

THE FLUSHING EFFECT AND EXPRESSION OF FOLLICLE STIMULATING HORMONE
RECEPTOR VARIANTS IN SHEEP

by

JACQUELYN M. HAND

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Approved by:

Major Professor
Timothy G. Rozell

Abstract

An increase in pre-mating dietary energy positively influences ovulation and lambing rates, and this practice is known as nutritional flushing. The mechanisms of flushing, however, are still unknown. Increasing dietary energy approximately two weeks before breeding likely increases the production of insulin-like growth factor I (IGF-I) within the ovary, which stimulates the synthesis of follicle stimulating hormone receptor (FSHR). Several alternatively spliced transcripts of the FSHR have been identified in sheep. Each variant form is believed to be produced according to the stage of follicle development. This study was carried out to evaluate expression patterns of the FSHR variant forms (FSHR-1, FSHR-2 and FSHR-3) in the sheep ovary in response to different flushing diets.

For this experiment, yearling Rambouillet ewes (n=93) were allocated among 6 different energy type treatment diets, either prairie or alfalfa hay based, for at least two weeks in combination with the insertion of a controlled internal drug releasing device (CIDR). Two of the treatment groups had commercially available block supplements provided and two had rolled corn supplemented. Mid-ventral laparotomy was performed on each ewe 3.5 to 4 days after CIDR removal. Follicles 4 mm and greater were aspirated and categorized as either medium (M; 4 to 6 mm) or large (L; > 6 mm). Total RNA was extracted from granulosa cells (GC) and reverse transcribed followed by qPCR of the resulting cDNA using specifically designed primer sets for each variant of the FSHR and for the LH receptor.

Changes in live weight were different ($P < 0.01$) between treatment diets but there were no statistical differences for NEFA concentrations between any of the treatments nor were there differences for body condition (mean = 3.0) or lambing rate. Therefore, it is likely a flushing response did not occur in this study.

Expression of FSHR-1 was different between M and L follicles ($P < 0.01$) and tended to be different for ewes fed alfalfa hay ($P = 0.05$). Overall mean expression of FSHR-3 was greater than expression of FSHR-1 or FSHR-2 ($P < 0.01$), although there was no difference between M and L follicles, or between treatment diets. The concentration of estradiol in follicular fluid was

not different between the treatment diets or follicle sizes nor was expression of lutenizing hormone receptor (LHR), indicating that follicles were similar developmentally.

The FSHR-1 form seemed to be the variant most likely to be involved in later stages of follicular development, and is potentially involved in follicle rescue. For all follicles, FSHR-3 was the more highly expressed form of the FSHR and may likely be essential throughout antral follicle development. Further research is required to determine the exact mechanism whereby initial energy status of ewes seems critical for the increased ovulation rate that occurs after energy supplementation (i.e. the flushing response).

Table of Contents

List of Figures	vi
List of Tables	vii
Acknowledgements.....	viii
Chapter 1 - LITERATURE REVIEW	1
Introduction.....	1
Requirements for Successful Reproduction.....	2
Processes of Reproduction.....	3
Hypothalamo-pituitary-gonadal Axis	3
Follicular Development	4
Differences in Follicular Development Among Livestock Species.....	9
Reproduction in the Sheep	10
Ovulation Rate	13
Factors that Influence Ovulation.....	13
Flushing Effect.....	15
Follicle Stimulating Hormone Receptor	18
Variant Forms of the Receptor.....	19
Signal Transduction of Variants	21
Chapter 2 - THE FLUSHING EFFECT AND EXPRESSION OF FOLLICLE STIMULATING HORMONE RECEPTOR VARIANTS IN SHEEP	23
Introduction.....	23
Materials and Methods.....	26
Animals and Treatment Diets	26
Controlled Internal Drug Releasing Devices (CIDRs)	27
Mid-Ventral Laparotomy	28
RNA Isolation and DNase Treatment.....	29
Reverse Transcription – Polymerase Chain Reaction.....	29
Quantitative Real-Time Polymerase Chain Reaction	29
Non-Esterified Fatty Acids	30

Follicular Fluid Estradiol Assay	30
Breeding Period	31
Statistical Analysis	31
Results.....	32
Body Condition Score.....	32
Effect of Diet on Live weight Change	33
Non-Esterified Fatty Acids	34
Number of Medium and Large Follicles.....	35
Gene Expression	36
Overall Gene Expression of Large and Medium Follicles.....	39
Estradiol Concentrations	40
Lambing Rate and Birth Weights.....	41
Discussion.....	42
Chapter 3 - References.....	48
Appendix A - Protocols	57
Appendix B - Reagents and Supplies.....	71

List of Figures

Figure 2.1 CIDR and surgery schedule.....	28
Figure 2.2 Initial body condition scores among treatment diets.....	32
Figure 2.3 Effect of dietary treatments on live weight change.....	33
Figure 2.4 Effect of dietary treatments on non-esterified fatty acids (NEFA).	34
Figure 2.5 Effect of dietary treatments on number of medium and large follicles.....	35
Figure 2.6 Effect of dietary treatments on FSHR variants and LH receptors in medium (4 to 6 mm) follicles.	37
Figure 2.7 Effect of dietary treatments on FSHR variants and LH receptors in large (> 6 mm) follicles.....	38
Figure 2.8 Relative expression of variant forms of FSH receptor and LH receptor in medium and large follicles.....	39
Figure 2.9 Concentration of estradiol in follicular fluid.....	40

List of Tables

Table 2.1 Treatment diets	26
Table 2.2 Block supplements	27
Table 2.3 Primers used for real-time PCR	30
Table 2.4 Lambing information	41
Table 3.1 Reagents and supplies.....	71

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Chapter 1 - LITERATURE REVIEW

Introduction

One of the basic requirements for successful reproduction in sheep and other livestock species is adequate nutrition. Many reproductive events are affected by nutrition, including gametogenesis and puberty (Foster and Olster, 1985). An increase in pre-mating dietary energy has been found to affect follicular development through increased ovulation rate (Gunn et al., 1979) and follicular size (Somchit et al., 2007). Many sheep production systems use the flushing effect as a method to increase lambing rates. Flushing involves increasing the available dietary energy prior to breeding. The physiological mechanism of flushing on reproductive performance remains largely unknown despite a number of recent studies.

The process of follicular development occurs before birth in many mammalian species and continues throughout the life of an animal. In the sheep fetal ovary, primordial follicles begin to appear around day 75 of a 150 day gestation period (McNatty et al., 1995), and the first appearance of antral follicles occurs by day 135 (Lundy et al., 1999). At birth, the mitotic activity of oogenesis is complete and the primary oocyte remains in a state of nuclear arrest, in which meiosis is incomplete until after puberty when the first LH surge occurs.

Follicular development is dependent upon endocrine support, specifically the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are produced and released from gonadotroph cells within the anterior pituitary. Follicle stimulating hormone has many functions in granulosa cells, including proliferation and differentiation effects. The diverse responses of granulosa cells to FSH during follicle growth (Wayne et al., 2007) is likely a consequence of the receptor to which FSH binds (Sairam and Babu, 2007).

Located on granulosa cells within the ovary, the well known FSH receptor (FSHR) was first cloned and characterized in rats as a large glycoprotein hormone receptor with seven transmembrane-spanning segments (Sprengel et al., 1990). Later, the G protein coupled form of the receptor (FSHR-1) also was cloned and sequenced in sheep (Yarney et al., 1993). Other studies revealed that several alternatively spliced forms of the FSHR existed, and likely had functional significance (Khan et al., 1993; Yarney et al., 1993). The FSHR-2 is believed to be a dominant negative form of the receptor that interferes with downstream signaling (Sairam et al.,

1996), while FSHR-3 resembles a growth factor type-I receptor (Sairam et al., 1997). The different variant forms are possibly expressed differently by granulosa cells at different stages of follicular growth (Sairam and Babu, 2007). Each variant is thought to couple to signal transduction pathways, allowing FSH to affect different stages of follicular growth and development depending on the receptor variant to which it binds (Ulloa-Aguirre et al., 2007).

The physiological mechanism of flushing likely involves the insulin-like growth factor system. Increasing dietary energy before breeding increases the production of insulin-like growth factor I (IGF-I) within the liver and ovary. Insulin-like growth factor I is involved in cellular proliferation and differentiation; more specifically, it is part of the insulin-like growth factor family that is important for folliculogenesis, steroidogenesis, and gonadotropin sensitivity (Monget and Monniaux, 1995). The subsequent accumulation of IGF-I within the ovary augments FSHR gene expression that is reinforced with a positive feedback loop between IGF-I and FSH (Zhou et al., 1997). Further research needs to be performed to examine the link between nutrition, likely through the IGF system, and FSHR variants in order to gain clearer insight into the precise mechanism of flushing.

Requirements for Successful Reproduction

Reproduction comprises many series of events beginning with an embryo that is developing a reproductive system to an animal that is able to produce viable gametes and is ready for copulation. The achievement of puberty, in which an animal acquires the ability to produce gametes and can successfully produce offspring, is one of the goals of reproduction. Once an animal has reached puberty, many factors influence the ability to successfully reproduce. Some are mediated by nutrition, stress, and timing of events (i.e. photoperiod).

Nutrition has an effect on all aspects of reproduction, including gametogenesis and puberty in both males and females. In natural conditions, the close association between nutrition and reproduction allows demanding processes of reproductive function, such as gestation and lactation, to be linked with food supply. This is a survival mechanism that takes advantage of certain times of the year when higher quality feeds are available and production of offspring becomes favorable. Sheep and goats are a prime example in that they breed during autumn

months to ensure that their offspring are born during the spring, making survival more likely. In the male, nutritional investment has fewer consequences than in the female (as reviewed by Scaramuzzi et al, 2006). Although nutrition in the male can influence reproductive processes such as sperm production, the consequences of poor nutrition are not life threatening as in the female; whereby a male may have less genetic input but a female could endanger the life of her embryo and herself. Folliculogenesis and ovulation rate are quite sensitive to nutrition, and nutritional manipulation can be used as a management strategy to improve reproduction.

The achievement of normal reproductive success can be prevented by stress. Stressors, such as behavioral interactions or heat stress, can be responsible for reduced reproductive efficiency. A hypothalamic-pituitary-gonadal axis is the basis of all reproductive function and follicular development and ovulation ultimately are regulated by the neuroendocrine control of the axis. This complex system is responsible for follicle growth and ovulation, and therefore is vulnerable to the effects of stress (Moberg, 1991).

The female is dependent on a series of reproductive events to occur to ensure reproductive success. The timing of each of these events is critical in such a way that if one event is disrupted, the result will be failed reproduction. Photoperiod is a classic example of timing reproductive events. Species that use photoperiod to synchronize breeding activity enhance the likelihood that the correct timing of reproductive activity (including follicle growth and ovulation) will occur because the time of year assures optimum growth and development of offspring and also supports lactation of the mother (as reviewed by Rosa and Bryant, 2003).

Taken together, there are many ways reproduction can be affected. Proper nutrition, decreased stress, and manipulation of timing of events all are well established and successful ways to maximize reproductive efficiency in livestock animals.

Processes of Reproduction

Hypothalamo-pituitary-gonadal Axis

Reproductive function requires integration of the hypothalamus, the pituitary, and ovaries to regulate development of gametes and their supportive structures. Regulation of reproductive functions involves collaboration of the nervous and endocrine systems, as reviewed by Senger

(1999). Afferent sensory nerves respond to an external stimulus such as a visual or tactile cue, or an internal stimulus such as dietary energy status, and synapse within the spinal cord. The neurons then transduce the signal in the form of an action potential directly to the hypothalamus, where neurohormones are released into a specialized portal system that carries them to capillary beds within the anterior pituitary. One such neurohormone, gonadotropin releasing hormone (GnRH), is a decapeptide synthesized from neurons within the surge center, located within the preoptic portion of the hypothalamus, and the tonic center, which is part of the ventromedial nucleus of the hypothalamus. The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are produced and released from gonadotroph cells within the anterior pituitary. Follicle stimulating hormone is responsible for growth of follicles on the ovary in the female and spermatogenesis in the male. Luteinizing hormone causes ovulation of the follicle and production of progesterone in the corpus luteum (CL) by the female and production of testosterone in the male.

Follicular Development

In the female, ovulation of a viable gamete requires successful completion of follicular development which occurs in a predictable, repeatable sequence of stages from primordial to primary, secondary, tertiary, early antral and preovulatory follicles. Primordial follicles, being the least developed, are characterized by having an oocyte surrounded by a thin, flattened layer of undifferentiated granulosa cells. Junctional complexes begin to form, such as gap and zonula adherens-like junctions, that connect adjacent granulosa cells and the oocyte to granulosa cells processes, respectively (Fair et al., 1997), so that these cells can communicate. The mechanisms and signals that selectively initiate when resting primordial follicles become activated and enter the growth phase have yet to be elucidated. Initiation of follicular growth (activated primordial follicles), however, can be detected by identifying a marker known as proliferating cell nuclear antigen (PCNA) (Wandji et al., 1996).

In most mammals, the ovarian reserve of oocytes is completely established before birth and comprises a reservoir where most of the oocytes are dormant and non-growing the majority of an animal's life (Fortune, 2003). The germ cells of the oocyte become surrounded by somatic pregranulosa cells, and the pool of resting primordial follicles will gradually be depleted

throughout the lifespan of the female as a result of either attrition or initiation of growth (Erickson, 1966). The activation of quiescent primordial follicles starts the process of folliculogenesis, which takes place as a continuous process. Within the ovary, a hierarchy exists among follicles according to functional status. The granulosa cells of developing follicles undergo extensive proliferation and differentiation, the fate of which is governed not only by gonadotropins but also other endocrine and local growth factors. Paracrine and autocrine factors are secreted by the oocyte and surrounding somatic cells while primordial follicles transition from a quiescent state into developing primary follicles, and may involve both stimulatory and inhibitory influences.

Small preantral follicles are not responsive to gonadotropin stimulation initially, and respond well to autocrine and paracrine factors. Local peptide factors in pre-granulosa cells such as kit ligand (KL), and fibroblast growth factor-2 (FGF-2) which upregulates KL, have been found to stimulate primordial to primary follicle transition in rats (Nilsson and Skinner, 2004). Bone morphogenetic protein 15 (BMP15) and KL were demonstrated to form a negative feedback response between the oocyte and granulosa cells, respectively, that appeared to stimulate mitotic activity of granulosa cells (Otsuka and Shimasaki, 2002). In addition, primordial follicles have been found to synthesize steroidogenic regulatory factors (Logan et al., 2002), as well as anti-Mullerian hormone (AMH), a member of the transforming growth factor β (TGF- β) superfamily. AMH has an inhibitory role in the initiation of primordial follicle growth. Mice that had a deletion of the AMH gene experienced an increased rate of primordial follicle recruitment resulting in a relatively early depletion of their primordial stock (Durlinger et al., 1999). The expression of AMH has been detected in granulosa cells from primary through preantral stages but is absent in primordial follicles indicating that growing follicles have an inhibitory feedback response on resting primordial follicles at the level of pregranulosa cells (Knight and Glister, 2006).

Transition of the quiescent primordial follicles into primary follicles is characterized by the development of flattened pre-granulosa cells into cuboidal granulosa cells, the continued growth of the oocyte, and initial development of pieces of the zona pellucida between the oocyte and granulosa cells (Fair et al., 1997). Primary follicles begin to express FSH receptors (Tisdall et al., 1995), however, there is evidence that FSHR may not be coupled to adenylyl cyclase in the early stages of development (Babu et al., 2000). Nevertheless, the involvement of FSH in

preantral follicles exists, possibly by accelerating the rate of selection (Campbell et al., 2003). In sheep, once the gonadotropin responsive stage is reached, the rate of follicle growth occurs quickly; taking about 5 days to increase from 0.8 mm to 2.5 mm, whereas primordial follicle growth through antrum formation and gonadotropin responsiveness can last about 210 days (Downing and Scaramuzzi, 1991). Once follicles reach 2.5 mm, rapid growth takes place within 2 days and this is when the greatest amounts of FSHR can be found. Upon antrum formation, FSH receptors can be found on granulosa cells.

An expanding number of factors, other than hormones, are being studied; many of which are known to have an effect of some magnitude on follicular growth and development. Two specific growth factors are thought to have a larger regulatory role than other factors and therefore have been studied in greater detail. Bone Morphogenetic Protein 15 (BMP15) and Growth and Differentiation Factor 9 (GDF9), members of the TGF- β superfamily, utilize intragonadal signals that are likely to be key regulators for the transition of primary to secondary follicles (Dong et al., 1996; Galloway et al., 2000) in sheep (Juengel et al., 2002; Brawtal et al., 1993). While both are localized specifically to the oocyte, expression of the GDF9 gene precedes that of BMP15 (Bodensteiner et al., 1999; Galloway et al., 2000). In sheep, GDF9 mRNA was found to be present beginning in primordial follicles with continued expression throughout follicular growth (Bodensteiner et al., 1999), whereas BMP15 mRNA is present beginning in the primary stage (Galloway et al., 2000), when follicles begin to grow. In contrast, many high ovulation rate species, such as mice and pigs, are believed to only require either GDF9 or BMP15 for follicle development and which one is used is species specific. Both genes are critical for maintenance of folliculogenesis; BMP15 has a large influence on ovulation rate and GDF9 is required for oocyte development. Sheep immunized against either GDF9 or BMP15 experienced arrested follicular development in follicles transitioning from the primordial to the primary stage (Juengel et al., 2002; McNatty et al., 2007).

Primary follicles will continue to develop into secondary follicles, which are characterized by a bilayer of cuboidal granulosa cells and the formation of the zona pellucida. Gap junctions can then form between granulosa cells and the oocyte (Fair et al., 1997). Theca cells begin to form and align along the basement membrane and appear elongated and are few in number (Braw-Tal and Roth, 2005) and this tissue does not have the ability to produce steroids until the tertiary stage (Logan et al., 2002). Differentiation of granulosa cells can be partially

attributed to the autocrine actions of activin. The activin protein is secreted by granulosa cells and its actions have been confirmed to increase the number of FSH receptors as well as increase the responsiveness of granulosa cells to FSH and LH (Zhao et al., 2001). As secondary follicles develop FSH receptors, acquisition of receptivity by the follicle begins its dependence on gonadotropic support. The expression of FSH receptors caused by differentiation of granulosa cells is thought to be augmented by local insulin-like growth factor I (IGF-I) action; likewise, FSH augments IGF-I receptor expression (Zhou et al., 1997). Unlike IGF-I, activin has a unique role in the propagation of FSH receptors in that it is able to influence FSH receptor expression in the absence of FSH (Zhao et al., 2001).

Tertiary follicles develop from secondary follicles and are characterized by multiple layers of cuboidal granulosa cells and the formation of a small fluid filled cavity, or antrum, surrounding the oocyte. Thecal cells become prominent and provide structural integrity for the follicles and develop blood vessels necessary for endocrine support. Receptors for LH are produced during this time and the response of LH permits theca interna cells to become androgenic (steroidogenically active) (Hedin et al., 1987). Consequently, it is the paracrine action of inhibin that augments LH mediated androgen production (Hsueh et al., 1987). Large preantral follicles exhibit expression of the genes for steroidogenic acute regulatory protein (StAR), steroidogenic enzymes such as side chain cleavage enzyme (SCC), Cytochrome P450 17 α -hydroxylase/17-20 lyase (17 α OH), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and cytochrome P450 aromatase, and LH receptors in theca interna cells (Logan et al., 2002). Conversion of cholesterol into androgens by SCC takes place upon cholesterol transportation from the outer to the inner mitochondrial membrane by StAR followed by the regulatory action of 17 α OH to convert pregnenolone to dehydroepiandrosterone (DHEA), as reviewed by Stocco (2001). DHEA is converted to androstenedion by 3 β -HSD, and androstenedione to testosterone by 17 β -hydroxysteroid dehydrogenase (17 β -HSD). Testosterone will then cross the thecal cell membrane into granulosa cells where it can be converted into estradiol by aromatase (Bogovich et al., 1986; Xu et al., 1995). Estradiol can be secreted from the follicle, through the blood, and to the hypothalamus where it stimulates GnRH synthesis and secretion.

The preovulatory follicle utilizes angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and angiopoietin to aid in the generation of new blood vessels required for follicular maturation and

corpus luteum (CL) development. As FSH concentrations become elevated to support follicle growth, LH receptors, originally only located on thecal cells, also become present on granulosa cells. Thus, only the follicle(s) that has LH receptors on its granulosa cells can continue to grow in an environment with much greater LH than FSH available. As a result, in order to ensure proper secretion of gonadotropins at the proper time, the regulation of FSH secretion is tightly controlled through the complex interaction of a number of hormones.

Negative regulation of FSH in the late stages of follicular development must occur in order to ensure fertilization of only one ovum in mono-ovular species and a limited number of ova in poly-ovular species. This can be accomplished by the heterodimer inhibin, a member of the TGF- β superfamily. The main action of inhibin on FSH is within the pituitary. As concentrations of FSH increase, inhibin is stimulated to increase causing negative feedback on secretion of FSH from the anterior pituitary. Maintenance of follicles and premature luteinization is prevented by activin by inhibiting oxytocin and FSH-stimulated progesterone production (Shukovski and Findlay, 1990). As the dominant follicle(s) emerges, inhibin and estradiol suppress the growth of subordinate follicles by decreasing FSH secretion. Secretion of FSH diminishes and the follicles which are able to receive support of LH at this point will continue development. The suppression of FSH and increase of local factors, such as inhibin, activin, and IGF-I, permits the dominant follicle(s) to survive while subordinate follicles become atretic.

Activin, also a member of the TGF- β superfamily, is a homodimeric protein and has a primary role within the pituitary where it is synthesized. Upon binding to the type II activin receptor, ActRIIA and ActRIIB, activin utilizes a serine/threonine-specific protein kinase pathway within gonadotroph cells to increase synthesis of FSH β -subunit mRNA (Bilezikjian et al., 2004). Once the activin receptors are activated, a signal cascade is initiated beginning with the phosphorylation of activin receptor-like kinase 4 (ALK4), a type I receptor (Bilezikjian et al., 2004) and continuing through the second messengers Smad3 and Smad4. The Smad3/Smad4 complex binds with the transcription factor Pitx2 and the entire unit attaches to binding elements on the promoter region of the FSH β -subunit gene within the nucleus resulting in transcription (Suszko et al., 2008). Activin is also responsible for stimulating its antagonist, inhibin. In order to confine the signals of activin, FSH induces inhibin and follistatin. Produced within the ovary, inhibin travels to the pituitary and confines the signals of activin; while follistatin, a glycoprotein monomer, binds to activin preventing it from binding to its receptor (Lerch et al., 2007).

Differences in Follicular Development Among Livestock Species

In the female adult, the process by which follicles develop and ovulate is well established. In order to ovulate a viable oocyte, however, several developmental changes must occur during embryonic development. In most livestock species, such as sheep, cattle, and pigs, the mitotic activity of oogenesis is completed prenatally. In rats and mice the oocytes do not complete mitosis until a few days after birth (Peters, 1969). The primordial germ cells proliferate to become primary oocytes and enter meiosis I. Recent findings in mice indicate that a retinoic acid responsive gene, stimulated by retinoic acid gene 8 (Stra8), is required for entry of female and male germ cells into meiosis (Baltus et al., 2008). The primary oocyte is then in a state of dictyate, or nuclear arrest, until after puberty when the first LH surge occurs; after which meiosis I resumes. Meiosis II is initiated after ovulation and is completed by the time of fertilization. Unlike female germ cells, male germ cells do not enter meiosis until after puberty. A proposed hypothesis by Koubova et al. 2006 is that the enzyme cytochrome p450 (CYP26) prevents Stra8 expression by mediating retinoic acid metabolism in embryonic testis (Koubova et al., 2006).

In livestock species, as well as primates and humans, follicular development begins before birth and continues in an asynchronous manner over the animal's lifetime. In contrast, rodent species do not begin follicle development until shortly after birth whereby waves of follicle growth are synchronous. Somatic pre-granulosa cells interact with primordial germ cells to constitute a population of primordial follicles (Bezard et al., 1979). Primordial follicles consist of an oocyte with a flattened layer of squamous granulosa cells. Some follicles leave the primordial resting pool and continue to grow while other follicles are just beginning to develop, creating a large array of preantral and early antral follicles developing over an extended period of time during gestation. This asynchronous formation of follicles occurs in many species. Primordial, primary, and secondary follicles begin to appear around days 75, 100, and 120, respectively, of gestation in the fetal sheep ovary (McNatty et al., 1995), with the first appearance of antral follicles by day 135 (Lundy et al., 1999). By day 135, the majority of germ cells are in primordial follicles (91%) and the rest are growing follicles (up to early antral follicles). In cattle (280 day gestation), the primordial, primary, and secondary follicles were

observed at days 90, 140, and 210, respectively (Russe and Stolla, 1979); while in humans, primordial and primary follicles appear on days 130 and 170, respectively, of gestation .

Waves of follicular growth occur during the estrous cycle in many species. The waves were first observed from the ovaries of cattle collected at slaughter by Rajakoski (Rajakoski, 1960), where it was noted that the number and diameter of follicles were continuously changing throughout the estrous cycle. Only later by the use of real-time ultrasonography (Sirois and Fortune, 1988) was the sequential development of individual follicles observed in the same animal and follicular waves confirmed. During the cow estrous cycle, the follicular activity was determined to be limited to two or three waves (Ginther et al., 1989). Studies on the presence (Souza et al., 1997) or absence (Schrick et al., 1993) of follicular waves in sheep have since been performed, with controversial results. Evans et al. 2000, provided evidence that the number of follicles within varying size classes fluctuated throughout the estrous cycle, and that the follicular waves were preceded by transient increases in FSH (Evans et al., 2002). Thus it appears that FSH stimulates the emergence of follicular waves in sheep. A possible explanation for the apparent lack of follicular waves in previous studies (Schrick et al., 1993) stems from the idea that sheep have two to four follicular waves during a relatively short estrous cycle, and therefore experience increased turn-over of follicles (Evans et al., 2000). Occurrence of follicular waves is not limited to the estrous cycle. Intermittent waves have also been recorded during pregnancy and the postpartum anestrous period (Ginther et al., 1996) and during the prepubertal period in cattle (Evans et al., 1994).

Reproduction in the Sheep

All animals are affected by environmental conditions in some manner, and usually respond by adapting to these conditions. In sheep, fluctuations in seasonality and photoperiod are the primary environmental changes that affect the reproductive processes. Seasonal breeding is an evolved reproductive strategy. In order to ensure optimum growth and development of offspring, reproductive activity is restricted to the best time of year, promoting births to occur during seasonally favorable conditions (Wayne et al., 1989). Reproductive seasonality in the ewe is characterized by a period of estrus activity, a typical estrous cycle of 17 days, and a period of anestrous in which sexual activity has ceased. The anestrous female has insufficient production of GnRH such that gonadotropin secretion is reduced; therefore on the ovaries of an anestrous

female neither preovulatory follicles nor a functional CL exists. Although follicular growth and regression continues in a manner similar to that observed during the luteal phase of the estrous cycle, follicles retain the ability to produce steroids, and LH continues to be released but at a lower frequency than during cyclicity (Martin, 1984).

The transition from anestrous to cyclic is controlled by photoperiod. Melatonin is the primary substance produced by pinealocytes that respond to photoperiod. It is synthesized and secreted only during the night and acts by stimulating or inhibiting GnRH secretion. The retina perceives light and dark as photoreception. In the anestrous ewe, long photoperiods are perceived and the retinal nerves transmit signals to the suprachiasmatic nucleus (SCN) in the hypothalamus. Excitatory neurons are stimulated to fire to the superior cervical ganglion (SCG) and ultimately travel to the pineal gland where inhibitory neurons continue to fire rendering pinealocytes unable to release melatonin (Bittman and Karsch, 1984). By contrast, during the fall when the dark hours are longer, as for sheep which are considered to be short-day breeders, the excitatory pathways are less active and the pineal gland has less inhibitory effects on the production of melatonin. More melatonin is released from pinealocytes and cyclicity resumes. Following seasonal anestrus, the first ovulation is not preceded by behavioral estrus; ovulation is silent.

Silent ovulation is a result of lower gonadotropin levels during anestrous as the brain is not accustomed to the effects of hormones. Upon the formation of the first CL, which is short-lived due to poor gonadotropin support, progesterone must 'prime' the brain in order that it becomes sensitized to estradiol. The primed brain then responds to estradiol secreted by the follicle in a more sensitized manner than it had in the previous cycle, such that it is now responsive to the physiological effects of estradiol. Therefore when the second follicular phase occurs, the response to estradiol will result in a display of behavioral estrus, as reviewed by Senger, (1999).

Temperature also plays a role in the reproductive patterns of sheep. In the fall, during moderate ambient temperatures, ewes will cycle and conceive to ensure giving birth during the spring. During high ambient temperatures such as the summer months, sheep are seasonally anestrous. This is an adapted survival mechanism since elevated body temperatures in the pregnant female can lead to embryonic death. Sheep breeds originating from temperate climates in mid to higher latitudes experience greater photodependence that controls the timing of

reproductive activity because of the seasonal changes in day length. Tropical breeds tend to be aseasonal, breeding at all times of the year, as a result of decreased variance of day length and availability of food (as reviewed by Rosa & Bryant, 2003).

The seasonality of sheep is a consequence of the endogenous circannual rhythm and environmental cues. In fact, seasonality has been found to persist in the absence of photoperiodic influence. Sheep that were exposed to constant day light or had a pinealectomy or superior cervical ganglionectomy (Lincoln et al., 1989) continued to experience seasonality, but were not synchronous with the natural breeding cycle. Therefore, it is believed that the neuroendocrine activity of seasonality in sheep is produced by an endogenous circannual rhythm and that photoperiod synchronizes this rhythm, rather than creates it (as reviewed by Rosa and Bryant, 2003).

Like the ewe, rams also manifest a response to seasonality. Spermatogenic activity and sexual behavior is continual, however the extent varies with the season. Between August and November, production of spermatozoa and intensity of sexual behavior is dramatically increased (Lincoln and Davidson, 1977). Also, sexual activity is achieved sooner in the ram than the ewe. Because follicles continue to grow in the female during anestrus, which allows ovulation to occur within a few days after the resumption of estrus, the earlier achievement of sexual activity in the ram allows sufficient time for the completion of spermatogenesis, which takes approximately 45 days (Rosa and Bryant, 2003).

The limited period of time that sheep are reproductively functional can significantly limit the ability of a sheep producer to provide market lambs during particular seasons. Fortunately, out-of-season breeding programs exist. The most widely used protocol in Australia and New Zealand is a controlled internal drug releasing device (CIDR; EAZI-BREED CIDR protocol). Recently, as of September 2010, the FDA approved the use of CIDRs in the United States. This progesterone insert is applied for 14 days and acts by simulating luteal function. Thus, when it is removed, the onset of estrus will occur within 1 to 3 days. The use of CIDRs helps producers control seasonality by enabling sheep to continue reproductive activity and lambing all year long.

Ovulation Rate

Factors that Influence Ovulation

Reproductive efficiency is largely dependent on ovulation rate. Hormonal signals that communicate between the pituitary and the ovary as well as local communication within the ovary between follicles and the oocyte determine the efficiency of ovulation rate. Ovulation rates in sheep have been found to be influenced by several factors including hormones, genetics, nutrition, photoperiod, and even presence of a ram.

Genetic differences for ovulation rate have been identified in several breeds of sheep including Booroola, Inverdale, Merino, Cambridge, and Belclare. The growth factor genes, BMP15 and GDF9, are thought to be required for normal ovarian follicular development (Juengel et al., 2002). When expressed individually, BMP15 and GDF9 form non-covalent homodimers; however while co-expressed (BMP15/GDF9) produce a heterodimer (Liao et al., 2003). Expression of both is essential in sheep, whether it be individually as homodimers or as an active heterodimer protein. The interactions of BMP15 and GDF9 with their target cells are believed to be by type I and II serine-threonine kinase receptors; BMP15 binds with activin-like receptor kinase 6 (ALK6) and BMPR-II (Moore et al., 2002), and GDF9 binds to ALK5 and BMPR-II (Vitt et al., 2002). There are currently five known naturally occurring point mutations of the BMP15 gene and one in the GDF9 gene (Galloway et al., 2000; Hanrahan et al., 2003). These point mutations can cause either increased ovulation rate or infertility.

Within the ovary, BMP15 has multiple biological functions that influence ovulation rate, including stimulating granulosa cell mitosis and expressing stem cell factor (SCF) in granulosa cells (Otsuka and Shimasaki, 2002). Also, expression of FSH receptor mRNA was found to be inhibited by BMP15, therefore is thought to be a negative regulator of FSHR and consequently inhibits FSH action (Otsuka et al., 2001). Studies in Inverdale ($FecX^1$) sheep that are heterozygous for the BMP15 mutation ($FecX^1/FecX^+$) exhibit increased ovulation rates (Galloway et al., 2000). A possible explanation for this unique phenotype is that reduced levels of BMP15 result in increased levels of FSHR resulting in the production of more developing healthy follicles (Otsuka et al., 2001). In contrast, Inverdale ewes homozygous for the BMP15 mutation ($FecX^1/FecX^1$) had arrested follicular growth at the primary stage of development, thus, did not ovulate (Galloway et al., 2000). Since BMP15 has been found to stimulate granulosa cell

proliferation and inhibit FSH dependent differentiation, it is proposed that in primary follicles, which are FSH independent, follicles have an absence of BMP15 and granulosa cells cease to proliferate (Otsuka et al., 2001).

Ewes with mutations of the GDF9 gene (FecG^H) are similar to those with BMP15 mutations in that multiple biological functions may occur. Two copies of (FecG^H) results in infertility and in contrast, heterozygotes exhibit increased ovulation rate (Hanrahan et al., 2003). Also, ewes heterozygous for both of the gene mutations (FecX^B/FecG^H) have an even higher ovulation rate (Hanrahan et al., 2003). However, heterozygous mutant ewes seem to secrete similar plasma concentrations of FSH and LH as wild-type ewes; although heterozygous ewes did have an increased responsiveness to LH (with respect to cAMP synthesis) (Shackell et al., 1993). Nevertheless, studies using Booroola gene mutations (Fec^B) indicate slightly elevated levels of FSH and suggest that the gene maybe operating at the level of the pituitary (Webb et al., 1999). The oocyte is most likely the regulator of growth and differentiation of surrounding somatic cells. Oocyte production of BMP15 and GDF9 influences endocrine responsiveness of adjacent cells and therefore ultimately controls the number of mature, ovulatory follicles. Although regulation of BMP15 and GDF9 is interesting, it is not useful in production systems to control ovulation rate.

The most widely recognized method for inducing estrus and increasing ovulation rate in sheep is by the ram effect, or the response of ewes to the unaccustomed presence of rams. During anestrus, ovulation ceases in response to a lack of frequency of pulsatile secretions of LH. The primary way to overcome anestrus in the sheep industry is by using the ram effect. This socially mediated effect can elicit a response from prepubertal, lactational, and seasonally anestrus ewes. The proximity of the males regulates ovarian activity in the female. Rams can induce either an acute or chronic effect on the females. Introduction of rams into an anestrus flock of ewes stimulates an acute response. These are short-term periods of increased high pulse frequency of LH leading to the induction of ovulation, and usually occur within a few minutes (Martin, 1984). Chronic effects, or continuous exposure of a ram, have been found to extend the duration of estrus in ewes (Oldham et al., 1979). However keeping rams in a ewe flock can increase the amount of time required to stimulate ewes to breed since they lack the sudden introduction of rams (Oldham et al., 1979). Within 50 hours of exposure to rams, most ewes are

responsive and elicit an endocrine response that leads to ovulation (Martin et al., 1986). The male effect also has an influence on other species such as cattle, goats, and pigs.

Flushing Effect

The effects of nutrition on reproduction are well known for many species. Nutrient intake affects follicular growth and development and ovulation rate through changes in body weight (BW) and body condition (BC) (Gunn et al., 1979). Increased dietary energy in sheep, or flushing, is a widely recognized phenomenon in which the nutrient intake of ewes is manipulated prior to breeding for the purpose of increasing lambing rates. Although genetic factors determine prolificacy, the degree to which an animal expresses their genetic potential is largely dependent on environmental influences. The observation that nutrition influences lambing rate and that ewes with good body condition give birth to more lambs dates to the 1800's. In the early 1900's, it became apparent that short term improvement of nutrition before and during mating was sufficient to increase lambing rates. The initial concept of 'flushing' included 2 to 3 weeks of an increased plane of nutrition. This high-level diet was found to increase body mass and was used to increase lamb crop (Clark, 1934). The associations between metabolic state and reproduction cause a shift in positive and negative energy balance that ultimately affect body weight and ovulation rate (Scaramuzzi et al., 2006) the result of which is a 'dynamic effect' with increased body weight and body condition.

Positive energy balance occurs when the nutrient requirement of an animal is less than the nutrient intake resulting in the storage of the excess nutrients. Similar to the dynamic effect, the 'static effect' is associated with increased body weight and ovulation rate, however, it is elevated, in a plateau sense, rather than gradually increasing (Scaramuzzi et al., 2006). These effects have been found to have a great influence on reproduction in that the high-level nutrition increases ovulation rate by increasing the number of gonadotropin dependent follicles (Rhind and McNeilly, 1986). Although the persistence of positive energy balance will inevitably increase body weight, folliculogenesis will be stimulated by the effects of nutrient intake before an increase in body weight is detectable. Therefore, the absence of detectable changes in body weight, or the 'acute effect', is typical within the first week of flushing.

Forages, such as alfalfa, are used to reach net energy of maintenance (NEm). The NEm includes NE for basal metabolism which regulates physiological processes such as muscular activity, tissue repair, and maintenance of ionic concentration gradients. Roughage alone is usually not enough to meet the energy needs of most livestock species. Supplementing the hay diet with grain helps to achieve maintenance and many times reaches above maintenance, or net energy of gain (NEg). When sheep receive energy in excess, NEg, the resulting energy can be used for growth, lactation, production of wool, reproductive processes and many other physiological needs. Additional supplementation using components such as soybean oil, distiller's grains, and soybean meal can be used to further increase the energy and protein levels in an animal's diet, as reviewed by the Sheep NRC (1985).

The gonadotropins FSH and LH are key hormones in follicular development however, their role in relation to nutrition is apparently minimal. Concentrations of FSH were not different in cattle fed various levels of hay and concentrate (Gutierrez et al., 1997; Armstrong et al., 2001). The number and size of small follicles recruited increased within the high dietary treatment groups, indicating that the effects of increased dietary intake are most likely independent of circulating concentrations of FSH (Gutierrez et al., 1997). Similar results have been found in sheep (Munoz Gutierrez et al., 2002).

The normal pattern of LH secretion occurs in pulses that stimulate follicular growth however, undernutrition suppresses LH secretion. In one study, ovariectomized cows that were undernourished had similar LH concentrations as those cows that were undernourished but did not have ovariectomies (Richards et al., 1991), supporting the idea that reduced nutrient intake may suppress LH secretion as a direct effect of the hypothalamo-pituitary axis rather than as a mechanism of ovarian hormonal feedback.

In addition to gonadotropins, growth factors also have an influence within the ovary. In ruminants, somatotropin, also referred to as growth hormone (GH), influences the partitioning of nutrients. Somatotropin is a polypeptide hormone secreted from the anterior pituitary and interacts with somatotropin receptors located within the liver and the ovary and therefore may have an effect on reproductive function. Although the receptor for somatotropin is found within many reproductive tissues such as the pituitary, CL, follicle, and placenta, the greatest abundance of the somatotropin receptor lies within the liver where it stimulates the synthesis and release of IGF-I, depending on energy status of the animal, as reviewed by Lucy, 2000. The IGF-I secreted

from the liver acts on many tissues throughout the body, including the ovary. As an animal becomes undernourished, the liver fails to respond to secretion of GH. The result is decreased secretion of liver IGF-I causing a subsequent decrease in available IGF-I to the ovary. GH also increases ovarian IGF-I production, although it is not required for IGF-I expression within the ovary (Hernandez et al., 1989). Somatotropin also has an effect on the ovary by the somatotropic receptors IB and IC located within the CL (Lucy et al., 1993), oocyte and granulosa cells and thus may be involved in folliculogenesis (Kolle et al., 1998).

The ovarian IGF system is also likely involved in the nutritional regulation of follicular growth. Insulin-like growth factor-I is a growth factor with pleiotropic effects; in sheep, IGF-I appears to stimulate either proliferation or differentiation of granulosa cells depending on the developmental stage of the follicle. Insulin-like growth factor-I was found to stimulate proliferation in small (1 to 3 mm) follicles and production of progesterone from granulosa cells in large (> 5 mm) follicles (Monget and Monniaux, 1995). The specific pattern of IGF genes is also dependent on the species. In primates, IGF-I has a large influence on the initiation of growth of primordial follicles. In mice, IGF-I stimulates granulosa cells of secondary follicles to differentiate, whereas in sheep and goats the granulosa cells are still proliferating.

In a study using IGF-I knockout mice, ovaries without the IGF-I gene had significantly reduced levels of FSHR in granulosa cells, demonstrating that IGF-I probably augments FSHR expression (Zhou et al., 1997). Also, it has been found that FSHR expression induced by IGF-I reinforces the subsequent accumulation of FSH induced IGF-I receptor expression (Zhou et al., 1991), creating an intrafollicular positive feedback loop between these hormones through their receptors. Later experiments found that IGF-I alone does not induce FSHR mRNA expression but rather it was the synergistic actions of IGF-I with FSH in a dose- and time- dependent manner (Minegishi et al., 2000). In the same experiment, IGF-I was also found to prolong the stability of FSHR mRNA which could serve to control the physiological changes seen in FSHR expression during the estrous cycle.

The LH receptor is an important marker of FSH induced differentiation of granulosa cells and is crucial for the response of LH to induce ovulation. Therefore, it is worth noting that the synergizing actions of IGF-I and FSH also increase the expression of LH receptor mRNA (Hirakawa et al., 1999).

Follicle Stimulating Hormone Receptor

The FSHR is found within the reproductive tissues of both males and females and was first cloned and sequenced from rat sertoli cells (Sprengel et al., 1990). Reproductive activity of FSHR is controlled in response to secretion of FSH from the anterior pituitary. In females, the FSHR is found on granulosa cells of developing follicles. The binding of FSH to the FSHR stimulates granulosa cell division and steroidogenesis. The first form of the FSHR to be identified was the G protein-coupled form, now referred to as FSHR-1. Although the model of the basic FSHR gene has been demonstrated for years, it does not explain the findings of a truncated carboxy terminus (Yarney et al., 1997b) or the presence of a motif similar to that of a growth factor type receptor (Sairam et al., 1997).

Regulation of transcription is important for a gene so that specific functions may be mediated within a cell at specific times. Regulatory elements are DNA sequences that lie just upstream from the transcription start site and promoter region and function by binding to transcription factors. The upstream region of the ovine FSHR gene has been characterized to some degree (Sairam and Subbarayan, 1997; Xing and Sairam, 2001). The 5' flanking sequence represents a 2.1 kb region upstream of the ATG (+1) translation start site. Deletion studies using fragments of the ovine FSHR promoter region linked to the luciferase gene as a reporter revealed that the strongest promoter was at -200 to +163 relative to the transcription start site (Xing and Sairam, 2001). The transcription start site in sheep is reported to occur -163 bp upstream with reference to ATG (+1) (Sairam and Subbarayan, 1997); which is similar to the rat and the human with transcription occurring within the first 200 bp upstream ATG (+1), as reviewed by Herman and Heckert, 2007. The transcription start site lies within the initiator region (InR). The InR directs transcriptional effects by positioning the RNA polymerase II complex at the promoter (Heckert et al., 1992).

Although the 5'-region appears to lack typical promoter elements, such as TATA and CCAAT, several other transcription factors and regulatory elements have been identified. Most notably, the transcription factors upstream stimulatory factors 1 and 2 (Usf-1 and Usf-2) bind to the E-box and are believed to be the *trans*-acting proteins that induce FSHR transcription (Heckert et al., 1998). Other potential conserved regulatory elements have also been identified (Sairam and Subbarayan, 1997) such as sites that resemble estrogen response element (ERE-

like), CREB response elements (CRE-like), and activator protein-1 (AP-1) binding sites. The CRE-like elements were located at -673 to -666 and -1473 to -1466, relative to the transcription start site, and function as potential binding sites for the transcription factor CREB, which is activated by increased intracellular levels of cAMP. Other various transcription factor motifs were found such as germ cell-specific elements and methylation sites as well as a few that are specific to testis genes (Sairam and Subbarayan, 1997). An E-box element (5'-GGTCACGTGACC-3') was identified and shown to be required for FSHR expression (Heckert et al., 1998).

The glycoprotein hormones are large with respect to other ligands that bind G protein-coupled receptors. The N-terminal extracellular domain of the glycoprotein hormone receptors is correspondingly large as well; the FSHR extracellular domain consists of 348 amino acid residues (Sprengel et al., 1990). Although other regions of FSHR, such as the extracellular loops of the transmembrane domain, may contribute to binding FSH, the extracellular domain is the region that binds to most glycoproteins with high affinity. The extracellular domain is attached to the first transmembrane segment and contains three sites for N-linked glycosylation, although only two of those sites are actually glycosylated in some species (Davis et al., 1995). Removal of one or both of the carbohydrates will continue to allow binding of FSH, however their existence on the receptor is required for proper folding of the FSHR protein into a conformation necessary for high affinity binding (Davis et al., 1995). The extracellular domain is connected to a transmembrane domain by a hinge region. The transmembrane domain contains seven hydrophobic membrane spanning segments. The 264 residue membrane spanning domain comprises seven segments each containing 20-25 hydrophobic amino acids in an α -helix with each transmembrane connected by a loop of 10-22 amino acids (Simoni et al., 1997). The loops alternate between the cytoplasm and the extracellular surfaces of the membrane. The intracellular domain of the receptor is a hydrophilic segment that extends into the cytoplasm of the cell by 63 amino acids.

Variant Forms of the Receptor

The process of alternative splicing cuts or “splices” pre-mRNA at multiple sites forming various sequences from a single gene. The result of alternate splicing, together with translational

modifications, is the formation of a variety of different proteins that serve separate functions within a cell. The first evidence of alternative splicing of the FSHR protein in sheep was found in testicular cDNA (Yarney et al., 1993). Northern blot analysis revealed multiple transcripts of various sizes. Located within chromosome 3 (Montgomery et al., 1995), the gene encoding ovine FSHR is approximately 85,000-100,000 base pairs in length comprising 10 exons and 9 introns (Sairam and Subbarayan, 1997). The FSHR protein produces four known subtypes as a result of alternative splicing: FSHR-1, FSHR-2, FSHR-3, and FSHR-4. Segments of the N-terminus are similar and share the ability to bind FSH while the carboxy termini display differing topographies that affect the functional roles of the variants.

The full length FSHR-1 variant is the most well known and studied form of the FSH receptors. The FSHR-1 receptor gene contains 10 exons. The first 9 exons encode the large extracellular domain and exon 10 encodes the transmembrane domain, including the cytoplasmic and extracellular loops (Sprengel et al., 1990). It is a G protein-coupled receptor (GPCR) that codes for a protein of 695 amino acids; the first 18 residues coding the signal peptide and the mature protein consisting of 675 amino acids (Sprengel et al., 1990). As a part of the subfamily of GPCR, the large extracellular domain of FSHR contains multiple leucine-rich repeats (LRR) in successive α -helices and β -sheets (Smits et al., 2003). Binding of FSH and activation of the receptor are two distinct actions; whereby the extracellular domain constitutes binding that is specific and with high affinity, and the transmembrane domain induces a conformational change that activates a signal transduction through G proteins, as reviewed by Dias et al., 2002. The FSHR-1 is thought to have a steroidogenic and differentiation function (Sairam and Babu, 2007).

The FSHR-2 consists of 652 amino acid residues and has similar sequence homology with FSHR-1 but differs by the cytoplasmic tail, as the protein sequence diverges at residue 625, whereby the last 27 amino acids are a different sequence (Sairam et al., 1996). This is reflected by the truncation of exon 10 linked to an additional novel 11th exon.

The structural conformation of FSHR-3 is quite different from the other receptor forms. The FSHR-3 contains exons 1 to 8, as does FSHR-1, and is spliced at nucleotide 392. Exons 9 and 10 are missing, and the putative exon 11 is added. The FSHR-3 is 259 amino acid residues total (Khan et al., 1993). The amino sequence of FSHR-3 is analogous to FSHR-1 until the point of divergence at 223 residues, at which point the final 36 residues differ (Khan et al., 1993). The

functional role of the FSHR-3 variant is believed to be during proliferation and rapid cell growth (Sairam and Babu, 2007).

The FSHR-4 variant form of the receptor is 117 residues in length and arises from exons 1-4 and therefore makes up only a portion of the extracellular domain (Yarney et al., 1997a). It does not have any transmembrane domains and therefore is referred to as the soluble FSH receptor that acts by binding FSH in the cytoplasm (Sairam and Babu, 2007). The functional role of FSHR-4 in the cell is unknown; however, it is possible that when it binds to FSH it may prevent the binding of FSH to FSHR.

Signal Transduction of Variants

The ability of FSH to act upon specific cells types at different times during development implies the involvement of multiple signal transduction pathways. Occupancy of FSH to the extracellular N-terminal domain of FSHR induces a conformational change that causes the intracellular C-terminal end of the receptor to activate the heterotrimeric G_s protein. There is evidence that the carboxy tail contains a sequence of amino acid residues that connect to and stabilize the G protein so the G protein can bind to a sequence on the third intracellular loop of the transmembrane domain (Ulloa-Aguirre et al., 2007). Activation of G_s causes the dissociation of the G protein into an α -subunit and a β/γ heterodimer subunit. The α -subunit stimulates the effector adenylate cyclase followed by an increase in cAMP production which activates PKA. The activated PKA phosphorylates cyclic AMP response element binding protein (CREB); a transcription factor that binds to a particular sequence of DNA in order to initiate transcription. The cAMP/PKA/CREB pathway prompts transcription, and ultimately expression, of several genes known to be involved in follicular cell processes, such as aromatase and inhibin- α .

The truncation of exon 10 for FSHR-2 is believed to be responsible for the lack of signaling of this receptor. The FSHR-2 is able to bind to FSH with high affinity; however, it is unable to activate the G protein. One possible explanation is believed to be that FSHR-2 is linked to an inhibitory pathway, such that the binding of FSH to FSHR-2 activates inhibitory G proteins (G_i) (Sairam et al., 1996), or that the intracellular domain stabilizes the G protein with the intracellular loops of the receptor. Cotransfection of FSHR-1 and FSHR-2 into FSH treated HEK 293 cells revealed a lack of production of cAMP (Sairam et al., 1996). Therefore, FSHR-2 is known as the “dominant negative” form of the FSHR.

The FSHR-3 acts in a cAMP-independent manner. Because FSHR-3 lacks exon 10, which codes for the heptahelical transmembrane domain, a single transmembrane domain exists and the FSHR-3 protein more closely resembles a growth factor type I receptor (Sairam et al., 1997), which is known to utilize the mitogen-activated protein kinase (MAPK) signaling pathway. It has been demonstrated that stimulation of FSHR-3 by FSH activates this pathway, and more specifically the extracellular-regulated kinase (ERK) signaling cascade (Babu et al., 2000).

Binding of FSH to FSHR-3 activates the effector Ras, which then initiates the ERK cascade. The cascade consists of three kinases: MAPKK, which activates a MAPK/ERK kinase (MEK), which activates MAPK. The Raf isoforms, ERK1/ERK2, are also regulated by Ras. Identification of the concensus sequence PVILSP (PXnS/TP) on the carboxy terminal end within FSHR-3, but not FSHR-1 or FSHR-2, indicates the coupling of FSHR-3 to the MAPK pathway, as this sequence is commonly seen for phosphorylation of MAPK (Gonzalez et al., 1991). The ability of FSH to cause DNA synthesis in granulosa cells during early follicle development at a time when FSH does not cause cAMP synthesis or steroidogenesis leads to the idea that FSHR-3 is the primary variant form used in cell signaling (Sairam and Babu, 2007).

Chapter 2 - THE FLUSHING EFFECT AND EXPRESSION OF FOLLICLE STIMULATING HORMONE RECEPTOR VARIANTS IN SHEEP

Introduction

The practice of flushing has been used for nearly a century by sheep producers because of its effectiveness in increasing lambing rates. Flushing involves increasing dietary energy approximately two weeks prior to exposure to a ram, and positively influences ovulation and lambing rates (Gunn et al., 1979). Although this practice has been successful in increasing lamb crops, the mechanisms responsible have yet to be elucidated. One possible mechanism may be through the insulin-like growth factor system and follicle stimulating hormone receptor (FSHR). Increased levels of nutrition result in greater production of insulin-like growth factor-I (IGF-I) (Thissen et al., 1994). Within the ovary, IGF-I has a synergistic effect with follicle stimulating hormone (FSH) that stimulates production of the follicle stimulating hormone receptor (Minegishi et al., 2000). Dietary intake has been found to regulate mRNA encoding IGF binding proteins (IGFBPs) (Armstrong et al., 2001) and thus to alter function by directly regulating the bioavailability of IGF-I.

Successful reproduction occurs when follicles on the ovary proliferate, differentiate, and then ovulate. Regulation of follicle growth is primarily controlled by FSH and luteinizing hormone (LH). The ability of FSH and LH to exert their specific effects is due to the production of the respective receptors at the appropriate time during follicle development. Luteinizing hormone receptor (LHR) mRNA has been found to increase in granulosa cells with increasing follicle size (Abdennebi et al., 1999). Therefore in the later stages of follicular development LHR approaches maximum expression. In sheep, the FSHR gene is expressed in follicles beginning at the primary stage (Tisdall et al., 1995).

The FSHR was first cloned and described in the rat as a G protein-coupled receptor (Sprengel et al., 1990) and later FSHR mRNA was described in sheep as a 2085 bp open reading frame (Yarney et al., 1993) with 10 exons and 9 introns. Upon binding FSH, FSHR primarily activates the cAMP/PKC/CREB pathway; however, several other signal transduction pathways

are utilized in granulosa cells (GC) during follicular growth and the ability of multiple pathways to operate ensures proper proliferation and differentiation of GC (Ulloa-Aguirre et al., 2007).

Several alternatively spliced FSHR transcripts have been identified in sheep (Khan et al., 1993; Yarney et al., 1993; Sairam et al., 1996) including the previously described G protein-coupled form (referred to as FSHR-1), FSHR-2 (the putative dominant negative receptor) and FSHR-3 (the growth factor type-I receptor). The FSHR-2 has a similar exon structure as FSHR-1 except that exon 10 is truncated and is spliced to an additional exon 11 (Yarney et al., 1997b). The truncation of exon 10 is likely responsible for the loss of intracellular signaling upon binding to the ligand. Although FSHR-2 bound FSH with high affinity, it was unable to activate the G protein itself, and prevented co-transfected FSHR-1 from doing so upon treatment with FSH (Yarney et al., 1997b).

The first 8 exons of FSHR-3 are identical to those for FSHR-1; however, FSHR-3 lacks exons 9 and 10, and exon 8 is spliced directly to exon 11 (Sairam et al., 1997). The FSHR-3 variant is thought to act in a cAMP-independent manner by utilizing the mitogen-activated protein kinase (MAPK) pathway, and more specifically, the extracellular-regulated kinase (ERK) signaling cascade (Babu et al., 2000). Regulation of the ERK cascade is controlled by the GTP binding protein, Raf, and its activation of the effector Ras. Activation of MAPK has also been found to be regulated by an increase of Ca^{2+} into the cell (Babu et al., 2000). Upon binding to FSHR-3, FSH induced a dramatic Ca^{2+} influx into granulosa cells (Babu et al., 2000; Touyz et al., 2000). These studies demonstrate that, following Ca^{2+} influx and subsequent activation of the ERK signaling cascade, granulosa cells proliferate and this series of events is likely the result of FSH action directly through FSHR-3.

The variant forms of the FSHR are possibly expressed differently by granulosa cells at different stages of follicular growth (Sairam and Babu, 2007). Each variant is thought to couple to different signal transduction pathways allowing FSH to affect different stages of follicular growth and development depending on which receptor variant it binds (Ulloa-Aguirre et al., 2007).

Nutrition has a role as a potential manipulator of reproductive processes. Ovulation rate and the number of preovulatory follicles that undergo atresia are likely influenced by supplementation of energy (Munoz Gutierrez et al., 2002). Crude protein (CP) supplementation,

however, was not found to influence follicular dynamics, either by number of follicles (Martin et al., 2010) or by metabolic hormones such as IGF-I (Armstrong et al., 2001).

This study was performed to evaluate the effects of short-term nutritional supplementation on expression of the variant forms of the FSHR. Given that nutritional status can positively affect follicular dynamics (Gutierrez et al., 1997), and that expression of the FSHR variants seems to be stage specific, we postulated that nutritional supplementation (specifically, flushing in sheep) may be linked to the abundance of particular forms of FSHR splice-variants. The expression of FSHR-3 may be increased as a result of greater dietary energy, and expressed in greater abundance during early follicle growth. Expression of the LHR by granulosa cells was used as indicator of developmental status of aspirated follicles. The aim of this study was to determine if various dietary energy and protein supplemented diets influenced the number and sizes of follicles on the ovary, and the expression patterns of the FSHR variant forms in follicles of a predetermined developmental status at a particular stage of development.

Materials and Methods

Animals and Treatment Diets

The use of all animals and procedures was approved by the Kansas State University Institutional Animal Care and Use Committee. Yearling Rambouillet ewes, *Ovis aries*, (n=93) were used for this study. On day 0, live weight and body condition score (scale: 1 to 5) were recorded for each ewe and dietary treatments were initiated. Ewes had free access to water. All ewes were housed at the KSU Beef Cattle Research Center for the duration of treatment (24+ days), prior to surgery. Six treatment diets were designated as follows: 1) PH; 2) PH+B1; 3) PH+C; 4) AH; 5) AH+B2; 6) AH+C (Table 2.1 and 2.2). Ewes were stratified by body weight and randomly assigned to treatment diets as follows: the six lightest ewes were randomly designated between the six treatment diets followed by randomization of the next six lightest and so forth. Two different block supplements or rolled corn were used to provide energy or protein supplementation for each hay-type based diet. One day before surgery, ewes were taken to the surgery suite at KSU's Weber Hall.

Table 2.1 Treatment diets

Treatment	Ingredient	% AsFed (hay)	DMI kg/animal/day
PH	Prairie hay	66.66	2.13
	Alfalfa hay	33.33	1.07
	Total	100.00	3.20
PH+B1	Prairie hay	66.66	1.69
	Alfalfa hay	33.33	0.84
	Block supplement 1	--	0.06
	Total	100.00	2.53
PH+C	Prairie hay	66.66	2.51
	Alfalfa hay	33.33	1.26
	Dry rolled corn	--	0.45
	Total	100.00	3.77
AH	Alfalfa hay	100.00	3.33
	Total	100.00	3.33
AH+B2	Alfalfa hay	100.00	3.94
	Block supplement 2	--	0.22
	Total	100.00	3.94
AH+C	Alfalfa hay	100.00	3.69
	Dry rolled corn	--	0.23
	Total	100.00	3.69

Table 2.2 Block supplements

Ingredient	Supplement 1 (% composition)	Supplement 2 (% composition)
Cooked Mix	72.18	89.22
Soybean oil	4.46	15.23
Beet Molasses ¹	95.54	84.77
Base Mix	27.77	10.86
Vitamin A, 30 KIU/g	1.14	2.80
Trace Minerals	1.82	5.37
Limestone	0.66	20.66
Vitamin D3, 30 KIU/g	0.11	0.58
Monocalcium phosphate, 21% P	11.60	32.27
Vitamin E, 20 KIU/lb	0.78	6.16
Selenium, 0.06%	1.02	11.49
Soybean oil	78.20	4.15
Distiller's grains with solubles	4.67	-----
Urea, 45% N	-----	16.52
Mcal/kg	1.84	2.16
% CP	18.11	12.62
% TDN	73.18	79.17

¹Moisture was evaporated from molasses, before mixing with the base mix

Controlled Internal Drug Releasing Devices (CIDRs)

Each ewe was fitted with a CIDR (EAZI-BREED™ CIDR®; Pfizer, New York, NY) in order to synchronize estrus and follicular development by maintaining a steady release of progesterone, thereby keeping the ewes in a continual luteal phase until removal of the CIDR. The CIDRs were inserted for 14 days in each ewe and surgeries were timed to occur 3.5 to 4 days after CIDR removal. Ewes were divided among 5 different surgery days such that 3 to 4 ewes were taken out of a treatment group 12 to 18 hours prior the assigned surgery day (18 to 20 surgeries per day). In order to allow each ewe precisely 14 days under the control of a CIDR, staggered times for CIDR insertion and removal were used (Figure 2.1)

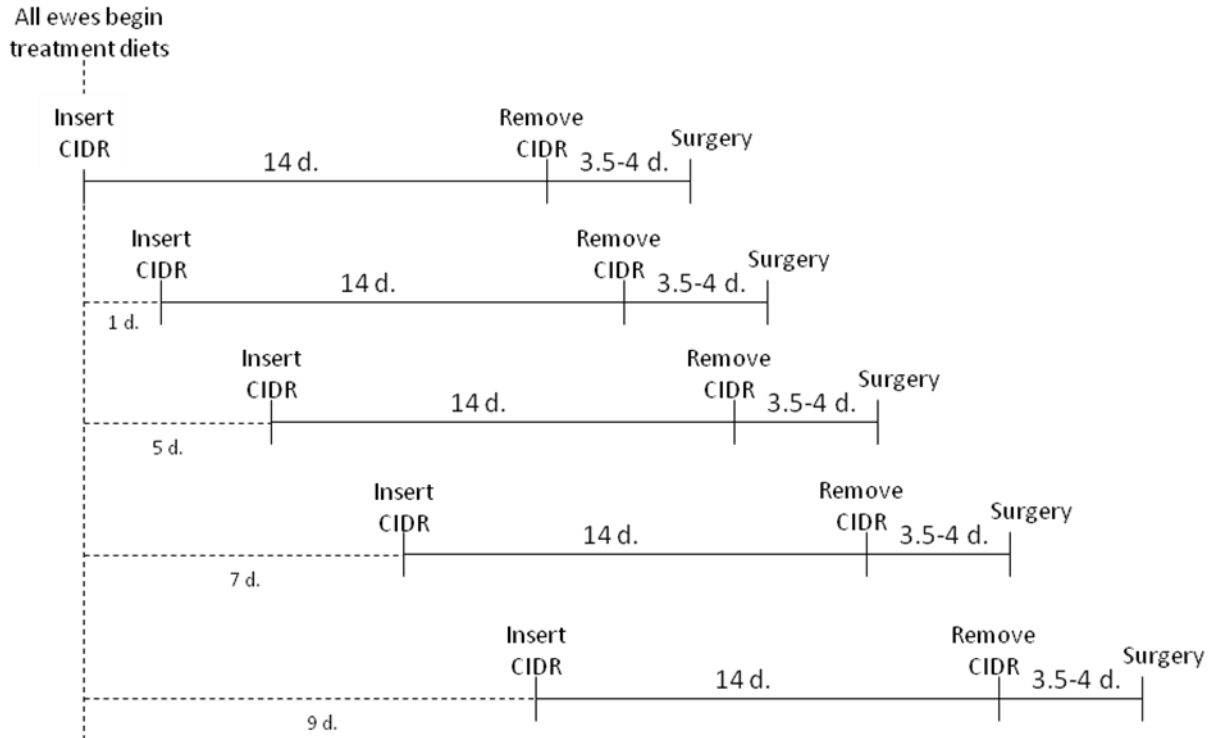


Figure 2.1 CIDR and surgery schedule.

Ewes were divided among 5 different surgery days such that 3 to 4 ewes were taken out of a treatment group upon removal of a CIDR and 3.5 to 4 days prior to surgery.

Mid-Ventral Laparotomy

Ewes were removed from feed and water 24 hours prior to mid-ventral laparotomy. All ewes received procaine penicillin G (22,000 U/kg BW) subcutaneously. A combined anesthetic of Xylazine (0.05 mg/kg bd wt) + Butorphenol (0.025 mg/kg) + Ketamine (1 mg/kg) was given intravenously followed by a subcutaneous injection of lidocaine HCL (2%) on the ventral midline. Each ewe was placed in dorsal recumbency and the limbs were immobilized. The abdomen was clipped and prepared aseptically with Betadine scrub (Purdue Pharma L.C.; Stamford, CT) and alcohol in alternating cycles. Sterile drapes were positioned and a caudal ventral midline incision (about 10 cm) was made for exposure of the ovaries. All follicles of approximately 4 mm or greater were identified and counted, and the number of each size category (medium: 4 to 6 mm; large: > 6 mm) was recorded. Follicles ≥ 4 mm in diameter were aspirated using a 25-gauge needle and 1-cc syringe and the aspirate placed in a sterile 1.5 mL microcentrifuge tube on ice. The linea alba and skin were closed in simple continuous suture

patterns. Ewes from each surgery day were allowed to recover in an enclosed pen for 5 days and fed a maintenance diet of corn silage, ground alfalfa, and distiller's grain.

The follicular fluid was centrifuged for 2 minutes at 2300 x g and the supernatant was aspirated and placed in a 0.5 mL microcentrifuge tube and stored at -20°C for subsequent assay of estrogen. The pellet was covered with 0.5 mL PBS and resuspended, then centrifuged for 1 min at 2300 x g, after which the supernatant was aspirated and discarded. Each granulosa cell pellet was resuspended in 1 mL of Trizol® Reagent (Invitrogen™; Carlsbad, CA) and stored at -80°C for RNA extraction.

RNA Isolation and DNase Treatment

All GC samples in TRIzol® Reagent were allowed to thaw on ice prior to RNA isolation according to the manufacturer's instructions (protocol in Appendix A). Total extracted RNA was DNase treated using a TURBO DNA-free™ kit (Ambion; Austin, TX) according to the manufacturer's instructions (protocol in Appendix A) to remove genomic (complete set of) or cellular (specific for cell function) DNA. All DNase treated RNA was stored at -80°C prior to reverse transcription and polymerase chain reaction.

Reverse Transcription – Polymerase Chain Reaction

Ribonucleic acid was converted to cDNA using the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen™; Carlsbad, CA) according to the manufacturer's instructions (protocol in Appendix A). A master mix was prepared for each sample including 8 µL total RNA as a starting template, 10 µL 2X RT Reaction Mix (including oligo(dT)s and random hexamers), and 2 µL RT Enzyme Mix. Each sample was incubated at 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min, in order to activate the reverse transcriptase, anneal the primers, and elongate the resulting cDNA. Following reverse transcription, samples were placed on ice for at least 1 min. To remove template RNA, *E. coli* RNase H was added (1 uL) to each sample and incubated at 37°C for 20 min. All cDNA was stored at -20°C until use for real-time PCR (qPCR).

Quantitative Real-Time Polymerase Chain Reaction

A master mix was prepared for each primer set using 10 µL Platinum® SYBR® Green qPCR SuperMix-UDG from Invitrogen™ (Carlsbad, CA) per well. Forward and reverse primers were used at 250 nM and ROX Reference Dye at 50 nM. All primers (Table 2.3) were designed

using Express® 3.0 software (Applied Biosystems; Foster City, CA) and synthesized by Applied Biosystems (Carlsbad, CA). Bovine beta-actin was used as the reference gene. Template mixes were prepared by diluting 1 µL cDNA with 4 µL nuclease-free water, to bring the final reaction volume of each well to 20 µL. Real-time PCR was performed using an ABI 7500 Fast Machine (Applied Biosystems) programmed as follows: 50 °C for 2 min hold, 95°C for 2 min hold, 40 cycles of: 95°C for 15 sec, and 60°C for 30 sec.

Table 2.3 Primers used for real-time PCR

Target	Accession No.	Primer Name	Sequences 5' to 3'	Primer (bp)	Amplicon (bp)
Beta-Actin	NM_001009784	oBeta-Actin Forward	GTCATCACCATCGGCAATGA	20	88
		oBeta-Actin Reverse	CGTGAATGCCGCAGGATT	18	
FSHR-1	L07302	oExon 10-1 Forward	CATTCACTGCCCACTTTTCATC	24	84
		oExon 10-1 Reverse	TGAGTGTGTAATTGGAACCATTGGT	25	
FSHR-2	NM_001009289	oExon 10/11-1 Forward	CAGGAACTCCGCAGGGATT	20	72
		oExon 10/11-1 Reverse	TGATTGCAGATGAGCCCAACA	21	
FSHR-3	L12767	oExon 8/11-0 Forward	CAGTAATTTGGAAGAACTGCCTAATG	26	80
		oExon 8/11-0 Reverse	AACAGTGCAGCAGTGGAGACA	21	
LHR	L36329	oLHR Forward	AGATTGCTAAGAAAATGGCAGTCCTCT	28	82
		oLHR Reverse	GCAGCTGAGATGGCAAAGAAAGAGA	25	

Non-Esterified Fatty Acids

Blood samples were taken from each ewe 12 to 18 hours prior to surgery via jugular venipuncture for analysis of non-esterified fatty acid (NEFA) concentrations. Serum was separated by centrifugation at 1,500 x g for 15 min at 4°C, then decanted into a 5 mL polypropylene tube and stored at -20°C until assay. Quantitative non-esterified fatty acid concentration was determined using HR Series NEFA-HR (2) kit (Wako Diagnostics; Richmond, VA). Samples were assayed in duplicate. Inter-assay CV = 12.7%; intra-assay CV = 5.9 %.

Follicular Fluid Estradiol Assay

Follicular fluid was aspirated at surgery and placed in a sterile 1.5 mL microcentrifuge tube on ice. The follicular fluid was centrifuged for 2 minutes at 2,300 x g and the supernatant was aspirated and placed in a 0.5 mL microcentrifuge tube and stored at -20°C. Estradiol radioimmunoassay (RIA) was performed using an estradiol double antibody kit (Siemens Medical

Solutions; Malvern, PA) according to the manufacturer's instructions (protocol in Appendix A). Inter-assay CV = 10.2%; intra-assay CV = 10.0%.

Breeding Period

Approximately 6 months after surgery and before the fall breeding period, ewes were placed in the same treatment diet for 4 weeks (rather than 2, in order to attempt to increase intake of the supplement blocks during the flushing period). Following the 4-week flushing period, all ewes were group-housed, placed on a maintenance diet, and were exposed to 3 Suffolk rams of proven fertility. As ewes lambled in the spring, lamb weights and number of lambs born per ewe were recorded within 12 hours of birth.

Statistical Analysis

Data were analyzed with SAS software (SAS Institute; Cary, NC). The analysis of treatment diets on all covariates used the mixed procedure (PROC MIXED) with a diagonal covariate structure because of the assumption that all observations were independent. Fixed effects included treatment diet and follicle size. No random effects were used in the model. The least square means of all genes and the number of follicles included an interaction term for diet and follicle size such that size of the follicles could be accounted for within each treatment.

Relative expression ($2.0^{-\Delta C_t} \times 1000$) values to bovine β -actin were used to analyze real-time PCR results of gene expression prior to SAS analysis. The effect of treatment diet on relative gene expression in combination with follicle size (M or L) was determined using the mixed procedure. Overall gene expression was evaluated using PROC UNIVARIATE to determine differences between the genes as dependent variables. For all analyses, the level of significance was set at $P \leq 0.01$ (rather than $P \leq 0.05$) to account for the assumption that all ewes had the same intake and thus each ewe could serve as an experimental unit.

Results

Body Condition Score

Initial ewe body condition scores (BCS) were determined at the initiation of treatment diets (day 0). No statistical differences ($P = 0.04$) for BCS between the treatment diets were detected (Figure 2.2). Mean body condition of the ewes was 3.0.

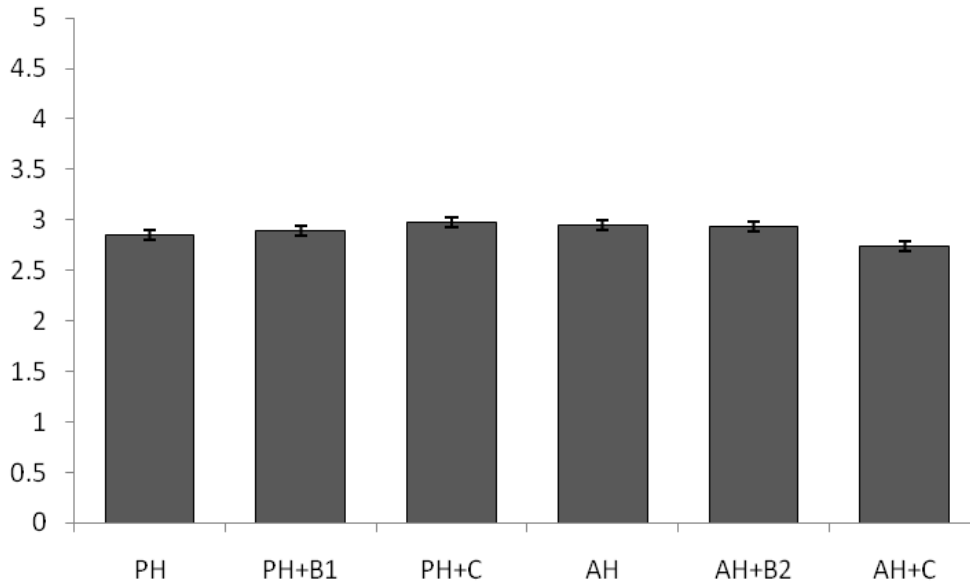


Figure 2.2 Initial body condition scores among treatment diets.

Initial body condition scores (BCS) were recorded for each ewe prior to assigning treatment groups. Yearling Rambouillet ewes ($n=93$) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). CIDRs were inserted at the time of initiation of dietary treatments (day 0). Data are presented as least square means of $BCS \pm SEM$.

Effect of Diet on Live weight Change

Changes in ewe body weights (weight after at least 2 weeks on diet – initial weight) were different ($P < 0.01$) between diets (Figure 2.3). Ewes consuming the prairie hay based diet (PH = 1.36 ± 0.51 kg) or prairie hay plus block-1 (PH+B1 = 0.28 ± 0.49 kg) had reduced body weights at week 2 compared to ewes fed alfalfa hay based diets (AH = 5.12 ± 0.47 kg; AH+B2 = 4.05 ± 0.49 kg; AH+C = 5.48 ± 0.51 kg).

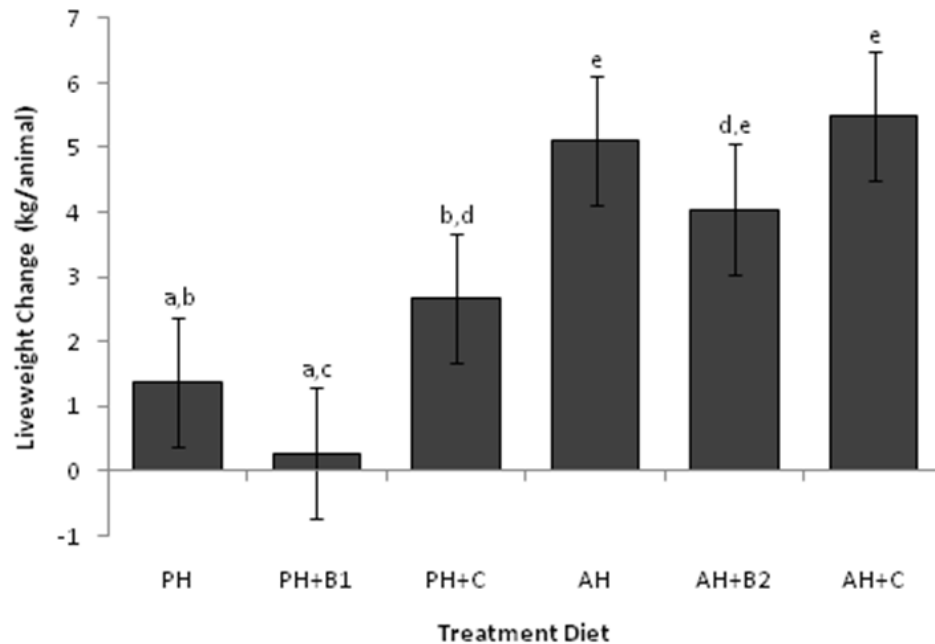


Figure 2.3 Effect of dietary treatments on live weight change.

Yearling Rambouillet ewes (n=93) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). CIDRs were inserted at the time of initiation of dietary treatments (day 0), and ewes were individually weighed. Data are presented as least square means of live weight change (weight after at least 2 weeks on diet – weight approximately 14 hours prior to surgery; weight in kg) \pm SEM. Means with differing superscripts are different ($P \leq 0.01$).

Non-Esterified Fatty Acids

Non-esterified fatty acid (NEFA) values in serum were not different ($P = 0.26$) at 12 hours prior to surgery for ewes within or between each of the treatment diets.

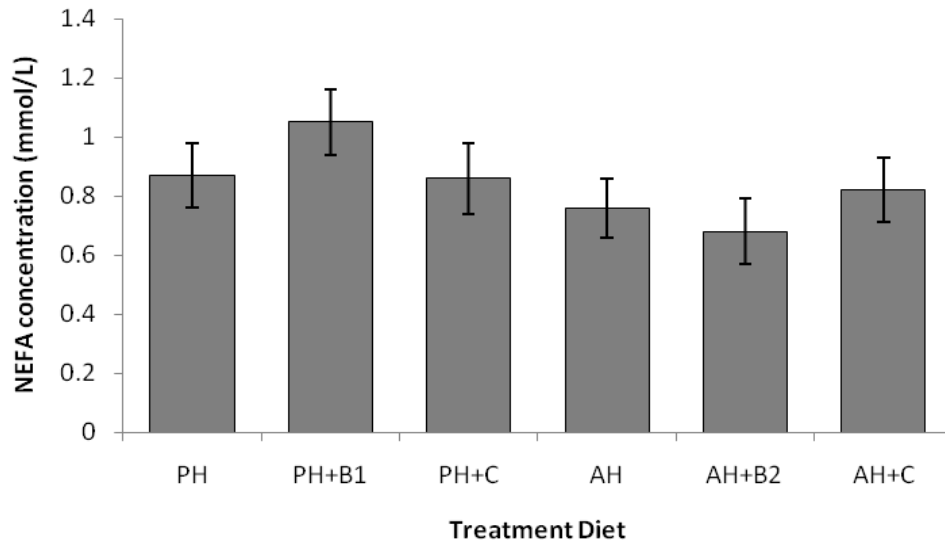


Figure 2.4 Effect of dietary treatments on non-esterified fatty acids (NEFA).

Yearling Rambouillet ewes (n=93) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). CIDRs were inserted at the time of initiation of dietary treatments (day 0). Data are presented as least square means of NEFA (mmol/L) \pm SEM. NEFA was assessed by taking jugular blood samples approximately 12 hours prior to surgery.

Number of Medium and Large Follicles

All follicles of greater than approximately 4 mm were measured at the time of surgery and classified as either medium (M; 4 to 6 mm) or large (L; > 6 mm). Numerically, ewes fed prairie hay appeared to have greater numbers of medium follicles (1.00 ± 0.19) than large follicles (0.41 ± 0.19 ; $P = 0.29$; LSM, $P = 0.03$; Fig 2.5). The effect of diet ($P = 0.45$) or the effect of follicle size ($P = 0.99$) on the number of follicles was not significant.

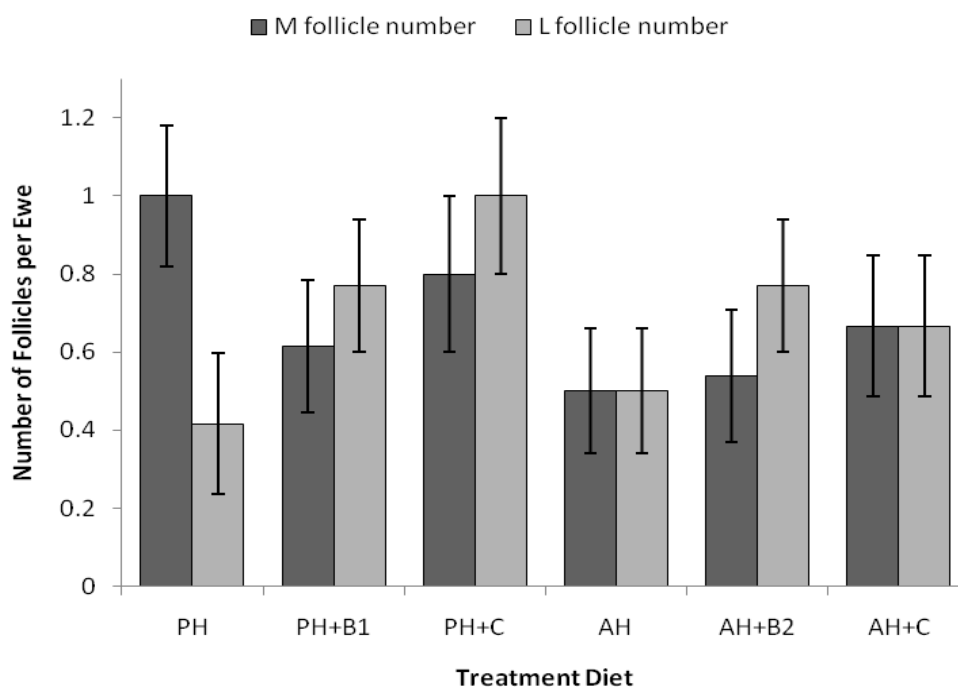


Figure 2.5 Effect of dietary treatments on number of medium and large follicles.

Yearling Rambouillet ewes (n=93) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). All medium and large follicles were measured and counted at the time of surgery. Follicles 4-6 mm in diameter were classified as medium (M) and follicles > 6 mm were classified as large (L). Data are presented as least square means of the diets \pm SEM.

Gene Expression

FSHR-1

Expression of FSHR-1 was greater ($P < 0.01$) in large follicles than in medium follicles but was not different ($P = 0.13$) between treatment diets. Ewes that consumed alfalfa hay tended ($P = 0.05$; LSM, $P < 0.01$) to have the greatest expression of FSHR-1 (43.32 ± 8.08 relative to β -actin) in large follicles (Fig. 2.7). Relative abundance of FSHR-1 (Fig. 2.6) in medium follicles was consistent across all diet types with no significant differences across treatments.

FSHR-2

Numerically, ewes fed alfalfa hay plus rolled corn appeared to have greater expression (LSM for AH+C in L follicles, $P < 0.01$) of FSHR-2 in large follicles (Fig. 2.7) as compared to ewes fed the other diets. However, overall abundance of FSHR-2 was not different between large and medium follicles ($P = 0.26$) or between the treatment diets ($P = 0.24$).

FSHR-3

The mean FSHR-3 variant expression relative to β -actin was greater than FSHR-1 or FSHR-2 ($P < 0.01$; Fig. 2.8). The FSHR-3 variant tended ($P = 0.07$; LSM, $P < 0.01$) to be different between large and medium follicles. In medium follicles, FSHR-3 was expressed at similar levels across all treatments (Fig 2.6). The greatest abundance of FSHR-3 in large follicles (Fig 2.7) tended ($P = 0.09$) to be for ewes consuming prairie hay and prairie hay plus corn, and tended ($P = 0.09$) to be lower for the ewes fed alfalfa hay alone and alfalfa hay plus block-2.

LHR

Expression of LHR relative to β -actin was not different ($P = 0.42$) between treatment diets or between medium and large follicles ($P = 0.79$).

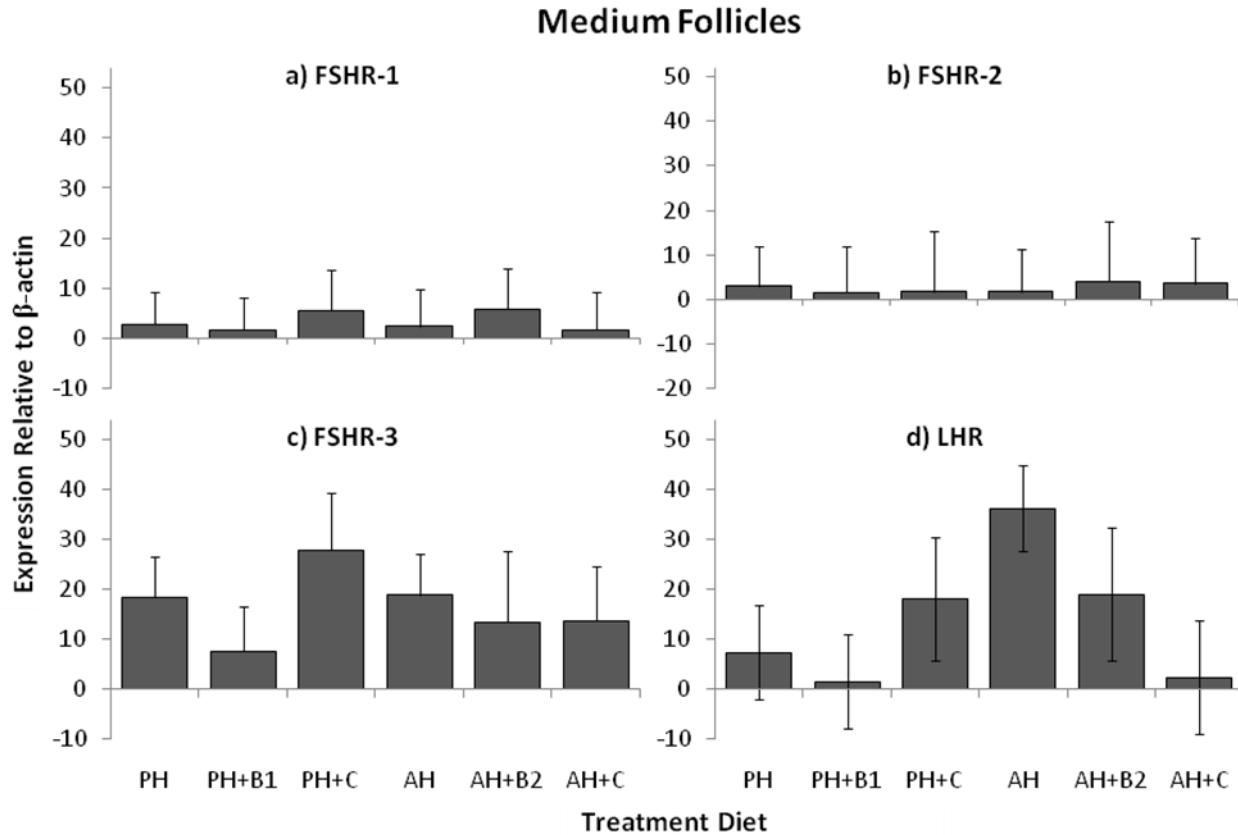


Figure 2.6 Effect of dietary treatments on FSHR variants and LH receptors in medium (4 to 6 mm) follicles.

Yearling Rambouillet ewes (n=93) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). Follicular fluid and granulosa cells were collected at surgery. Total RNA was extracted and gene expression was evaluated by reverse-transcription and real-time PCR using primers specific to each of the variant forms of the FSHR or to the LHR. Results are expressed in arbitrary units relative to β -actin ($2.0^{-\Delta C_t} \times 1000$) \pm SEM. The number of follicles (n) used in each treatment diet is as follows, and is the same for each gene: PH, n = 10; PH+B1, n = 6; PH+C, n = 6; AH, n = 7; AH+B2, n = 5; AH+C, n = 7.

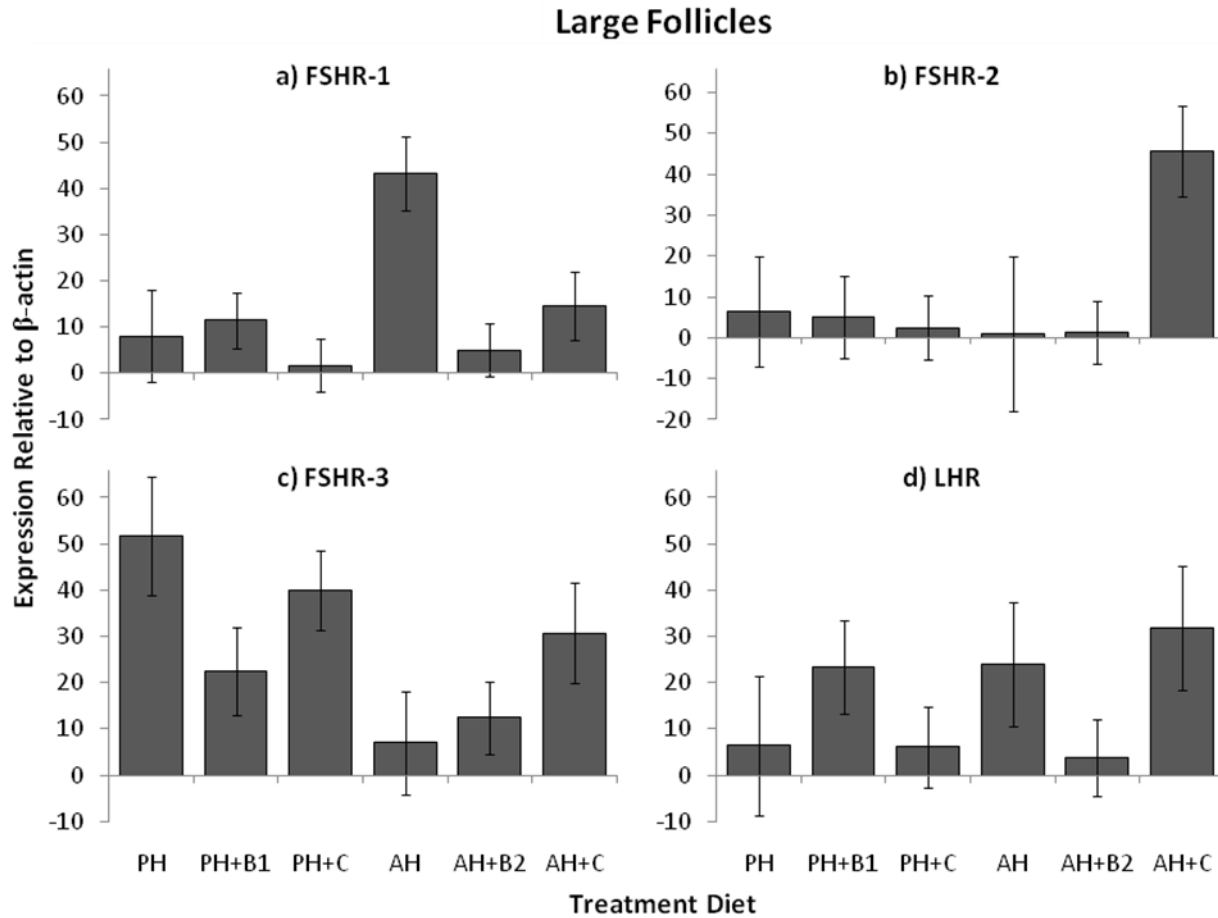


Figure 2.7 Effect of dietary treatments on FSHR variants and LH receptors in large (> 6 mm) follicles.

Yearling Rambouillet ewes (n=93) were fed one of the following treatment diets for at least 14 days: prairie hay (PH), prairie hay plus block supplement-1 (PH+B1), prairie hay plus rolled corn (PH+C), alfalfa hay (AH), alfalfa hay plus block supplement-2 (AH+B2), or alfalfa hay plus rolled corn (AH+C). Follicular fluid and granulosa cells were collected at surgery. Total RNA was extracted and gene expression was evaluated by reverse-transcription and real-time PCR using primers specific to each of the variant forms of the FSHR or to the LHR. Results are expressed in arbitrary units relative to β -actin ($2.0^{-\Delta C_t} \times 1000$) \pm SEM. The number of follicles (n) used in each treatment diet is as follows, and is the same for each gene: PH, n = 4; PH+B1, n = 10; PH+C, n = 8; AH, n = 7; AH+B2, n = 10; AH+C, n = 8.

Overall Gene Expression of Large and Medium Follicles

Overall expression relative to β -actin of FSHR-3, regardless of treatment diet, was greater ($P < 0.01$) than FSHR-1 or FSHR-2 (Fig 2.9). Expression of LHR tended to be different than expression of FSHR-1 ($P = 0.05$) and FSHR-2 ($P = 0.02$) but was not different than expression of FSHR-3.

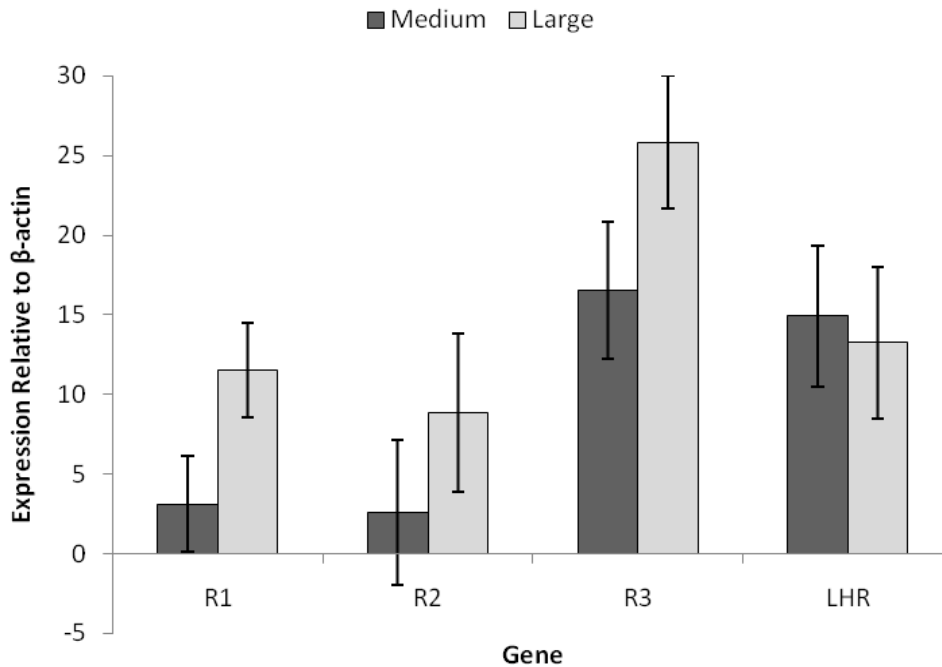


Figure 2.8 Relative expression of variant forms of FSH receptor and LH receptor in medium and large follicles.

Follicular fluid and granulosa cells were collected from follicles 4 to 6 mm in diameter (M) or from follicles greater than 6 mm (L) at the time of surgery. Total RNA was extracted and gene expression was evaluated by reverse-transcription and real-time PCR using primers specific to each of the variant forms of the FSHR or to the LHR. Results are expressed in arbitrary units relative to β -actin ($2.0^{-\Delta C_t} \times 1000$) \pm SEM.

Estradiol Concentrations

The average concentration of estradiol from follicular fluid was not different ($P = 0.56$) for any dietary treatment or for medium or large follicles ($P = 0.62$; Fig 2.10).

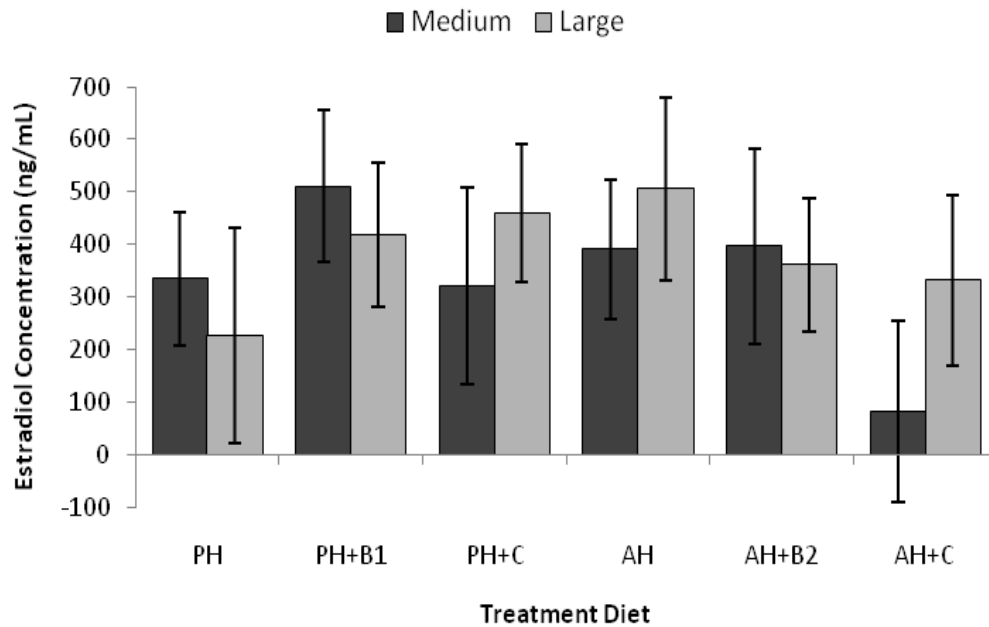


Figure 2.9 Concentration of estradiol in follicular fluid.

Follicular fluid and granulosa cells were aspirated at surgery, 3.5 to 4 days after CIDR removal. Samples were centrifuged and supernatant follicular fluid was removed for estradiol radioimmunoassay. Data are presented as least square means of follicular fluid estradiol concentrations (ng/mL) from each of the diets \pm SEM.

Lambing Rate and Birth Weights

The overall lambing rate was 1.16. The interaction between number of lambs born and lamb birth weights was significant ($P < 0.01$). Lamb birth weights between treatment diets were not significantly different between treatments ($P = 0.37$). The numerically highest birth weights occurred in the prairie hay plus corn treatment (5.52 ± 0.93 kg) and the numerically lowest birth weights occurred in the prairie hay plus block treatment (3.08 ± 0.93 kg). Overall mean birth weight was 5.04 kg.

Table 2.4 Lambing information

Diet	*No. of Ewes	*No. Lambs Born	Lambing Rate	Birth Weight (kg)
Total	54	63	1.16	5.04
PH	9	11	1.22	4.73
PH+B1	10	12	1.20	3.08
PH+C	9	10	1.11	5.52
AH	6	6	1.00	4.90
AH+B2	9	11	1.22	4.30
AH+C	7	9	1.28	5.15

*Unable to obtain tag numbers from four ewes. Four lambs were born from the non-tagged ewes and were therefore unable to be designated into the proper treatment group.

Discussion

The process of flushing involves increasing dietary energy in sheep prior to breeding, and results in increased ovulation (and subsequent lambing) rates. Although flushing has been used as a production practice for many years, the precise physiological mechanisms whereby increased dietary energy causes an increased ovulation rate are unknown. This study was undertaken to determine if increased ovulation in response to flushing involves altered expression of variant forms of the FSH receptor. In order to examine FSHR expression in response to nutritional flushing, six treatment diets varying in dietary energy and protein were fed to ewes and the quantity and type of FSHR variants produced by the resulting follicles was subsequently evaluated. Base diets were chosen to closely approximate conditions faced by sheep producers. Thus, prairie hay was chosen to mimic fall pasture conditions in the Great Plains of the United States, and alfalfa hay to simulate the lush pastures typically found in other common sheep-producing regions of the world. Both prairie hay and alfalfa hay were supplemented with nothing, a commercially available block supplement or rolled corn in order to create a range of dietary energy conditions that would subsequently allow comparisons to be made regarding the role of energy on FSH receptor expression and on follicular development.

In order to determine whether sufficient energy was provided to ultimately allow a flushing response to occur, NEFA and live weight were assessed. The NEFA values indicated that ewes fed all treatment diets had similar levels of utilization of fatty acids. Had there been sufficient available energy (enough to increase ovulation rate), the ewes fed the prairie hay based diets would be expected to have had increased NEFA concentrations from the increased catabolism of adipose tissue as a result of the intake of lower energy-value hay. As in another study (Faulconnier et al., 2007), blood samples for the present experiment were collected for NEFA analysis once during the trial, however they were not collected after a fasting period and thus may not have accurately reflected fat catabolism in the ewes.

Lambing rates did not change in response to the six different diets fed in the present study, and thus it is likely that ovulation rate (although not directly assessed) was also unchanged. This finding is in agreement with Thomas et al. (1987) who found that ewes supplemented with corn 12 days prior to fall breeding had increased live weight, body condition and ovulation rate as compared to non-supplement ewes; however, there was no difference in

subsequent lambing rate. Ewes in the present study were assessed a mean initial body condition of 3.0 (on a scale of 1 to 5) and ewes in good body condition (2.5 to 3.0) have not been found to have any significant increases in ovulation rate with pre-mating flushing (Russel et al., 1969; Gunn et al., 1984). In contrast, ewes in moderate to poor body condition (≤ 2.0) have been found to have increased ovulation rates in response to nutritional flushing that increased their body condition score at least 0.5 to 1.0 as compared to ewes maintained at a moderately-good condition of 2.5 (Gunn et al., 1984). Thus, it is possible that ovulation and lambing rates did not increase in this study due to ewes being over-conditioned at the start of the flushing period, a situation that is likely due to the insulin-like growth factor-1 (IGF-1) system.

Insulin-like growth factor-I is secreted from the liver and acts on many tissues throughout the body, including the ovary. As an animal begins to transition to a positive energy balance, increased secretion of liver IGF-I causes an increase in available IGF-I to the ovary in addition to increased local production of ovarian IGF-I (Lucy, 2000). Armstrong et al. (2001) reported a direct effect of dietary intake on the IGF system, specifically, a decrease of mRNA for the IGF binding proteins 2 and 4 (IGFBP-2 and -4) in small antral follicles of cattle. These binding proteins act by suppressing the actions of IGF-I and IGF-II, and thus decreased expression of IGFBP-2 and -4 should allow greater activity of IGF-I and II by allowing more intrafollicular IGF to be available in times of increasing energy availability.

The ovarian IGF system contributes to the nutritional regulation of follicular growth and in sheep appears to stimulate either proliferation or differentiation of granulosa cells depending on the developmental stage of the follicle (Monget and Monniaux, 1995). These investigators found that IGF-I stimulated proliferation of granulosa cells in small follicles (1 to 3 mm) and promoted steroidogenesis in large follicles (> 5 mm). All follicles collected in the current study were between 4 and 8 mm (rather than < 3 mm), and thus were in similar developmental stages in regards to potential IGF-1 activity.

There is also a possible association between IGF-I and FSHR. In a study using IGF-I knockout mice, ovaries without the IGF-I gene had significantly reduced levels of FSHR in granulosa cells, demonstrating that IGF-I probably augments FSHR expression (Zhou et al., 1997). A critical level of FSHR is required for follicular maturation and steroidogenesis, therefore ovarian IGF-I may enhance FSH responsiveness by enhancing FSHR expression. Insulin like growth factor-I alone does not induce FSHR mRNA expression but rather FSH and

IGF-I appear to exert synergistic effects on FSHR production in a dose- and time-dependent manner (Minegishi et al., 2000). Insulin like growth factor-I also was found to prolong the stability of FSHR mRNA, which could serve to control the physiological changes caused by altered FSHR expression during the estrous cycle (Minegishi et al., 2000).

Although these and other results provide evidence that increased dietary nutrients likely increase FSH receptor production, possibly through increased bioavailability of IGF-I, it is unknown which variant form of the FSHR may be involved. Recent studies have revealed possible altered patterns of expression of FSHR variants at different physiological stages of follicular development in cattle (Rozell et al., 2005) and in sheep (Sullivan et al., 2009). The ability of FSH to act upon specific cell types at different times during development may require the involvement of multiple FSH receptor types and coupling to different signal transduction pathways. Previous studies have illustrated that FSHR transcripts that include exon 11 (FSHR-2 or FSHR-3) are more highly expressed in small follicles whereas the transcripts containing exon 10 (FSHR-1) are expressed in greater abundance in large follicles in sheep (Sullivan et al., 2009) and possibly in cattle (Rozell et al., 2005).

In this study, follicles were collected 3.5 to 4.0 days after CIDR removal. In sheep, follicles have been found to require approximately 5 days to increase from 0.8 mm to 2.5 mm (Downing and Scaramuzzi, 1991). Once follicles reach 2.5 mm, rapid growth takes place within the next 2 days and this is when the greatest overall quantity of FSHR can be found (Tisdall et al., 1995). Upon antrum formation, FSH receptors can be detected on granulosa cells. As FSH concentrations become suppressed during later stages of follicle growth, LH receptors, originally only located on thecal cells, also become present on granulosa cells of the largest follicles. Therefore, developmentally, we wanted to capture follicles that had reached full gonadotropin responsiveness by obtaining a maximum number of FSH receptors, but were likely not yet dominant (LH-dependent), as well as follicles that had just been selected (FSH-dependent). Thus, we could assess both quantity and type of FSHR variants at a specific stage of FSH-dependent growth and any differences in FSHR expression should be due to differences in available energy and not to dramatic developmental differences between follicles.

If FSHR-3 is primarily involved in early follicular growth (as suggested by Sairam and Babu, 2007) it would be expected to decrease by the developmental stage observed in this study. The FSHR-3, however, was not decreased for any treatment diet. The finding that FSHR-3

continued to be expressed in great amounts during a stage of development when FSHR-1 is typically expressed in large amounts implies that FSHR-3 could be constitutively activated in later stages of follicular development and may not be as sensitive to nutritional or metabolic changes. The FSHR-3 variant also may be important for FSH to continue utilizing the MAPK pathway beyond granulosa cell proliferation and differentiation, which has been found to create a more efficient signal transduction than the activation of the MAPK pathway through FSHR-1 (Babu et al., 2000).

Follicles were aspirated at surgery 4 days after CIDR removal. Sheep have two to four follicular waves during the estrous cycle, and therefore may experience increased turn-over of follicles (Evans et al., 2000) as compared to cattle which may have one to three follicular waves (Ginther et al., 1989). Upon removal of a CIDR, estrus in sheep will occur within approximately 24 to 72 hours (mean = 48 hours) during a 17-day estrous cycle. After the onset of estrus, ovulation in the ewe will take place within approximately 30 hours (Quirke et al., 1981). Our goal was to aspirate follicles prior to the first ovulation after removal of the CIDR to ensure most follicles aspirated were from the same wave. Collection of follicles from a different wave, say the first follicular wave after ovulation, could result in a weak form of dominance of the largest follicles (Evans, 2003) which may alter FSH receptor expression. Observation from the present study, however, showed that expression of FSHR-3 was not different between medium and large follicles, which may be a result of the similar developmental stages of the follicles regardless of size, and/or inaccuracies in measurement of follicle sizes. Further, production of estradiol was not different between the treatment diets or follicle sizes nor was expression of LHR different and these results also support the idea that follicles were developmentally similar. In contrast, expression of FSHR-1 increased as follicle size increased. This is in agreement to previous reports in which it was found that the primary functional expression of FSHR-1 takes place closer to achievement of dominance when granulosa cells have differentiated and are steroidogenically active (Sairam and Babu, 2007; Tisdall et al., 1995).

The number of follicles within the ovary is generally not increased by flushing, per se, but rather flushing increases ovulation rate by preventing atresia of growing follicles (Haresign, 1981; Munoz Gutierrez et al., 2002). Similarly, in this study ewes fed alfalfa hay had numerically similar numbers of medium and large follicles and increased expression of FSHR-1 from large follicles (as compared to medium follicles). Thus, it is possible that increased follicle

rescue or prevention of atresia that occurs during flushing, may involve FSHR-1. Considering the possible roles of ovarian IGF-I (as a stimulator of proliferation and differentiation and an augmenter of FSHR expression) and the expression pattern of FSHR-1 (steroidogenesis and possible follicle rescue in response to FSH), it seems plausible that IGF-I may have the greatest influence on FSHR-1 as compared to other FSHR variants. However, it was not possible (due to limited sample amounts and the priority of assessing expression of all FSHR variant types) to examine IGF-I expression or concentration in follicular fluid of follicles collected in this study.

Although follicles were categorized as either medium or large based on apparent size, they were likely in similar developmental stages with regards to receptor expression due to the timing of CIDR removal, and the variation in the timing of ovulation (24 to 72 hours) after CIDR removal. This becomes evident when observing the patterns of LHR expression between the two sizes of follicles; there were no significant differences in LHR expression between medium and large follicles, as would be expected if large follicles were truly at a LH- and not FSH-dependent stage of development.

In a study by Sairam et al. (1996), human embryonic kidney (HEK-293) cells co-transfected with both FSHR-1 and FSHR-2 failed to activate adenylate cyclase in response to treatment with FSH, in contrast to HEK-293 cells transfected only with FSHR-1. The FSHR-2 variant has thusly been termed the “dominant negative” form of the FSH receptor and somehow functions to reduce FSH responsiveness (Sairam et al., 1996). Expression of FSHR-2 during follicular development could therefore cause follicles to lose FSH responsiveness, a scenario that, if it occurred prior to attainment of LH receptors on granulosa cells, could accelerate the atresia process. Because there are other well-established factors that reduce the production of FSH during later stages of follicle development such as inhibin and estradiol, the FSHR-2 receptor may serve a relatively minor role. The present study was designed to select healthy (FSH responsive) follicles in which the role of FSHR-2 would potentially be minimal. If FSHR-2 does serve to decrease FSH responsiveness in sheep granulosa cells, then it would be expected to be expressed in greater amounts later in development (beyond when follicles were selected in this study). Therefore the reduced amounts of FSHR-2 across treatments seem logical; however, it is only apparent from this experiment that FSHR-2 did not seem to be affected by different dietary treatments.

It was hypothesized that adding supplemental energy would increase ovulation rate through altered FSH responsiveness of healthy follicles via increasing expression of FSH receptors (FSHR-1 and/or FSHR-3), yet no changes in lambing rates were observed in this study. The proposed mechanism for the flushing response is that increased levels of energy intake will increase plasma IGF-I concentrations (Armstrong et al., 2001) and nutritional supplementation will increase ovulation rate by decreasing the number of large preovulatory follicles that undergo atresia (Munoz Gutierrez et al., 2002). The effects on follicular development, however, are more likely to be a result of a metabolic signaling mechanism whereby the environment for growth becomes more favorable, rather than by a quantitative supply of nutrients (Scaramuzzi et al., 2006). Therefore a possible mechanism for flushing may be through stimulating IGF-I by energy supplement, which supports the growth and differentiation of large, healthy follicles and thereby increases ovulation rate and ultimately lambing rate.

In summary, a flushing response was not achieved, as there were no differences in the number of follicles or lambing rate. Expression of the FSHR-1 variant form was not different between ewes fed the treatment diets but was different between medium and large follicles and it may be possible that increased expression of FSHR-1 as follicles proceed through development allows them to avoid atresia. Because the mechanism of flushing is known to reduce the number of follicles undergoing atresia, it is possible that FSHR-1 is the primary form of the FSH receptor involved in the flushing response. Overall expression of FSHR-3 was greater than the other FSHR variant forms and therefore, the FSHR-3 variant may be essential throughout antral follicular development. From our results and others, it appears likely that involvement of the IGF-I/FSH/FSHR system in the ovary is dependent on metabolic changes that occur only when ewes transition from a negative to a positive energy balance, and not simply in response to an increase in energy available. Further research will be necessary to elucidate the role of IGF-I in FSH receptor variant expression. In addition, further studies in which ewes begin the flushing period at lower body condition scores (mean 2.0 to 2.5), and in which energy differences between treatment diets are more pronounced, would be very helpful in understanding the role of FSH receptor variants on the increased ovulation rate seen after nutritional flushing.

Chapter 3 - References

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Appendix A - Protocols

Aspiration Protocol

- Consider each follicle from each ovary on a ewe one sample.
- Measure the largest follicles and record on ovary chart.
- Aspirate follicular fluid from each follicle using a 1 cc syringe and a 22 gauge needle.
- Place follicular fluid from a follicle into a 1.5 mL microcentrifuge tube.
- Place tube in microcentrifuge.
- Centrifuge 2 minutes at 2300 rcf (xg)
- Aspirate supernatant and place in clean .5 mL microcentrifuge tube – freeze in -20°C
- Cover each cell pellet with .5 mL PBS.
- Resuspend by finger-flicking or gentle pipetting.
- Centrifuge 1 minute at 2300 rcf (xg)
- Aspirate and discard PBS (leave a small amount to avoid disturbing the cell pellet).
- Add 1 mL Trizol to each sample.
- Resuspend the cell pellet by finger-flicking.
- Freeze in -80°C until RNA extraction.

RNA Extraction from TriZol samples

- Thaw Trizol samples (containing cell lysates in 1 mL Trizol) at room temp for 3-5 minutes on ice.
- While these are thawing, measure approximately 1 mL of Chloroform:Isoamylalcohol (24:1) and 1 mL of 2-Propanol (Isopropanol) into two separate mixing tubes and leave (capped) in the hood.
- Add 200 μ L Chloroform:Isoamylalcohol to each Trizol sample (put excess ChCl_3 into Hazardous Waste container).
- Mix vigorously by hand for 15 seconds.
- Incubate 3 minutes at room temp (mix by hand several times while incubating).
- Take capped samples to the microcentrifuge.
- Centrifuge for 15 minutes at full speed.
- Label new tubes while this is spinning.
- Take samples back to the hood and aspirate aqueous fraction (clear liquid at the top) being careful to avoid the white protein layer).
- Transfer to new tube – try to get about 500 μ L of aqueous fraction removed.
- Cap the old tubes and discard the remaining pink phase into the Haz. Waste bag in the hood.
- Add 500 μ L Isopropanol to each sample (put excess in Haz. Waste container).
- Incubate 10 minutes at room temp.
- Take samples to microcentrifuge.
- Centrifuge for 10 minutes at full speed (16000 \times g).
- Take samples back to the hood and carefully remove the supernatant liquid and discard (into Hazardous Waste bag).
- Add 1 mL 75% EtOH (Ethanol and water) to the precipitate and vortex.
- Centrifuge for 5 minutes at full speed (align all tubes the same way, so you know where your pellet should form).
- Preheat ~500 μ L ddH₂O at 65°C (the water bath).
- Aspirate EtOH carefully, keeping away from pellet.
- Air dry pellets for 5-10 minutes.

- Add 15 μL preheated ddH₂O and finger-flick to mix.
- Incubate 10 minutes at 65°C.
- Freeze extracted total RNA in the -80°C freezer.

Combining Follicles Protocol

For follicles that have been stored in 75% EtOH. Combine while finishing RNA extraction:

Two follicles:

- After adding 15 μL of ddH₂O to tube 1, mix by pipetting many times
- Incubate for 10 min at 65⁰C, meanwhile, spin tube 2 then remove EtOH and air dry
- Resuspend RNA in tube 1 by pipetting many times
- Transfer RNA from tube 1 to tube 2, and pipette many times
- Incubate tube 2 for 10 min at 65⁰C
- Pipette many times in tube 2 then transfer back to tube 1 and pipette
- Transfer RNA from tube 1 back to tube 2 and pipette many times
- Continue with DNase treatment

Three follicles:

- Add 5 μL of ddH₂O to each follicle tube
- Incubate all 3 tube for 10 min at 65⁰C
- Pipette each tube many times to resuspend RNA
- Add contents of tube 1 to tube 2, then pipette the 10 μL many times
- Add contents of tube 2 back to tube 1, and pipette
- Add contents of tube 1 to tube 3, and pipette the 15 μL many times
- Add contents of tube 3 to tube 2, and pipette
- Add contents of tube 2 back to tube 1, and pipette
- Add contents of tube 1 back to tube 3, and pipette

Four follicles:

- Add 3.75 μL of ddH₂O to each follicle tube
- Incubate all 4 tubes for 10 min at 65⁰C
- Pipette each tube many times to resuspend RNA
- Add contents of tube 1 to tube 2, then pipette the 7.5 μL many times
- Add contents of tube 2 to tube 3, and pipette the 11.25 μL many times

- Add contents of tube 3 back to tube 1, and pipette
- Add contents of tube 1 to tube 4, and pipette the 15 μL many times
- Add contents of tube 4 to tube 3, and pipette
- Add contents of tube 3 to tube 2, and pipette
- Add contents of tube 2 to tube 1, and pipette
- Add contents of tube 1 back to tube 4, and pipette

DNase Treat RNA Protocol – TURBO DNA-*free*

- Use 0.5 mL microcentrifuge tubes
- Prepare Master Mix (on ice):

	<u>1 tube</u>	<u>X? tubes</u>
10X Turbo DNase Buffer	1.2 μ L	____ μ L
TURBO DNase	0.5 μ L	____ μ L
ddH ₂ O	<u>2.8 μL</u>	____ μ L
	4.5 μ L	

- Add 4.5 μ L master mix to each tube.
- Add 7.5 μ L RNA to each tube and finger-flick.
- Incubate 40 minutes at 37°C (can use waterbath).
- Vortex the DNase inactivation reagent.
- Add 4 μ L DNase inactivation reagent to each tube and finger-flick.
- Incubate 3 minutes at room temp.
 - Finger-flick several times to disperse the inactivation reagent.
- Centrifuge for 1.5 minutes at 9300 rcf (xg).
- Carefully transfer the supernatant containing the RNA into a new tube (should be about 10-12 μ L).
 - Avoid the inactivation reagent, as it can interfere with downstream reactions
- Store in the -80°C freezer. DNase treated RNA is unstable.

RT-PCR Protocol - SuperScript III First-Strand Synthesis SuperMix

Use this kit to convert RNA to cDNA.

Starting material: 10 pg to 1 µg DNase treated (total) RNA.

- Combine the following kit components in a 0.2 mL or 0.5 mL thin-walled PCR tube on ice. For multiple reactions, a master mix without RNA may be prepared:

	<u>1 rxn</u>	<u>x? rxns</u>
2X RT Reaction Mix	10 µL	___ µL
RT Enzyme Mix	2 µL	___ µL
RNA (10pg to 1µg total RNA)	x µL	___ µL
ddH ₂ O	<u>to 20 µL</u>	___ µL
	20 µL	

- Gently mix tube contents and incubate at 25°C for 10 minutes.
- Incubate at 42°C for 50 minutes.
- Terminate the reaction at 85°C for 5 minutes.
- Chill on ice.
- Add 1 µL of *E. coli* RNase H and incubate at 37°C for 20 minutes.
- Use 1 µL of undiluted cDNA to prepare template for qPCR or store the reaction at -20°C until use.

**Real-Time PCR Protocol - Platinum SYBR Green qPCR SuperMix-UDG
with ROX**

For multiple reactions, prepare a master mix of common components.

- Prepare a 96-well plate map with template and primer set information.
- Label 0.5 mL microcentrifuge tubes with primer set name, template name, or master mix.

The following volumes have been scaled down to give a 20 μ L reaction volume; prepare the following on ice:

Primer Masermixes:

<u>Component</u>	<u>Per reaction</u>	<u>x? rxns</u>
Platinum SYBR Green	10 μ L	__ μ L
Forward Primer (250 nM)	.25 μ L	__ μ L
Reverse Primer (250 nM)	.25 μ L	__ μ L
ROX Reference Dye	.4 μ L	__ μ L
ddH ₂ O (to 15 μ L)	<u>4.1 μL</u>	__ μ L
	15 μ L per well	? μ L total

For each template, prepare a dilution:

<u>cDNA template</u>	<u>Per well</u>	<u>x? wells</u>
cDNA template	1 μ L	__ μ L
ddH ₂ O (to 5 μ L)	<u>4 μL</u>	__ μ L
	5 μ L per well	? μ L total

- Once the primer master mixes and template are prepared, take them (on ice) to Colleen's lab. Load the 96-well plate according to the map prepared. Add 15 μ L primer master mix and 5 μ L diluted cDNA template per well for a final reaction volume of 20 μ L.
- Program the ABI Prism 7500 as follows:
 - 50°C for 2 minutes hold (1 cycle)
 - 95°C for 2 minutes hold (1 cycle)
 - 40 cycles of:
 - 95°C, 15 seconds
 - 60°C, 30 seconds

Estradiol Radioimmunoassay Protocol

A. Diagnostic Products Corporation (DPC) Estradiol Double Antibody Kit (# KE2D1) with Modifications.

Dilute ovine follicular fluid from small and medium-sized follicles 1:100 in zero calibrator prior to assay. Follicular fluid from large follicles may need dilution at 1:500.

- Estradiol Antiserum (# E2D1) 1 vial of lyophilized estradiol antiserum, raised in rabbits. Add 10 mL of Milli-Q-water. Store refrigerated: stable at 4°C for 30 days after reconstitution.
- ¹²⁵I Estradiol (# E2D2) 1 vial of iodinated synthetic Estradiol in liquid form, 1 mL, 3.5 µCi. Store refrigerated: stable at 4°C for 30 days after opening, or until the expiration date marked on the label.
- Estradiol Calibrators/Standards (# E2D3-9). One set of seven glass vials, labeled A through G, of Estradiol calibrators, with preservative. The zero calibrator A (also referred to as RIA Buffer or Estradiol Calibrator Matrix) contains 6 mL, and the remaining calibrators B through G contain 3 mL each. Store refrigerated: stable at 4°C for 30 days after opening.
- Precipitating Solution (# N6), 110 mL. One vial consisting of goat anti-rabbit- gamma globulin and dilute PEG in saline. Store refrigerated: stable at 4°C for 30 days after opening.

B. RIA Standards and Reagents

- Assay Standards: Standards come ready for use in the kit. Note: The concentration of unknown samples run in 100 µL volumes will be read off the standard curve at pg/mL values. If sample dilutions are made, the concentration needs to be adjusted.

<u>Standard Vial labeled</u>	<u>Concentration at 100 µL per tube</u>
A. 0 pg/ml (Maximum Binding)	0 pg/ml
B. 5 pg/ml	2.5 pg/ml
C. 10 pg/ml	5 pg/ml
D. 20 pg/ml	10 pg/ml

E. 50 pg/ml	25 pg/ml
F. 150 pg/ml	75 pg/ml
G. 500 pg/ml	250 pg/ml

- Estradiol Antiserum: At least 10 minutes before use, reconstitute vial by adding 10 mL Milli-Q water or bring to room temperature (RT) if stored at 4°C. Mix by gentle inversion.
- Quality controls: To ensure assay is running properly:
 - QC1 - Use standard C - 100 µL = 5 pg/mL (four tubes-2 for beginning and end of run)
 - For Inter-assay controls (CV between assays):
 - Make the following dilutions using an ovine follicular fluid (oFF) standard:
 - QC2 - 1:100 dilution of oFF - (495 µL RIA calibrator matrix + 5 µL oFF standard (four tubes))
 - For Inter-assay controls (CV within an assay):
 - A second set of quality controls can be placed at the end of an assay greater than 200 tubes.

C. RIA Protocol

Note: Rainin pipettes are used for standard and sample preparation. Eppendorf repeater pipette is used for the addition of Estradiol antiserum, ¹²⁵I-Estradiol, and Precipitation Solution.

- Set up:
 - a. Label 12 x 75 mm polypropylene tubes:
 - Total Count (TC, tubes 1 and 2)
 - Non-Specific Binding (NSB, tubes 3 and 4)
 - Maximum Binding (MB, tubes 5 and 6)
 - Standards (tubes 7-18; at least three additional standards can be added to the lower end of the curve, if desired)
 - Quality Controls (QC1, tubes 19 and 20; QC2, tubes 21 and 22)
 - Samples will start at tube 23 and will be run in duplicate
 - b. Place tubes in racks on trays covered with lab bench paper.

- c. Bring all samples and RIA components, except Precipitating Solution, to RT before use.
 - d. TC tubes are left empty at this point.
 - e. To the NSB tubes, add 100 μL RIA calibrator matrix.
 - f. To the MB tubes, add 100 μL RIA calibrator matrix.
 - g. To the standard tubes (7-18), add 100 μL of each standard concentration (2.5 to 250 pg/mL , B to G) in ascending order, in duplicate.
 - h. To QC1 and QC2, add 100 μL of the indicated quality control.
 - i. To tubes 23 and above, add samples. Total volume is 100 μL . Any dilutions are made in RIA calibrator matrix.
- Assay
 - a. Add 30 μL of Estradiol Antiserum to all tubes except TC and NSB tubes (tubes 1-4). Vortex and incubate at RT for 2 hours.
 - b. Add 75 μL of ^{125}I -Estradiol to all tubes. Cap TC tubes and set aside until counting.
 - c. Vortex and incubate at RT for 1 hour.
 - d. Write out amount (0.024 $\mu\text{Ci/tube}$) of radioactivity, date, and initials on Rad tape and place on the covered tray containing the tubes.
 - e. Add 1 mL of *cold* Precipitating Solution to all tubes except TC tubes. Vortex and incubate at RT for 10 minutes.
 - f. Centrifuge all tubes, except TC, at 3,100 $\times\text{g}$ for 15 minutes at 4°C.
 - g. Take tubes (in centrifuge racks) to the radioactive hood. Pour supernatant into metal pan. Keeping tubes inverted, rap sharply on paper towels, then move to dry paper towels. Every 5 minutes for ~15 minutes (3 times), rap the inverted tubes sharply on the paper towels and move to dry towels. If no hanging droplets are present, proceed to counter.
 - h. Count tubes for 1 minute in Gamma Counter.
 - i. Dispose liquid in bound ^{125}I radioactive liquid waste carboy (0.02 $\mu\text{Ci/tube}$) and paper in solid bound ^{125}I radioactive waste can (0.004 $\mu\text{Ci/tube}$).
 - j. Use Assay Zap software and Estradiol.azm method to calculate Estradiol concentration in samples.

Progesterone Radioimmunoassay Protocol

Siemens Medical Solutions Diagnostics Progesterone COAT-A-COUNT Assay (#TKPG1)

All components must be at room temperature before use.

Prepare ovine follicular fluid samples prior to assay by diluting 1 μL follicular fluid in 49 μL zero calibrator.

- Plain tubes: Label four uncoated 12 x 75 mm polypropylene tubes TC (total counts) and NSB (nonspecific binding) in duplicate.

Coated tubes: Label fourteen Progesterone Ab-coated tubes with standard curve concentrations, starting with 0 ng/mL in duplicate. At least two additional standards can be added to the lower end of the curve, if desired. Label additional Ab-coated tubes, also in duplicate for controls and samples.

- Pipette 100 μL of the zero calibrator into the NSB and 0 ng/mL tubes, and 100 μL of each of the standard curve calibrators into its designated tubes. Pipette 100 μL of each control and unknown samples into the tubes prepared.

Because progesterone has a tendency to adsorb to plastic and even more so to glass, it is important to dispense each sample into the very bottom of each tube. In addition, it is also important to coat the pipette tip by rinsing a few times in the sample before making the transfer to the tube. Also, change tips between each standard and sample. Samples expected to exceed 40 ng/mL should be diluted in the zero calibrator before assay.

- Add 1.0 mL of ^{125}I Progesterone to every tube. Vortex.
No more than 10 minutes should elapse during dispensing of the tracer. Set the TC tubes aside for counting; they require no further processing.
- Incubate for 3 hours at room temperature.
- Decant thoroughly.

Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant the contents of all tubes (except for TC) in the radioactive hood (in an appropriate container) and allow them to drain on paper towels for 2 to 3 minutes. Strike tubes sharply on paper towels to remove residual droplets.

- Count for 1 minute with Gamma Counter.

RNA Quality Protocol – Agilent 2100 Bioanalyzer with Agilent 6000 Pico Kit

Preparing the Gel-Dye Mix:

- Allow the RNA 6000 Pico dye concentrate to equilibrate to room temperature for 30 min.
- Vortex RNA 6000 Pico dye concentrate for 10 seconds, spin down and add 65 μ L of the filtered gel to the 1 μ L of dye.
- Vortex solution well. Spin tube at 13000 xg for 10 min at room temperature. Use prepared Gel-Dye mix within one day. While tube is spinning, place samples in water bath for 2 min.
- Denature 1.5 μ L RNA aliquots at 70°C for 2 minutes, then place on ice.

Loading the Gel-Dye Mix:

- Put a new RNA 6000 Pico chip on the chip priming station.
- Pipette 9.0 μ L of gel-dye mix into the well marked G – with the circle around it.
- Make sure the plunger is positioned at 1 mL and then close the chip priming station (will have to push down hard – until hear a click).
- Press the plunger until it is held by the clip.
- Wait for exactly 30 seconds, then release the clip.
- Wait for 5 seconds. Slowly pull back plunger to 1 mL position.
- Open chip priming station and pipette 9.0 mL of gel-dye mix into the wells marked G (no circle).
- Discard the remaining gel-dye mix.

Loading the Agilent RNA 6000 Pico Marker

- Pipette 9.0 μ L of the RNA 6000 Pico Conditioning Solution (white) in the well marked CS.
- Pipette 5 μ L of RNA 6000 Pico marker (green) in all 11 sample wells and in the well marked ladder.

Loading the Diluted Ladder and Samples

- Pipette 1 μL of the heat denatured and aliquoted ladder in the well marked ladder.
- Pipette 1 μL of sample in each of the 11 sample wells. Pipette 1 μL of RNA 6000 Pico Marker (green) in each unused sample well.
- Put the chip in the adapter of the IKA vortexer and vortex for 30 seconds at the indicated setting (2400 rpm).
- Run the chip in the Agilent 2100 bioanalyzer within 5 minutes.

Appendix B - Reagents and Supplies

Table 0.1 Reagents and supplies

Item	Company	Catalog Number
<u>RNA Isolation</u>		
TRIzol Reagent	Invitrogen	15596-026
Chloroform	Fisher Scientific	C606-4
Isoamyl Alcohol	Sigma	I-3643
2-propanol (Isopropanol)	Fisher Scientific	A464-4
Ethanol		
Nuclease-Free Water	Ambion	AM9937
<u>DNase Treatment</u>		
TURBO DNA-free	Ambion	AM1907
<u>Reverse Transcription</u>		
SuperScript III	Invitrogen	11752-050
<u>Quantitative PCR (Real-Time)</u>		
Platinum SYBR Green with Rox	Invitrogen	1173-038
<u>Estradiol assay</u>		
Estradiol Double Antibody Kit	Siemens Medical Solutions	KE2D1
<u>Progesterone assay</u>		
COAT-A-COUNT	Siemens Medical Solutions	TKPG1
<u>RNA Quality</u>		
Agilent RNA 6000 Pico Kit	Agilent Technologies	5067-1513
<u>Other</u>		
Comfort Grip Gloves (small)	Fisher Scientific	19-167-030B
RNase-free Barrier Filter Tips all sizes (10 µL – 1000 µL)	Ambion	AM12640 - AM12665
Microcentrifuge Tubes (0.5 & 1.5 mL)	Fisher Scientific	
DEPC-Treated Water	Ambion	AM9922