition to the effect of leptin on kisspeptin, leptin also participates in other complex energy homeostasis pathways. Leptin has been shown to affect anorexigenic neurons in the hypothalamus by stimulating proopiomelanocortin (POMC) expression resulting in α- melanocyte stimulating hormone (MSH) release and satiety in mice (Forbes et al., 2001). Additionally, leptin inhibits orexigenic neuropeptide Y (NPY) neural circuits in mice (Jang et al., 2000) and rats (Kohno et al., 2007); decreased NPY is

associated with decreased feeding behavior in rodents. Increasing NPY activity generally has an inhibitory effect on gonadotropin secretion in ovariectomized sheep (McShane et al., 1992). Agouti-related protein (AgRP) is a 132 amino acid signaling protein that is released from the same neurons as NPY in the ARC. Agouti-related protein has been found to suppress the anorexigenic activities of α-MSH in mice (Yang et al., 1999). Infusion of insulin has been shown to decrease NPY expression in rats (Schwartz et al., 1992), and to decrease Agouti-related protein immunoreactivity in rats (Breen et al., 2005).

A discussion of leptin would be incomplete without mention of ghrelin; the 27 amino acid hormone synthesized and secreted from epithelial cells of the stomach. Generally, ghrelin opposes effects of leptin. Ghrelin is negatively correlated with body mass index and is sensitive to short- and long-term energy deficiency (for review, see, Tena-Sempere, 2007). Ghrelin has been shown to increase before programmed feeding events in sheep (Sugino et al., 2002; Sugino et al., 2004). However, infusion of ghrelin into either the lateral ventricles or intravenously failed to induce a change in voluntary feed intake which suggests that ruminants may not be as responsive to the orexigenic properties of ghrelin as many other species (Iqbal et al., 2006). Ghrelin has also been shown to inhibit LH release in rats (Fernandez-Fernandez et al., 2004) and in monkeys (Vulliemoz et al., 2004).

Prolactin

Prolactin (PRL) is a 199 amino acid protein hormone synthesized and secreted from the adenohypophysis that has a variety of functions including irreplaceable roles in reproduction and modulatory roles in metabolism and growth. Prolactin receptors come in a variety of isoforms and expression is variable between tissues and physiological conditions which may mediate the variety of effects of PRL (for review, see, Egli et al., 2010). Prolactin is spontaneously released from lactotrophs of the adenohypophysis. Its release from lactotrophes is dependent on the hypophysiotropic factor known as dopamine which is an inhibitor of prolactin secretion. Estradiol causes increased PRL gene expression that increases PRL synthesis and storage in the lactotrophes, but does not in itself cause PRL secretion into systemic circulation. In addition to the negative control of PRL by dopamine, oxytocin and β-endorphin have been found to stimulate the release of PRL (for review, see, Norman and Litwack, 1997).

Prolactin is most known for its essential role in mammary organogenesis and lactogenesis; however, PRL influences many other reproductive processes as well. Prolactin remains at a relatively low concentration throughout the estrous cycle, except for a surge-like release coincident with the LH surge before ovulation in ewes (Butler et al., 1972). A variety of stimuli can cause elevated PRL concentrations including stimulation of the cervix, suckling (Grosvenor et al., 1979), photoperiod (Dahl, 2008) and acute stress (Nicoll et al., 1960). High concentrations of PRL are known to inhibit reproduction by decreasing GnRH secretion (Cohen-Becker et al., 1986), and elevated PRL concentrations inhibit estradiol synthesis through blocking aromatase enzyme

activity (Tsai-Morris et al., 1983). Similarly, low concentrations of PRL have been associated with infertility in women (Kauppila et al., 1987), and rodents (Horseman et al., 1997; Ormandy et al., 1997).

Insulin-like Growth Factors

Insulin-like growth factor I (IGF-1) and insulin-like growth factor II are pluripotent factors that have high sequence homologies to insulin. Insulin-like growth factors are expressed in a variety of tissues though the liver is the predominant source. Generally, these factors elicit anabolic actions through the insulin-like growth factor 1 receptor. Insulin-like growth factor was originally identified as a sulfation factor necessary for the incorporation of sulfate into cartilage (Salmon and Daughaday, 1990). Since then, IGF-1 has been found to stimulate growth and regulate a number of cellular functions through endocrine and paracrine actions under the control of growth hormone (Le Roith et al., 2001). Insulin-like growth hormone binding proteins are a large family of proteins that bind IGF affecting the bioavailability of IGF in tissue.

Insulin-like growth factor plays an important role in ovarian function by stimulating both cell differentiation and growth (Monniaux and Pisselet, 1992). It was found that FSH, LH and cyclic adenosine monophosphate (cAMP) agonists (Hatey et al., 1992) and estradiol (Bley et al., 1992) can stimulate IGF-1 mRNA expression in granulosa cells. Insulin-like growth factor 1 is important for sensitizing developing follicles to FSH, stimulating steroidogenesis; however during dominance, IGF-1 concentrations are known to rise in the largest follicle and fall sharply in smaller follicles in response to LH in monovular species (for review, see, Ginther et al., 2001). In ewes

and gilts it is known that IGF-1 concentrations are higher in females with higher ovulation rates (for review, see, Hunter et al., 2004). Insulin-like growth factor 1 and its receptor have also been shown to be important to cellular differentiation during luteinization (Talavera and Menon, 1991). IGF-1 increases progesterone synthesis through up-regulation of cytochrome p450 in large luteal cells (Adashi et al., 1985; Talavera and Menon, 1991).

Reproduction in Ewes

Seasonality

Seasonality is the process whereby reproductive and metabolic processes are synchronized to periods of the year with appropriate environmental conditions. Processes of late pregnancy and lactation present the female with exceptional energy demands, accordingly, reproductive behavior and gametogenesis are limited to periods of the year that result in timing energy demands to energy availability (for review, see, Malpaux, 2006). Sheep were first proposed to be seasonal breeders in 1937 by Marshal. Today, it is well accepted that the breeding season of sheep is initiated by an increase of LH pulse frequency that stimulates ovarian estradiol secretion, which in turn, stimulates the LH preovulatory surge and ovulation, followed by corpus luteum function and normal ovarian cyclicity (for review, see, Karsch et al., 1980). An important part of this process is a decrease in the sensitivity of the negative feedback effect of estradiol on LH secretion (Legan et al., 1977). However, many steroid independent pathways for the effect of

season have been proposed as well (Barker-Gibb and Clarke, 2000; Robinson et al., 1985).

Photoperiod is thought to be a major environmental cue for timing the breeding season in sheep. During the fall, when the relative amount of light is decreasing and the corresponding amount of dark is increasing, breeding activity is stimulated. Decreasing ratios of hours of light to hours of dark per day is perceived by the retina. The physiological pathway for photoperiod is thought to involve the following: retinal signals are transmitted through the suprachiasmatic nucleus of the hypothalamus to the superior cervical ganglia of the sympathetic nervous system, where they stimulate an increase of epinephrine release in the pineal gland that increases melatonin synthesis and release, that in turn stimulates GnRH and LH release (Karsch et al., 1984; Legan and Winans, 1981; Turek and Campbell, 1979). It was found that melatonin implants placed in to the mediobasal hypothalamus elicit an LH response in seasonally anestrous ewes; whereas, implants placed into other portions of the hypothalamus did not initiate the same response, suggesting that the site of action of melatonin on reproduction resides in the hypothalamus rather than in the pituitary (Malpaux et al., 1993). Subsequent studies have identified the premamilary area of the mediobasal hypothalamus to have the greatest density of melatonin binding sites (Malpaux et al., 1998). Interestingly, the pars tuberalis of the adenohypophysis binds melantonin with high affinity (Bittman and Weaver, 1990; Morgan et al., 1989); however, this region is likely not involved with mediating the effects of season because site directed melantonin treatment does result in an increased LH secretion (Malpaux et al., 1994).

Alterations in photoperiod have been shown to effect populations of KiSS neurons in ARC of sheep. Following the transition from long days to short days, the number of KiSS immunoreactive cells significantly increased in the ARC, but no change in KiSS immunoreactivity was found in the POA (Chalivoix et al., 2010). Interestingly, ovariectomized ewes exhibit seasonal fluctuations in KiSS-1 expression in the ARC that directly correlates with that observed in intact ewes (Smith et al., 2007), suggesting a gonadal steroid-independent method of initiating the breeding season. Furthermore, it was shown that administration of kisspeptin to estradiol-treated ovariectomized ewes during the non-breeding season causes a surge-like release of LH (Caraty et al., 2007). These findings highlight the importance of KiSS as a mediator of the effect of season on reproduction in sheep.

The seasonal rhythm of reproduction in sheep is most appropriately described as circannual (Karsch et al., 1989). Circannual refers to a pattern of physiological changes in an animal that take place over the course of approximately one year and is driven by an endogenous feature or characteristic. For a species to be considered circannual, the subjects must: 1) exhibit the cycle in experimental conditions for at least 2 yr in the absence of environmental cues, 2) the cycle length must deviate from 1 yr, and, 3) animals must desynchronize in time; which proves the absence of an environmental cue. In sheep, change in photoperiod is the major environmental signal to synchronize reproductive processes.

Effect of Nutritional Supplementation on Reproduction

It is well known that the practice of flushing or feeding a higher plane of nutrition to ewes immediately before the breeding season can increase ovulation rate in ewes. It is well known that flushing sheep causes a higher ovulation rate in ewes (Scaramuzzi and Radford, 1983), defined as the average number of ova released from a group of females (Scaramuzzi and Radford, 1983). An increase in body condition score from low to middle is associated with increased ovulation rates (Rhind and McNeilly, 1986). However, some reports have suggested that an increase in body weight (BW) alone does not cause increased ovulation rates possibly because little is gained from feeding well-fed ewes even more (Clark, 1934). Interestingly, increased body condition score (BCS) has been associated with a greater number of large follicles but a similar number of small follicles as ewes with lower BCS (McNeilly et al., 1991; Rhind and McNeilly, 1986).Other factors besides number of follicles may be affected by flushing as well. Rhind and McNeily (1998) reported an increase in aromatase activity within follicles of flushed ewes compared to un-flushed ewes.

It is not entirely clear how an increase in nutrition is physiologically translated into an increased ovulation rate. Studies on the effect of nutrition on reproductive rates of sheep have shown both an increase (Downing et al., 1995b; Rhind and Schanbacher, 1991) and no change in gonadotropin secretion (Downing et al., 1995a; Rhind et al., 1989; Rhind and McNeilly, 1986). It has been suggested that the very small differences in hormone concentrations or secretion patterns that mediate the change in ovulation rate may not be detectable by the methods used in previous reports (Scaramuzzi and Radford,

1983). It has been suggested that metabolic hormones such as glucose, insulin and leptin act directly on the ovary to enhance folliculogenesis independent of the effects of these metabolites and hormones on GnRH and gonadotropin secretion (Scaramuzzi et al., 2006).

The Biostimulatory Effect of Males

It is well known that exposing anestrus ewes to a novel ram will induce ovulation (for review, see, Delgadillo et al., 2009; Martin et al., 1986; Schinckel, 1954). The general process of induction of estrous in a female conspecific is known as the biostimulatory effect or male effect; more specifically, in sheep this process is known as the "ram effect". In this thesis I use the "biostimulatory effect of rams" in lieu of the "ram effect".

The biostimulatory effect of rams on ewes involves a relatively rapid increase in the pulsatile secretion of LH reflected as an increase in LH pulse frequency and basal LH concentrations (Martin et al., 1980; Poindron et al., 1980). This change in LH pulse frequency results in rapid follicular growth and maturation and ultimately in ovulation. Ovulation usually occurs within 2 to 3 d after introduction of rams and generally results in normal estrous cyclicity thereafter (Atkinson and Williamson, 1985). Interestingly, it has been reported that acute effects of rams on ewes do not involve changes in FSH secretion (Poindron et al., 1980).

Pheromones

The ram effect is mediated primarily through pheromones (Knight and Lynch, 1980; Knight et al., 1983). Karlson and Luscher (1959) defined a pheromone as a biochemical signal produced by one individual and perceived by another of the same species to elicit a response in the second individual (1959). Pheromones can be classified by their effects on the second individual as either "signaling" or "priming". Signaling pheromones cause specific and immediate changes in behavior of the individual that perceives the stimulus. On the other hand, priming pheromones cause physiological changes, typically mediated by alterations in endocrine events in the perceiving animal. The difference between the two classes of pheromones is that signaling pheromones cause a change in behavior, whereas priming pheromones cause changes in physiological processes, such as reproduction. The biostimulatory effect of rams is mediated primarily through a priming pheromone that induces ovulation in anestrous ewes (Rekwot et al., 2001).

Although the priming pheromone(s) produced by rams have not been identified specifically (Cohen-Tannoudji et al., 1994), it is known that substances that induce ovulation in ewes are found in wool and wax and not necessary in the urine of rams (Knight and Lynch, 1980). In goats, it is known that these substances are produced by location specific sebaceous glands and appear to be regulated by testosterone (Iwata et al., 2000). The locations of sebaceous glands that produce pheromones are only on heads and necks of male goats. However, injection of dihydrotestosterone into skin samples

collected from other regions can stimulate pheromone production, which suggests that 5α reductase expression dictates where pheromones are produced (Iwata et al., 2001).

Adrenal Response to the Biostimulatory Effect of Rams.

There are reports that adrenal output is related to reproductive processes in rodents (Nichols and Chevins, 1981). Furthermore, corticosterone, the dominant adrenal glucocorticoid in rodents, is elevated in female rats exposed to the urine of male rats (Mora and Sánchez-Criado, 2004). Similarly, female mice exposed to the excretory products of male mice exhibited increased corticosterone concentrations than in female mice not exposed to male urine (Marchlewska-Koj and Zacharczuk-Kakietek, 1990). Following these findings, it was reported that women directed to sniff androstadienone, a putative human pheromone, exhibited elevated salivary cortisol concentrations (Wyart et al., 2007). Tauck et al. (2007) reported increased cortisol concentrations over a 24-d period in cows exposed to bulls than in cows exposed to steers. Of significance to this thesis is the work of Tauck et al. (2007; 2010) who found that increasing cortisol pulse amplitude was related to a decreasing LH pulse amplitude in cows exposed to bulls; whereas, in cows exposed to steers this relationship was not present. These results indicate that a component of the biostimulatory effect of males involves alterations in adrenal glucocorticoids; however the function or the importance of these changes is not clear.

Sensation and Perception of the Biostimulatory Effect of Rams

Non-Olfactory Stimuli. There is evidence that non-olfactory stimuli from the ram can also induce ovulation in anestrous ewes. Olfactory bulbecotomy which render ewes anosmatic (Cohen-Tannoudji et al., 1986), and goats treated nasally with zinc sulfate solution (Chemineau et al., 1986) exposed to males exhibited a similar LH response as sensory-intact ewes and does, respectively. In 1988, Pearce and Oldham found that significantly more ewes separated from rams by a clear fence ovulated than ewes not exposed to rams; however, significantly more ewes that were allowed full physical contact with the rams ovulated than ewes separated from rams by a fence. Ewes exposed to still images of rams were also shown to exhibit an LH response, though much less intensely than ewes exposed to rams (Hawken et al., 2009). Furthermore, it is known that sheep can differentiate between images of familiar and unfamiliar individuals, as well as detect stress levels in the images these individuals (Tate et al., 2006). This means that sheep are capable of recognizing and differentiating other sheep, and can detect if another animal is under stress. Therefore, visual stimulation can be considered a component of the ram effect; however, it is not a substitute for other sources of stimulation.

Olfactory Stimuli. A ewe's perception of odors from rams is mediated by both the main and accessory olfactory systems. The main olfactory system originates in the main olfactory epithelium which is directly linked to the main olfactory bulbs. The main olfactory bulbs have both afferent and efferent connections to the amygdaloid nucleus, piriform cortex, anterior olfactory nucleus, and entorhinal nucleus. The accessory system

originates in the vomeronasal organ that is directly linked to the accessory olfactory bulb. The accessory olfactory bulbs are bi-directionally linked to the bed nucleus stria terminalis through the amygdala. The bed nucleus stria terminalis has projections within the preoptic area, though not directly to GnRH neurons in the area (for review, see, Martin et al., 1986). In 2006, Gelez and Fabre-Nys confirmed these pathways by identifying increased Fos immunoreactivity in both main and accessory olfactory bulbs, anterior olfactory nucleus, cortical and basal amygdale, dendate gyrus, ventromedial hypothalamus, piriform and orbitofrontal cortices in ewes exposed to rams than ewes exposed to other ewes. Although both the main and accessory olfactory systems are involved with mediating the perception of the male, the main olfactory system, particularly the cortical nucleus of the amygdala, is essential for an LH response to the male (Gelez et al., 2004).

Recently, it was observed that female goats exposed to the odor of male goats exhibit a change in multiple unit activity (MUA) of neurons in close proximity to KNDy neurons of the ARC (Okamura et al., 2010). It is thought that pheromonal stimuli are mediated by an increase in NKB that is known to alter multiple unit activity and stimulate kisspeptin expression. Interestingly, if the female is exposed to the odors of a male to soon after the preceding MUA volley there is no change in measured voltage in response to the odor. This observation indicates the presence of a refractory period wherein the KNDy system is insensitive to additional inputs (Okamura et al., 2010). Furthermore, these observation suggests a method whereby male pheromonal stimuli modulates activity of the putative GnRH pulse generator, and also suggests a mechanism for the

continuation of an oscillating stimulant leading to the well-known change in LH pulse frequency in response to the biostimulatory effect of males.

Factors Affecting the Efficacy of the Biostimulatory Effect of Rams

For many years it has been assumed that for the male effect to work ewes must be isolated from males before exposure. However, the little research that has been done in this area indicates that isolation of the sexes may not be as important as originally thought. Cohen-Tannoudji and Signoret (1987) found that ewes exposed to rams for 2 h on two consecutive days exhibited the same increase in LH pulse frequency on both days. Interestingly, cycling ewes exposed to rams that are replaced at 17-d intervals show increased mating synchronization (Hawken and Beard, 2009). These reports suggest that an unfamiliar ram may be a more important requirement than isolation from all rams.

A series of other factors summarized by the term “depth of anestrus” also dictate the effectiveness of a ram to stimulate ovulation in ewes. These factors have not yet been completely elucidated, but it is known that breed of ewe and temporal proximity to the natural breeding season are important factors related to the ram effect. Nugent et al. (1988) found that more Dorset ewes (a breed known to have an extended breeding season) exposed to rams ovulated in the spring than Hampshire ewes (a breed with a more limited breeding season) exposed to rams. In the same study, it was found that more Hampshire ewes exposed to rams ovulated in the summer than in the spring. These results indicate that as the natural breeding season nears the potential to respond to the biostimulatory effect of a ram increases.

Effect of Biostimulation on Metabolism

It has been suggested that the biostimulatory effect of males may also involve changes in metabolic hormone concentrations in females in addition to the well-known changes in reproductive hormones and status. Changes in non-esterified fatty acids (NEFA) have been reported in cow exposed to bulls. An initial report found that cows exposed to bulls had higher circulating concentrations of NEFA than cows not exposed to bulls (Landaeta-Hernández et al., 2004). Since then, Olsen (2009) reported lower concentrations of NEFA in cows exposed to bulls than in cows exposed to steers. Olsen (2009) suggested that the differences between the results of these two studies were likely the result of different proportions of cows cycling during the times that samples were collected. Ovulatory events are associated with increased cortisol concentrations; this increase in cortisol also stimulates lipolysis and alters carbohydrate metabolism. In the study by Landaeta-Hernandez et al. (2004) there was a high proportion of cows that had resumed ovulatory activity before the start of the experiment; whereas, in the study by Olsen (2009) all cows were anovulatory during the sampling period. Additionally, Olsen (2009) found that leptin concentrations were increased in cows exposed to bulls for 6 h and were increased over a longer period when cows were exposed for 12 h per day. These results indicate that the biostimulatory effect of bulls elicits a dose-dependent effect on metabolic hormone concentrations in postpartum, anovular, suckled cows. There are no studies in sheep that have investigated the possibility that the biostimulatory effect of rams on ewes may have a similar effect as that observed in the bovine during the transition from an anovular to an ovular condition.

CHAPTER 3

STATEMENT OF THE PROBLEM

The biostimulatory effect of rams can have a significant impact on reproductive processes of ewes. It is well known that exposing anestrous ewes to a novel ram will induce ovulation (for review, see, Delgadillo et al., 2009; Martin et al., 1986; Schinckel, 1954). Similarly, it is known that nutrition, more specifically, perception of energy balance is also a key regulator of reproduction in ewes, and targeted nutritional supplementation can stimulate reproductive efficiency. Tauck et al. (2007) identified changes in the hypothalamic- hypophyseal-adrenal axis related to the resumption of ovulatory activity in postpartum, anovular cows exposed to bulls. As discussed in the literature review, cortisol is known for its role in stress response and as a stimulator of processes that result in increased energy availability. It is possible that the biostimulatory effect of rams may be mediated in way related to perception of the energy balance, perhaps mediated by cortisol.

The goals of this research were 1) to determine if temporal patterns of cortisol concentrations are altered in ewes exposed to rams during the transition into the breeding season, and 2) to determine if ewes exposed to rams during the transition into the breeding season exhibit differences in cortisol, leptin, T3, T4, prolactin and IGF-1 concentrations.

CHAPTER 4

EXPERIMENT 1: CONCENTRATIONS OF CORTISOL AND LH IN VIRGIN EWES ACUTELY EXPOSED TO RAMS DURING THE TRANSITION INTO THE BREEDING SEASON

Introduction

The biostimulatory effect of rams on ewes is known to cause a relatively rapid increase in LH pulse frequency that accelerates resumption of seasonal ovulatory activity (Martin et al., 1980; Poindron et al., 1980). There are reports that the biostimulatory effect of males involves changes in adrenal cortical glucocorticoids in rodents (Marchlewska-Koj and Zacharczuk-Kakietek, 1990; Nichols and Chevins, 1981), humans (Wyart et al., 2007) and cattle (Tauck et al., 2007; 2010). Recently, Tauck et al. (2010) reported that cortisol pulse frequency decreased and pulse amplitude increased in postpartum, anovular, suckled cows exposed to bulls. It was suggested that alterations in hypothalamic-pituitary-adrenal axis activity may play a role in the physiologic mechanism of the biostimulatory effect of bulls to accelerate resumption of ovulatory activity in the bovine. It is not known if activation of the hypothalamic-pituitary-adrenal axis is involved with the "ram effect" in sheep. The objective of this experiment was to determine if temporal patterns of cortisol concentrations are altered in 18-mo-old virgin Targhee ewes exposed to rams during the transition into the breeding season. The hypotheses were that 1) exposing seasonally anovular ewes to rams would alter patterns

of cortisol concentrations, and 2) that these changes are related to changes in temporal characteristics of LH concentrations that accelerate the occurrence of ovulation.

Materials and Methods

Animals and Treatments

Thirty-five 18-mo-old virgin Targhee ewes that had been isolated from males since weaning the previous yr, were used in this study. Additionally, 3 sexually experienced, epididymectomized rams and 3 wethers that had been castrated before secondary sex characteristics developed, were also used in this experiment. This experiment was conducted at the Montana State University Fort Ellis Research and Teaching Facility. Animal care, handling, and protocols used in this experiment were approved by the Montana State University Agricultural Animal Care and Use Committee.

Jugular venous blood samples were collected from each ewe 10 and 15 d before exposure to males and assayed for progesterone (P4). All ewes used in this study had concentrations of progesterone less than 1.0 ng/mL on these 2 d and were considered to be anovular. Additionally, one sample from the intensive sampling day was also assayed for progesterone to assess whether ovulation had occurred in any of the ewes during the intervening time before exposure to males. Ewes were stratified by BW and assigned randomly to be exposed to rams (RE; n = 17) or exposed to wethers (NE; n = 18). Ewes within exposure type were then assigned randomly to an intensive sampling day; 1 (RE-1; n = 5, NE-1; n = 6), 2 (RE-2; n = 6, NE-2; n = 6), or 3 (RE-3; n = 6, NE-3; n = 6). Consecutive sampling days were 3 d apart; D1 was August 18, 2009.

Pre-treatment Handling

Each ewe received an indwelling jugular catheter 3 d before exposure to males. Catheters were 5 ¼" 16 gauge extended use catheters (Jorgenson Laboratories, Loveland, CO). Catheters were flushed twice daily with heparinized saline (10 IU/mL in 0.9% sterile NaCl solution) until the night before the ewes were exposed to either a ram or wether. Ewes in both exposure types were adapted to the conditions for housing and obtaining intensive blood sampling, penning, and handling by humans for 8 h/d during the 3 d before exposure on D 0.

Blood Sampling for LH and Cortisol

At 0830, (-2 h) RE and NE ewes were placed randomly into 1.5 m x 2 m pens (3 ewes/pen). Blood samples (~10 mL) were collected at 15-min intervals for 2 h. When the 0 min sample was obtained, rams or wethers were placed into pens holding ewes (1 ram or wether/3 ewes). Blood collection continued at 15-min intervals for 6 h. An equal volume of sterile saline solution (0.9%) was used to flush the catheters of each ewe after each blood sample was collected.

Blood samples were promptly cooled and stored overnight at 4^{o} C then centrifuged at 1,850 x *g* for 30 min. Sera was harvested and stored at -20^{o}C until assayed for LH and cortisol.

Luteinizing Hormone, Cortisol and P4 Assays

Concentrations of LH in serum samples were determined in duplicate by a liquid-liquid phase RIA (Niswender et al., 1969). The primary antibody was NIDDK anti-oLH-

1 AFP 192279RB and bLH AFP 11743B was used for the iodination and standards. Both assay reagents were obtained from the National Pituitary Program (NHPP) and Dr. A. Parlow (University of San Francisco, San Francisco, CA). Intra- and inter assay CV were 29.6 and 26.3% respectively.

Cortisol concentrations in serum samples were determined in duplicate by a solid-phase RIA kit (Siemens Healthcare Diagnostics, Los Angeles, CA). The intra- and inter assay CV were less than 10% in serum pools that contained 91 and 21.5 ng/mL.

Progesterone concentrations were determined in serum samples in duplicate by a solid-phase RIA kit (Siemens Healthcare Diagnostics, Los Angeles, CA). The intra- and inter assay CV were less than 5% in a serum pool that contained 2.4 ng/mL.

Characteristics of Temporal Patterns of LH and Cortisol Concentrations

Characteristics of temporal patterns of LH and cortisol included: 1) mean concentration, 2) baseline concentration, 3) pulse amplitude, 4) pulse frequency, and 5) pulse duration. For each hormone in each ewe, during each the pre-exposure sampling period, and the exposure period, a plot of hormone concentration over time was generated. Baseline concentrations were identified and the mean baseline concentration was the mean of these concentrations. Concentrations of LH or cortisol that were > 1 SD above the mean baseline concentration were considered as concentrations within a pulse of each hormone. Pulse amplitude (ng/mL) was calculated by subtracting the mean baseline concentration and the highest concentration in each pulse. Pulse duration (min) was the time that the concentration pattern deviated from baseline concentrations. Pulse

frequency (peaks/h) was number of peaks during the blood sampling period divided by the time of that period.

Statistical Analyses

Characteristics of temporal concentration patterns for LH and cortisol, including mean and baseline hormone concentration, as well as, pulse amplitude, frequency and duration and time to first pulse were analyzed using PROC GLM for a completely randomized design of SAS (SAS Inst. Inc., Cary, NC). The model included exposure type. Animal was the experimental unit. Means were separated using Bonferroni's tests.

Relationships between temporal patterns of cortisol and LH concentrations were determined by regressing characteristics of LH concentrations on characteristics of cortisol concentrations within exposure type using the PROC REGRESS procedure of SAS. Mean concentrations of LH were regressed on mean concentrations of cortisol and baseline concentrations of LH were regressed on baseline concentrations of cortisol and so on.

Results

Based on progesterone concentrations on the intensive sampling day, it was determined that 5 RE and 6 NE ewes had resumed luteal activity and were excluded from analyses. Additionally 1 ewe from the RE group was excluded from analysis because several characteristics of LH were statistically identified as outliers. Initial analyses

revealed no effect of intensive sampling day or its interactions on any dependent variable so data were pooled over sampling day.

Mean and baseline concentrations, pulse frequency and amplitude, and number of cortisol pulses did not differ between RE and NE ewes (Table 1). Cortisol pulse duration was longer ($P = 0.02$) in RE than in NE ewes (Table 1). Mean LH concentration, pulse amplitude and duration of LH did not differ between RE and NE ewes. However, baseline LH concentrations were greater ($P = 0.029$) in RE than in NE ewes. Additionally, LH pulse frequency and number of LH pulses were greater ($P = 0.0197$) in RE than in NE ewes (Table 2). Time until the first cortisol or LH pulse after introduction of males did not differ between RE and NE ewes (Table 2).

Table 1. Characteristics of temporal cortisol concentration patterns in virgin Targhee ewes during the first 6 h of exposure to rams (RE) or wethers (NE) during the transition into the breeding season

	Exposure type			
Item	NE	RE	SEM[a]	*P*- Value
n	12	11		
Mean, ng/mL	11.56	15.34	5.53	0.12
Baseline, ng/mL	4.62	4.68	0.023	0.95
Frequency, pulse/hr	0.75	0.72	0.19	0.71
Number of pulses	4.50	4.32	1.15	0.71
Amplitude, ng/mL	28.23	30.51	17.69	0.76
Pulse duration, min	62.26[b]	73.81[c]	11.07	0.04
Time to 1st pulse, min	58.75	46.36	38.15	0.44

[a] Standard error of mean.

[b,c] Values within rows differ.

Table 2. Characteristics of temporal LH concentration patterns in virgin Targhee ewes during the first 6 h of exposure to rams (RE) or wethers (NE) during the transition into the breeding season.

	Exposure type			
Item	NE	RE	SEM[a]	*P*- Value
n	12	11		
Mean, ng/mL	0.78	1.51	1.02	0.11
Baseline, ng/mL	0.46[a]	0.82[b]	0.75	0.03
Frequency, pulse/hr	0.85[a]	1.02[b]	0.17	0.02
Number of pulses	5.08[a]	6.14[b]	1.00	0.02
Amplitude, ng/mL	1.12	2.01	1.39	0.14
Pulse duration, min	51.73	52.12	13.05	0.94
Time to 1st pulse, min	48.19	33.31	1.92	0.188

[a] Standard error of mean.

[b,c] Values within rows differ.

There were no linear relationships for mean or baseline concentrations or pulse amplitude or frequency of LH regressed on mean or baseline concentrations or pulse amplitude or frequency of cortisol within exposure type. However, there was a tendency ($P = 0.057$) for a linear relationship between LH pulse frequency and cortisol pulse frequency in RE ewes but not in NE ewes (Table 3). Similarly, there was a tendency ($P = 0.057$) for a linear relationship between number of LH pulses and number of cortisol pulses in RE ewes but not in NE ewes (Table 3).

Table 3. Linear regression of LH pulse frequency and number of LH pulses on cortisol pulse frequency and number of cortisol pulses, respectively, within ewes exposed to rams (RE) and ewes exposed to wethers (NE) during the transition into the breeding season**.**

Variable	NE	*P*-Value	RE	*P*-Value
LH frequency on cortisol frequency				
Intercept	1.43	0.004	1.59	0.0002
Slope (pulses/hr)/(pulses/hr)	-0.809	0.101	-0.78	0.057
Number of LH pulses on number of cortisol pulses				
Intercept	6.43	<0.0001	9.52	0.0002
Slope (pulses/hr)/(pulses/hr)	-0.3	0.104	-0.78	0.0572

Discussion

The rational for this experiment was that changes in adrenal cortisol in ovular females are associated with the biostimulatory effect of males in other species other than the bovine as reported by Tauck et al. (2010). It is well known that cortisol can have a suppressive effect on reproduction. Therefore, it was of interest to determine if changes in temporal cortisol concentration patterns were associated with the biostimulatory effect of rams on ewes during the transition into the breeding season. More specifically, the purpose of this experiment was to determine if temporal concentrations of cortisol are altered in ewes during acute exposure to rams during the transition into the breeding season, and if alterations in temporal cortisol concentrations were related to temporal concentrations of LH.

Exposing ewes to rams during the transition into the breeding season in the present study caused an increase in cortisol pulse duration; however, other characteristics of cortisol concentrations patterns were not altered by exposure to rams compared to exposing ewes to wethers. This change in concentration pattern without a change in the overall magnitude of the signal may useful for understanding how cortisol may be able to modulate reproduction in ways other than the stereotypical negative effects. It is possible that this change in cortisol concentration patterns is beneficial in the sense that this particular concentration pattern is associated with hastened resumption of luteal activity (see Experiment 2).

In the present study we found that cortisol pulse duration was longer in ewes exposed to rams than in ewes exposed to wethers. This result is consistent with the findings of Tauck et al. (2010) who reported that cortisol pulse duration tended to increase in postpartum, anovular, suckled cows exposed to bulls compared with that in cows not exposed to bulls. Tauck et al. (2010) also reported lower cortisol pulse frequency in postpartum, anovular, suckled cows exposed to bulls than in cows not exposed to bulls. Results from the current study did not show decreased cortisol pulse frequency in ewes exposed to rams compared to ewes exposed to wethers. Cortisol pulse frequency was slightly lower in ewes exposed to rams than in ewes not exposed to rams; however, there was considerable animal variation associated with cortisol pulse frequency that precludes firm conclusion in this regard as to the effect of rams on cortisol pulse frequency in ewes.

Exposing ewes to rams during the transition into the breeding season increased LH pulse frequency and baseline LH concentration relative to ewes exposed to wethers. These results are consistent with findings of Martin et al. (1980) and Poindron et al (1980) in that seasonally anestrus ewes exposed to rams exhibited increased LH pulse frequency and a greater baseline concentration of LH. This is evidence of a change in the signal that LH conveys to the ovary. It is well known that LH pulse frequency is increased during the breeding season allowing for follicular maturation and ovulation (for review, see, Karsch et al., 1980).

Interestingly, in ewes exposed to rams, there was a tendency for a negative, linear relationship between LH pulse frequency and cortisol pulse frequency. This relationship was not observed in ewes exposed to wethers. This result indicates that there is something about exposing ewes to rams that is not only associated with alterations in hypothalamic-pituitary-ovarian axis and hypothalamic-pituitary-adrenal axis but is also associated with synchronization of these systems. Similarly, Tauck et al. (2010) found that as cortisol amplitude increased LH pulse amplitude decreased in postpartum, anovular, suckled cows exposed to bulls, and that this relationship was not present in cows not exposed to bulls. Together these results suggest that in anovular females, characteristics of LH and cortisol can be considered independent of each other. However, when females are exposed to males the relationship appears to change in a negative manner. Thus, the change in this relationship in ewes exposed to rams is very much like that reported by Tauck et al. (2010) in the bovine and may be a basic physiological response of females during transitions from anovular to ovular conditions.

It is not clear from the results of this study if changes in certain characteristics of cortisol patterns cause changes in LH patterns that lead to resumption of luteal activity, or if changes in cortisol are merely a “side effect” that does not in itself mediate changes in LH concentration patterns. Our data do show that the first LH pulse after exposing ewes to rams occurs sooner than the first pulse of cortisol after exposure to rams. However, this does not exclude the possibility that changes in cortisol affect changes in LH concentration patterns, but it certainly does not provide any evidence that changes in cortisol concentration patterns cause changes in LH concentration patterns.

CHAPTER 5

EXPERIMENT 2: RESUMPTION OF LUTEAL ACTIVITY AND CONCENTRATIONS OF CORTISOL, LEPTIN, TRIIODOTHYRONINE, THYROXINE, PROLACTIN, AND IGF-1 IN VIRGIN EWES EXPOSED CHRONICALLY TO RAMS

Introduction

Ewes exposed to novel rams during the transition into the breeding season resume ovulatory activity sooner than ewes not exposed to rams (for review, see, Delgadillo et al., 2009; for review, see, Martin et al., 1986; Schinckel, 1954). This biostimulatory effect of rams could be used to improve reproductive performance of ewes. Feeding an increasing plane of nutrition during the early breeding season also improves reproductive performance in ewes (Scaramuzzi et al., 1993). Furthermore, feeding a higher plane of nutrition is known to alter metabolic hormones concentrations (for review, see, Scaramuzzi et al., 2006), which is a method of assessing metabolic status. Therefore, it is of interest to determine if the biostimulatory effect of rams also causes a change in metabolic hormones concentrations over the course of an estrous cycle length in ewes.

The objective of this study was to determine if ewes exposed to rams during the transition into the breeding season exhibit differences in cortisol, leptin, T3, T4, prolactin, and IGF-1 concentrations. The hypotheses tested were that time until resumption of luteal activity, temporal patterns of cortisol, leptin, prolactin, T3, T4 and IGF-1 do not differ over a 20-d period among virgin ewes exposed to rams.

Materials and Methods

Animals and Treatments

Thirty-six 18-mo-old virgin Targhee ewes that had been isolated from males since weaning the previous year were used in this study. Additionally, 2 sexually experienced epididymectomized rams and 2 wethers that had been orchiectomized before secondary sex characteristics developed were also used in this experiment. This experiment was conducted at the Montana State University Fort Ellis Research and Teaching Facility. Animal care, handling, and protocols used in this experiment were approved by the Montana State University Agricultural Animal Care and Use Committee.

Blood samples were collected from each ewe 10 and 15 d before exposure to males and assayed for progesterone concentrations. All of the ewes had concentrations of progesterone less than 1.0 ng/mL, and were considered to be anovular. Ewes were randomly assigned to treatments; exposed continously to rams (RE; n=18), or exposed continously to wethers (NE; n = 18) from the introduction of males (0 d) until the end of the blood sampling period (22 d). Ewes were then randomly assigned to either a west or east pen within each exposure type, which were considered replicates within treatment (RE-west, n = 9; RE-east, n = 9; NE-west, n = 9; NE-east, n = 9). Ewes were then randomly assigned to 3 groups to start exposure to males on 1 of 3 days; day 1 (RE-1; n = 6, NE-1; n = 6), day 3 (RE-2; n = 6, NE-2; n = 6), or day 6 (RE-3; n = 6, NE-3; n = 6). Either a ram or a wether was introduced to the ewes on what was considered D 0 for each ewe. Thus, D 0 among ewes of different exposure was different. Day 0 for exposure start day 1 was August 18, 2009.

Ewes were weighed twice, 24 h apart, before the start of the experiment. The mean of these measurements was considered initial BW. Final BW was determined the same way at the end of the 20-d exposure period.

Facilities and Animal Care

During the experiment, ewes were housed in 4 pens approximately 10 m x 30 m in area. Pens within each treatment were separated by a fence draped with black tarpaulins to limit visual and tactile contact among ewes and males between pens. Pens between exposure types were separated by approximately 20 m and two fences, draped with black tarpaulins to limit visual, tactile and olfactory contact separated ewes within each exposure type (see figure 1). Additionally, ewes were habituated to these pens for 2wks before the start of the experiment.

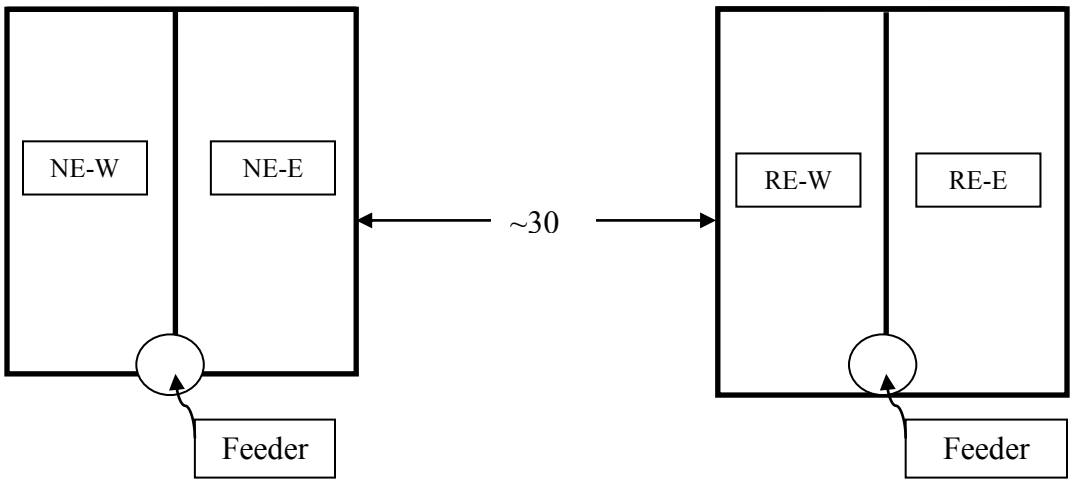

Figure 1. Facilities where ewes were housed during exposure to either rams (RE) or wethers (NE), with 2 pens per exposure type (E and W). Bold black lines denote fences draped with opaque tarpaulins.

During the adaptation period and the experiment, ewes and the respective males had free access to water, good quality sanfoin hay (~12.5% protein) and any pasture grasses within the pens and to salt blocks.

Blood Sampling Procedures

Blood samples were collected from each ewe by jugular venipuncture every other day from the start of the experiment (D 0) until D 22. Blood samples were collected from ewes from exposure start groups 1 and 3 on D 0, then on D 2, 4 and every other D until the end of the experiment. Blood samples were collected from exposure start group 2 ewes on d 0, d 1, d 3 and every other day until the end of the experiment. Blood samples were stored cool and allowed to clot, then centrifuged at 1,850 x *g* for 30 min. Sera was harvested and stored at -20°C until assayed for progesterone, cortisol, leptin, T3, T4, prolactin and IGF-1 concentrations.

Determination of Resumption of Luteal Activity

Progesterone concentrations patterns were used to determine the day of resumption of luteal activity. A rise in progesterone concentrations > 1.0 ng/mL in two consecutive samples provided evidence of resumption of luteal activity. The day before the rise was considered the day of resumption of ovulatory activity.

Progesterone Assay

Progesterone concentrations were determined in all serum samples in duplicate by a solid-phase RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA) according to

manufacturers specifications. The intra- and inter assay CV were less than 5% in a serum pool that contained 2.4 ng/mL.

Metabolic Hormone Assays

Concentrations of cortisol were determined in all serum samples collected during the experiment by the same method described in Experiment 1. Intra- and inter-assay CV were less than 10% in pooled ewe sera that contained 91 and 21.5 ng/mL.

Triiodothyronine and T4 were assayed by Dr. Dennis Hallford (New Mexico State University, Las Cruces, NM) in duplicate using solid phase RIA kits (Siemens Medical Diagnostics Los Angeles, CA, USA) validated for sheep serum (Richards et al., 1999; Wells et al., 2003, respectivly). Intra- and inter-assay CV for T3 were 9.3 and 12.6%, respectively. Intra- and inter-assay CV for T4 were 7.5 and 13.3%, respectively.

Prolactin and IGF-1 were assayed by Dr. Dennis Hallford (New Mexico State University, Las Cruces, NM) in duplicate using double antibody RIA validated for sheep serum (Berrie et al., 1995; Spoon and Hallford, 1989, respectively). Intra- and inter-assay CV for PRL were less than 10%, respectively; and intra- and inter-assay CV for IGF-1 were less than 10%, respectively.

Leptin was assayed by Dr. Duane Kiesler (University of Missouri, Columbia, MO), in triplicate using a competitive liquid-liquid phase, double-antibody RIA procedure described previously (Delavaud et al., 2000). The intra- and inter- assay CV were less than 5.0%.

Statistical Analyses

Initial analyses indicated that pen or day exposure began, or interactions of pen or day exposure began with independent variables did not affect any dependent variable. Therefore data across pen and day when exposure began were pooled for further analyses. When data from ewes that began exposure on different days was pooled, a new indicator for day of exposure was created. Samples collected on the day males were introduced to the females were classified as 0, the next sample was d 1.5, then 3.5 and so on until d 21.5.

Body weight at the beginning of the exposure period, at the end of exposure and change (final BW – initial BW) in BW were analyzed by one-way ANOVA using PROC ANOVA in SAS (SAS, Cary, NC). The model included exposure type, Means were separated using Bonferroni's Multiple Comparison test.

Interval to resumption of luteal activity was analyzed by one-way ANOVA, using PROC GLM of SAS. The model included exposure type. Means were separated using Bonferroni's Multiple Comparison tests. Proportions of ewes that resumed luteal activity by 7.5 and 11.5 d after exposure and by the end of the exposure period were analyzed by chi-square analyses using PROC FREQ of SAS.

Cortisol, PRL, IGF-1, T3, T4 and leptin concentrations were analyzed using the PROC MIXED for repeated measures analysis of SAS. The model included exposure type, day, and the interactions between these variables and compound symmetry was used for the analyses. Animal was the experimental unit with day after exposure as the repeated measure. Means were separated using Bonferroni's Multiple Comparison tests.

Results

There was no difference in body weight between RE and NE ewes at the beginning or at the end of exposure period. Ewes exposed to rams gained more (P = 0.0015) weight during the experiment than ewes not exposed to rams (Table 3).

Table 4. Mean initial, final and change in body weight for 18mo-old virgin Targhee ewes exposed to rams (RE) or wethers (NE) during 22d exposure period during the transition into the breeding season.

Variable	NE	RE	SEM[1]	*P*-Value
Initial BW	60.10	59.21	4.97	0.592
Final BW	60.91	63.31	4.27	0.101
Change BW	0.81[a]	4.10[b]	2.87	0.0015

[a,b]Values differ within row

[1]Standard error of the mean

Based on progesterone concentrations on d 0, it was determined that 5 RE and 6 NE ewes had resumed luteal activity and were excluded from analyses related to resumption of luteal activity. Ewes exposed to rams resumed luteal activity sooner (P = 0.04) than ewes exposed to wethers (Table 4). More (P = 0.02) RE ewes resumed luteal activity by 7.5 d after exposure than NE ewes (Table 4). However, by the end of the exposure period there was no difference between the proportion of RE and NE ewes that resumed luteal activity (Table 4).

Table 5. Resumption of luteal activity in anovular, virgin Targhee ewes exposed to rams (RE) or wethers (NE)[1]

Variable	RE	NE	SEM	X^2	*P*-Value
n	12	13			
Interval from exposure, d	3.5[a]	7.5[b]	4.4		0.04
% resumed luteal activity by d 7.5	100[a]	62[b]		5.76	0.02
% resumed luteal activity by d 11.5	100	92		0.96	0.32
% resumed luteal activity by d 21.5	100	92		0.96	0.32

[1]d 0 = introduction of males.
[a,b] Values within rows differ.

There were no differences between RE and NE ewes for temporal concentrations patterns of cortisol, T3, IGF-1, leptin or T3:T4 ratios. However, there was an exposure type by day interaction ($P = 0.05$) for T4 concentrations (Figure 1). Concentrations of T4 decreased in NE ewes between d 1.5 and 9.5; whereas, T4 in RE ewes decreased between d 1.5 and 5.5 with no further decrease until d 17.5. Concentrations of T4 did not differ between RE and NE ewes after d 9.5.

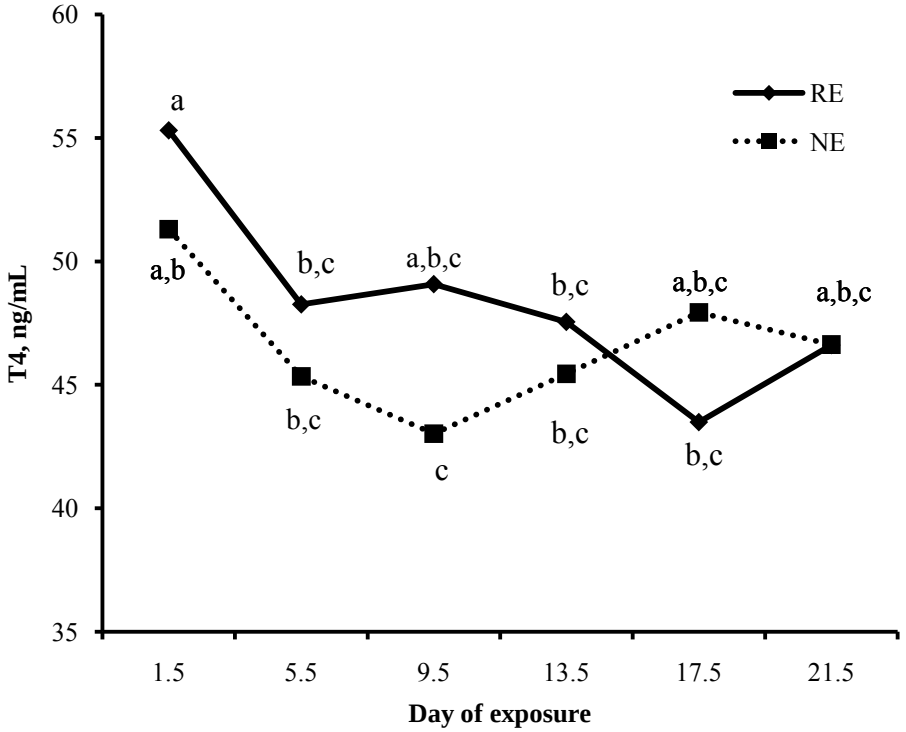

Figure 2. Least squares means for T4 concentrations of ewes after exposure to rams (RE; n = 18) and wethers (NE; n = 18). Exposure type by d interaction, $P = 0.05$. Means that share a common letter do not differ ($P > 0.05$). SEM = 5.7 ng/mL.

There was an exposure type by day interaction ($P = 0.01$) for PRL concentrations. This interaction appeared to be due to a decrease in PRL concentration in RE ewes between d 5.5 and 17.5; whereas, there was no change in PRL concentrations in NE ewes over this period (Figure 3).

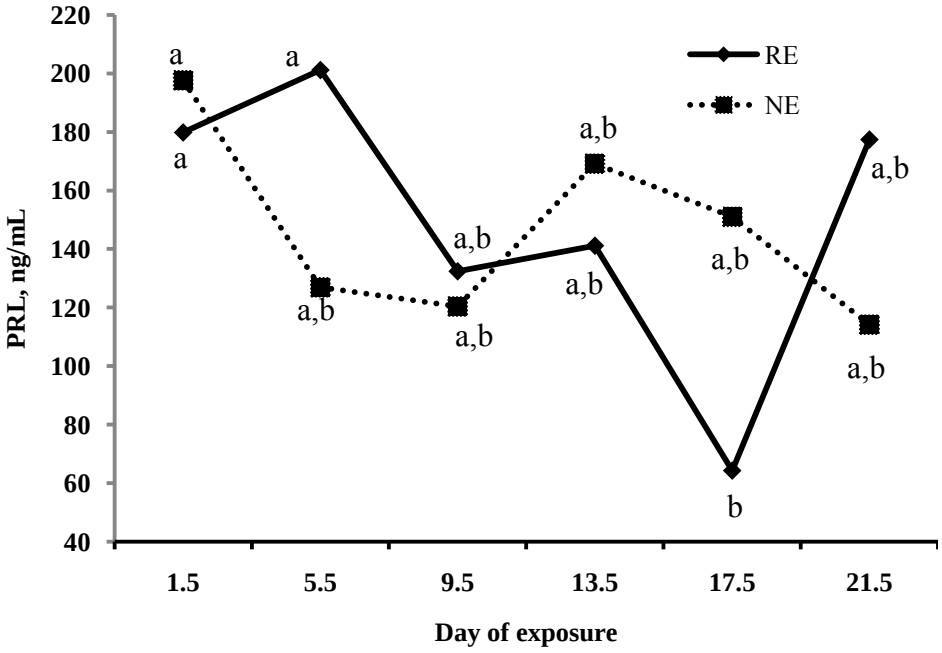

Figure 3. Least squares means for prolactin (PRL) concentrations of ewes after exposure to rams (RE; n = 18) and wethers (NE; n = 18). Exposure type by d interaction, $P = 0.01$. Means that share common letters do not differ ($P > 0.05$). SEM = 111.5 ng/mL.

Discussion

To determine if the biostimulatory effect of rams is associated with alterations in metabolic status, virgin ewes were exposed to rams during the transition into the breeding season. It is known that feeding a higher plane of nutrition to ewes before the breeding season results in increased ovulation rate. Furthermore, it is known that changes in nutrition result in changes in metabolic hormones concentrations. It was of interest to determine if the presence of a ram during the transition into the breeding season can have similar effects on metabolic status.

Ewes exposed to rams and ewes exposed to wethers did not exhibit a difference in concentrations of cortisol, leptin and IGF-1 in this experiment. Concentrations of each of these hormones did differ among days of exposure. These findings that cortisol concentrations in ewes exposed to rams do not differ from ewes exposed to wethers are not consistent with those of Tauck et al. (2007) who identified increased cortisol concentrations over time in cows exposed to bulls. From this experiment it does not appear that ewes chronically exposed to rams show increased HPA axis activity as in cows or that the large individual animal variation of ewe in the present study masked differences in cortisol concentrations between RE and NE ewes. Another point to consider in the present study with regard to changes in cortisol concentrations is that all of the RE ewes and a majority of the NE ewes exhibited ovulatory activity over the exposure period. Once cows began to show ovulation, cortisol concentrations did not differ between bull-exposed and non-exposed postpartum cows (Tauck et al., 2007).

In contrast to our findings that leptin concentrations did not differ between RE and NE ewes, Olsen et al. (2009) found that cows exposed to bulls exhibit alterations in temporal concentrations of leptin from cows not exposed to bulls. Results for IGF-1 of the present study are the first known report of IGF-1 concentrations in ewes exposed to rams during the transition into the breeding season. It is known that IGF-1 concentrations in ewes of high BCS are greater than in ewes of low BCS during early breeding season (Vinoles et al., 1999). It is interesting that RE ewes that gained BW during the experiment did not exhibit increased IGF-1 concentrations. It is possible that the amount

of BW gain in RE ewes was not sufficient to induce a change in BCS to cause a detectable difference in IGF-1 concentrations.

In this experiment, ewes exposed to males resumed luteal activity sooner than ewes not exposed to rams; these findings are consistent with numerous previous reports (for review, see, Delgadillo et al., 2009; for review, see, Martin et al., 1986; Schinckel, 1954).

The biostimulatory effect of rams is also associated with changes in certain metabolic hormones concentrations. Ewes exposed to rams exhibited a decrease in T4 during the first days of exposure; however, NE ewes did not exhibit a statistically significant decrease in T4 until 9.5 days of exposure. It should be noted that by day 11.5, the sampling period following the significant decrease in T4, there was no difference in percentage of ewes that resumed luteal activity. It should be noted that both RE and NE exhibited a general decrease in T4 concentrations during the sampling period, the more important feature maybe that this decrease occurred in RE ewes earlier than in NE ewes. This is an interesting finding, because it is the first known report that T4 is involved with the ram effect, or with the initiation of the breeding season. It is known that thyroid hormones are necessary to initiate seasonal inhibition of reproductive activity in ewes (Moenter et al., 1991) and rams (Parkinson and Follett, 1994). We did not observe a significant change in T3 concentrations or T3:T4 ratios between RE or NE ewes. Although there is a visual trend in these data for a decrease similar to that observed for T4, there was considerable individual animal variation that limited our ability to detect a significant difference with the number of ewes per exposure type. However, T3

concentrations roughly followed those of T4, and T3:T4 ratios were numerically lower in RE ewes. It may be that the acute (within the first few days after exposure) biostimulatory effect of rams is partially mediated or permitted by hastening a decrease concentrations of T4.

It is known that PRL concentrations in ewes follow a circannual rhythm and are highest around summer solstice and reach their nadir by winter solstice (Karsch et al., 1989). Furthermore, seasonal rhythms of PRL have been shown to be unaffected by seasonal rhythms of thyroid hormones (Billings et al., 2002). Thus, changes in both PRL and T4 suggest the biostimulatory effect of rams involves diverse changes in the neuroendocrinology of ewes during the transition into the breeding season. Ewes exposed to rams during the transition into the breeding season exhibited a decrease in PRL concentrations between d 5.5 and 17.5 of exposure; whereas, NE ewes did not exhibit a significant change in PRL concentrations during exposure. This decrease in PRL was likely not a stimulus for resumption of luteal activity because it occurred after most RE ewes resumed luteal activity. This hypothesis is supported by with previous conclusions in that the onset of the breeding season appears to be independent of decreasing PRL concentrations (Worthy et al., 1985). Nevertheless, the biostimulatory effect of rams was associated with alterations in PRL concentrations in ewes during the transition in to the breeding season in the present study.

Perhaps the most intriguing finding of the present study was that RE ewes gained significantly more weight than NE ewes over the 22-d exposure period. To our knowledge this is the first known report that BW of ewes can be altered by exposing

them to rams. It is not known how exposure to rams caused ewes to gain more weight than ewes exposed to wethers. Feed intake was not measured in this study, so knowing if ewes exposed to rams simply ate more and gained weight or if the presence of rams altered their metabolic status in a way that caused them to utilize energy more efficiently is not clear. Increased calorie intake is typically associated with increased T3 concentrations caused by increased conversion of T3 to T4 (for review, see, Hadley and Levine, 2006). The general decrease in T4 concentrations in ewes exposed to rams suggests that metabolic rate has decreased and perhaps this induced BW gain at the same level of feed. Presumably, this stimulus for a change in metabolic status in RE ewes appears to come from increased TRH, which would cause increased TSH, ultimately leading to increased T4.

In conclusion, exposing ewes to rams during the transition into the breeding season not only hastens resumption to luteal activity, but appears to change certain metabolic hormones concentrations that may be associated with a greater change in BW than ewes not exposed to rams. This is the first report that the biostimulatory effect of rams is associated other alterations in endocrinology and physiology of ewes during the transition into the breeding season.

CHAPTER 6

GENERAL DISCUSSION

In Experiment 1 of this thesis we reported that exposing ewes to rams during the transition into the breeding season involves changes in temporal cortisol concentration patterns. More specifically, we identified an increase in cortisol pulse duration. This finding is consistent with findings in postpartum, anovular, cows exposed to bulls (Tauck et al., 2010). Other characteristics of cortisol concentrations did not differ significantly, though many were numerically different between ewes exposed to rams or wethers. We also reported an increase in pulse frequency and baseline concentration of LH during this same period which is in agreement with numerous reports associated with the biostimulatory effect of rams, including those of Martin et al. (1980) and Poindron et al (1980). Similarly, we also identified a greater number of LH pulses during the intensive sampling period; this was expected because the length of the sampling period was the same for all ewes. Perhaps more interestingly, is that ewes exposed to rams exhibited a negative, linear relationship between LH pulse frequency and cortisol pulse frequency which was not observed in wether-exposed ewes. This suggests that when ewes are exposed to rams there is a change in neuro-circuits controlling the release of these hormones.

In Experiment 2, we found that the biostimulatory effect of rams involves relative long-term changes in PRL and T4 concentration patterns. More interestingly, we found the ewes exposed to rams gained more weight than ewes exposed to wethers during the transition into the breeding season. Together, these results show that exposure to a ram

during the transition into the breeding season does involve changes in metabolism and the primer pheromonal effect of the ram to increase the occurrence of cyclicity in ewes at the beginning of the breeding season.

It is known that during the breeding season GnRH neurons receive more synaptic input than during the non-breeding season (Xiong et al., 1997). Thyroxine is a known stimulator of morphological rearrangements within the brain during development (for review, see, Lehman et al., 2010b). It is possible that changes in T4 concentrations we observed in ewes exposed to rams represent signals to the hypothalamus to rearrange neuronal structure in preparation of the breeding season. Previous reports indicate that thyroidectomized ewes resume ovulatory activity at the beginning of the breeding season equivalently to thyroid intact ewes (Billings et al., 2002). However, T4 may be involved with the mechanism by which rams hasten the onset of the breeding season. An interesting future study would examine the effects of exposing thyroidectomized ewes to rams during the transition into the breeding season. With enough ewes, it would also be interesting to compare degree of neuronal rearrangement within the hypothalamus of thyroidectomized ewes to thyroid-intact or thyroid hormone replaced ewes exposed to rams.

Hypothesis for Biostimulatory Effect of Rams on GnRH release and Metabolic Status

In recent years, KNDy neurons have been proposed as the putative "gate keepers" for signals that regulate release of GnRH in the hypothalamus. As pointed out in the review of literature, KNDy neurons are sensitive to a number of signaling molecules,

including ovarian steroids and glucocorticoids. Furthermore, kisspeptin neurons also receive considerable synaptic input from nearby neurons as well. It should be noted that KiSS affects POMC and NYP/AgRP cells in the hypothalamus in addition to GnRH secreting neurons (Backholer et al., 2010). This suggests that KNDy neurons should not be considered exclusive modulators of reproduction, but perhaps central regulators of many bodily systems. The following diagram (figure 4; adapted from: Backholer et al., 2010; Hadley and Levine, 2006; Karsch et al., 1987; Lehman et al., 2010a; Maeda et al., 2010; Okamura et al., 2010) represents a hypothetical model based on the results of the present study for the relationship among central regulators of female reproductive and metabolic processes in ewes exposed to rams.

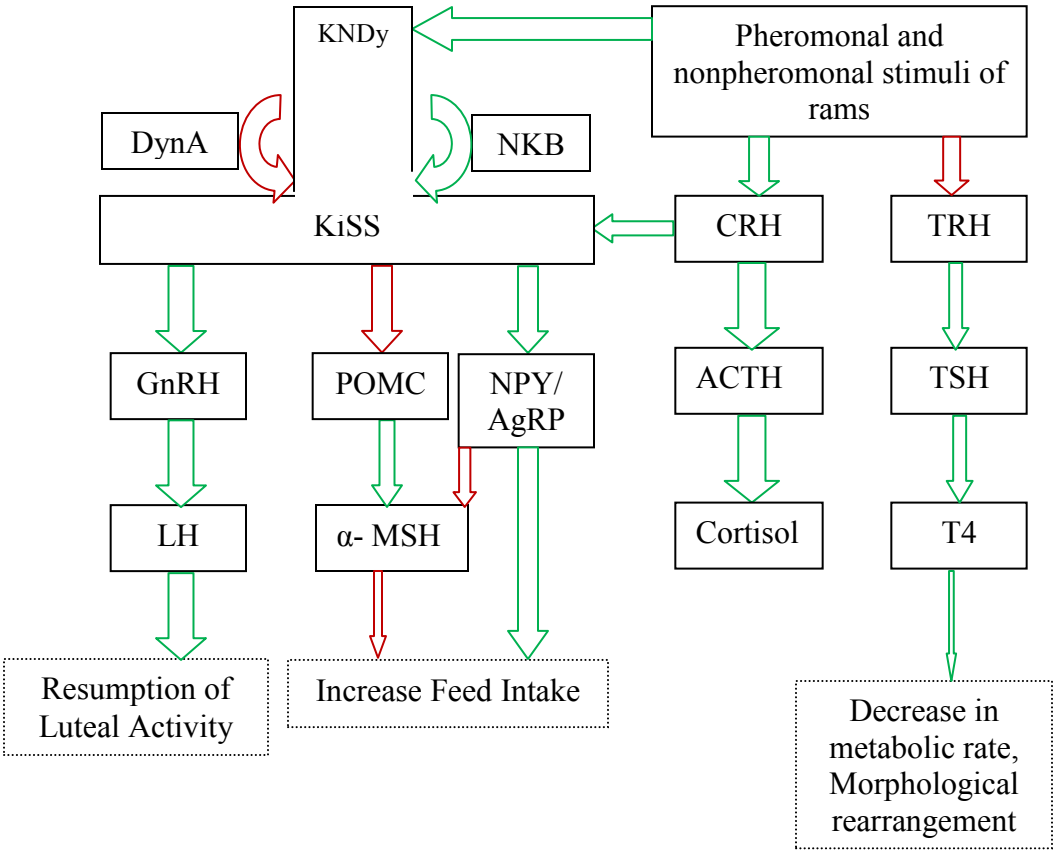

Figure 4. Hypothetical model for the relationship among hypothalamic regulators of reproductive and metabolic processes in ewes exposed to rams. Green arrows indicate a positive effect, red arrows indicate a negative effect, and width of the arrow indicates magnitude of the effect.

The above diagram depicts the numerous effects and pathways of exposing ewes to rams during the transition into the breeding season. Recently Okamura et al. (2010) showed that exposing does to bucks increases MUA in ARC, and those alterations in MUA correlate with release of kisspeptin. It is known that increased KiSS expression during the transition into the breeding season stimulates GnRH release (Chalivoix et al., 2010), causing an increase in LH pulse frequency and ultimately resumption of luteal activity (Karsch et al., 1993). We propose that our results that ewes exposed to rams resumed luteal activity sooner than ewes not exposed to rams support this mechanism.

Recently, Backholer et al. (2010) showed that increased kisspeptin causes decreased POMC and increased NPY expression in the hypothalamus of rats. This is interesting because POMC through conversion to α-MSH, is known to reduce feeding activity in mice. In addition, increased NPY expression is known to increase feeding activity in rodents (Jang et al., 2000; Kohno et al., 2007). If kisspeptin is increased in ewes exposed to rams, and the relationship among kisspeptin, POMC and NPY in ewes is similar to that in rodents, then this could explain how ewes exposed to rams gained more weight than ewes exposed to wethers. However, in the present experiment feed intake of ewes for the two exposure types was not measured. So whether or not activation of this pathway is associated with the difference in gain of BW between ewes in these exposure types cannot be evaluated. We also identified changes in temporal cortisol concentrations in ewes during the first 6 h of exposure to rams. It is not clear if cortisol facilitated changes in kisspeptin or if perhaps activation of these other pathways caused changes in cortisol concentration patterns. We suggest evaluation of this pathway would be an important "next step" in determining the physiological pathway whereby rams stimulate ovulatory activity in anovular ewes during the transition into the breeding season.

Both RE and NE ewes exhibited a general decrease in T4 throughout the exposure period; however, T4 concentrations appeared to decrease sooner in ewes exposed to rams than in ewes exposed to wethers. Decreased T4 can be thought of as a decrease in metabolic rate. Thus, it is possible that RE ewes gained more weight than NE ewes because they had a lower metabolic rate for a greater proportion of the exposure period. Additionally, the action of T4 is known to mediate morphological rearrangement of

neurons. The change in T4 that we observed could be related to hastened signals in ewes exposed to rams to prepare the hypothalamus for the breeding season.

The majority of neuroendocrine research to date involves colocalizing antigens with immunocytochemistry on thick sections. This process, though quite fruitful, has limitations, namely the restriction to identify only a few antigens at a time. As suggested, only antigenic molecules can be identified and the efficacy of identifying these molecules is limited to the sensitivity of the antibodies and biotinylation processes.

Mass spectrometry (MS) is a technique that could be used to better evaluate the complexity of the hypothalamus. Mass spectrometry could be used in two ways: 1) matrix assisted laser ionization/desorption imaging of hypothalamic sections; and, 2) global profiling of intra- and extracellular endogenous metabolite and protein/peptide content. Used in these ways, mass spectrometry can provide identification of numerous molecules as well as information related to the special distribution throughout the brain. Furthermore, the results from mass spectrometry can be made quantitative, yielding concentrations of numerous molecules from relatively small amounts of tissue or extract. However, use of MS is not without challenges. First this technique requires access to expensive equipment and the expertise to use it in an effective manner and challenges have also been identified in analyzing membrane bound proteins with MS. Nevertheless, MS is a potential method to probe the complexity of the hypothalamus.

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