



Effect of excess degradable intake protein on ovarian steroids, oviductal proteins and early embryonic development in ewes  
by Jie Weng

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science  
Montana State University  
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**Abstract:**

The objective of this study was to determine if feeding a diet high in degradable intake protein during the estrous cycle alters blood urea nitrogen (BUN), ovarian steroids, oviductal proteins, or early embryonic development in sheep. Ewes were fed either a control (C) diet composed of mixed-grass hay that provided 100% of the NRC protein requirement for maintenance or the C diet plus a protein supplement (high protein; HP) which provides 200% of maintenance. Estrous cycles were synchronized with intravaginal sponges containing a progestogen. One half of the ewes on each diet had their oviducts and uterine horns removed on either Day 2 or 4 after estrus and breeding to fertile rams. Jugular blood samples (10 ml) were collected daily from each ewe beginning at Day 2 of the synchronized estrous cycle and continuing until surgery. Right and left ampulla (AMP), isthmus (IST), and uterine horn (UTH) segments were flushed with Delbecco's PBS. Flushings were flash frozen in liquid N<sub>2</sub>. Protein in flushings was assayed using the BCA method. Serum samples were assayed for BUN, progesterone, and estradiol-17 $\beta$ . Blood urea nitrogen concentrations were higher ( $P < .05$ ) in HP ewes than C ewes during the synchronized cycle. Progesterone concentrations during the synchronized cycle did not differ ( $P > .10$ ) between ewes on the HP and C diets. Estradiol-17 $\beta$  concentrations were higher ( $P < .05$ ) in C ewes than in HP ewes during the periovulatory period. Feeding of the HP diet during the first 4 days of the next cycle did not affect ( $P > .10$ ) AMP, IST, or UTH protein contents or concentrations but reduced ( $P < .05$ ) estradiol-17 $\beta$  concentrations, increased ( $P < .05$ ) the rate of progesterone secretion between Days 2 and 4 after breeding, and delayed ( $P < .05$ ) the rate of passage of early embryos through the oviducts. Feeding mature ewes excess degradable intake protein during a synchronized estrous cycle and during the first 4 days after breeding may contribute to embryonic loss by altering the ovarian steroid milieu after ovulation. Altering the steroid environment may delay embryo passage through the oviduct and result in a temporal asynchronization between the embryo and uterus. This in turn may affect the process of maternal recognition of pregnancy and result in embryonic death.

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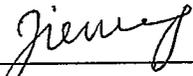
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## ABSTRACT

The objective of this study was to determine if feeding a diet high in degradable intake protein during the estrous cycle alters blood urea nitrogen (BUN), ovarian steroids, oviductal proteins, or early embryonic development in sheep. Ewes were fed either a control (C) diet composed of mixed-grass hay that provided 100% of the NRC protein requirement for maintenance or the C diet plus a protein supplement (high protein; HP) which provides 200% of maintenance. Estrous cycles were synchronized with intravaginal sponges containing a progestogen. One half of the ewes on each diet had their oviducts and uterine horns removed on either Day 2 or 4 after estrus and breeding to fertile rams. Jugular blood samples (10 ml) were collected daily from each ewe beginning at Day 2 of the synchronized estrous cycle and continuing until surgery. Right and left ampulla (AMP), isthmus (IST), and uterine horn (UTH) segments were flushed with Delbecco's PBS. Flushings were flash frozen in liquid N<sub>2</sub>. Protein in flushings was assayed using the BCA method. Serum samples were assayed for BUN, progesterone, and estradiol-17 $\beta$ . Blood urea nitrogen concentrations were higher ( $P < .05$ ) in HP ewes than C ewes during the synchronized cycle. Progesterone concentrations during the synchronized cycle did not differ ( $P > .10$ ) between ewes on the HP and C diets. Estradiol-17 $\beta$  concentrations were higher ( $P < .05$ ) in C ewes than in HP ewes during the periovulatory period. Feeding of the HP diet during the first 4 days of the next cycle did not affect ( $P > .10$ ) AMP, IST, or UTH protein contents or concentrations but reduced ( $P < .05$ ) estradiol-17 $\beta$  concentrations, increased ( $P < .05$ ) the rate of progesterone secretion between Days 2 and 4 after breeding, and delayed ( $P < .05$ ) the rate of passage of early embryos through the oviducts. Feeding mature ewes excess degradable intake protein during a synchronized estrous cycle and during the first 4 days after breeding may contribute to embryonic loss by altering the ovarian steroid milieu after ovulation. Altering the steroid environment may delay embryo passage through the oviduct and result in a temporal asynchronization between the embryo and uterus. This in turn may affect the process of maternal recognition of pregnancy and result in embryonic death.

## INTRODUCTION

The oviduct is an essential structure of the female reproductive tract. It is the site for ova transport, sperm capacitation, fertilization, and transport and development of the early embryo. After fertilization, the newly formed embryo is transported through the isthmus to the uterus. During transport the embryo undergoes its first cleavage divisions. The period during which the embryo transits the oviduct and enters the uterus lasts about 3 days for the ewe, 3.5 days for the cow, 2 days for the sow (Bearden and Fuquay, 1984a). By the time the embryo reaches the uterus, it is a blastocyst. The successful completion of these early divisions is essential to the survival of the embryo. The oviduct provides the site and the medium for these crucial steps.

Many factors affect reproductive efficiency of domestic ruminants. Dietary nutrients and their influence are among the most important of these factors. Nutrition can affect many reproductive processes in ruminants. For example, feeding ruminants a high plane of nutrition accelerates age at puberty, decreases postpartum interval, and alters placental size. On the other hand, feeding a low plane of nutrition has opposite effects. The mechanisms by

which nutrition changes reproductive processes are not well understood or even known. There is the possibility that changing the nutritional intake of ruminant females alters very early events of the reproductive cycle. These events occur in the oviduct. Thus, the oviduct may be a target for the effect of nutrition on reproduction.

Specifically, this thesis examines the hypothesis that the effect of nutrition on reproduction may be mediated by the oviductal environment in which embryos grow immediately after fertilization. An understanding of the role of nutrition in altering oviductal function may result in the discovery of novel mechanisms whereby specific nutrients alter reproductive efficiency in mammals. This in turn would allow us to develop techniques to optimize embryonic survival, fetal development, and birth weight in ruminants.

The present review focuses upon: a) the physiology of reproduction of ewe with emphasis on the physiology and histomorphology of the oviduct, b) the endocrinological control of oviductal function, c) the role of oviductal secretion in embryo development, and d) the influence of nutritional plane and/or dietary nutrients on reproductive efficiency.

## REVIEW OF LITERATURE

Functional Anatomy and Physiology  
of the Ewe Oviduct

The oviducts are a pair of convoluted tubes extending from near the ovaries to and connecting the uterine horns. Oviducts of ewes are approximately 15 to 19 cm long and are divided into three functional segments: 1) the infundibulum, a funnel-shaped opening near the ovary, 2) the ampulla, or middle segment, and, 3) the isthmus, the narrow proximal portion of oviduct connecting the oviduct to the uterine lumen (Hafez, 1993a).

The infundibulum, which includes the fimbria, is that portion of the tube adjacent to the ovary. This region is responsible for guiding ovulated eggs into the opening of the middle segment or ampulla of the oviduct. The ampulla is from 3 to 5 mm in diameter and accounts for about half of the oviductal length. It is thin-walled with many easily distensible mucosal folds (Ellington, 1991). Once the egg is deposited in the ampulla, it is transported toward the uterus by two distinct methods. Wavelike contractions of the muscularis layer push the egg along the lumen, while the ciliary action of the epithelial cells also moves the

egg. Not all the epithelial cells are ciliated, however. The non-ciliated cells are secretory in nature and provide the oviductal fluid found in the lumen. This medium provides for fertilization, nourishment of the egg, and many other processes that will be discussed later in this review.

The ampullary-isthmic junction in ewes is difficult to locate anatomically. The isthmus contains fewer mucosal folds and a much narrower lumen than the ampulla, and it delays the ovum several hours during transport. Fertilization occurs at this junction. Sperm arrive at the site of fertilization through the isthmus, moving in much the same way as the egg. The movement of sperm is aided by flagellum (Hafez, 1993b).

The isthmus is thick walled and smaller than the ampulla, being 0.5 to 1 mm in diameter. Sperm capacitation and molecular changes on the egg occur in this region of the oviduct (Hafez, 1993a). The isthmus joins the uterine horn at the utero-tubal junction.

### Functional Histology

Histologically, the oviduct is divided into three cell layers. The outer layer, basically connective tissue, is

the tunica serosa. The middle layer is the tunica muscularis, which includes both circular and longitudinal smooth muscle layers. The longitudinal muscle layer is closer to the lumen compared to the circular muscle layer. The innermost layer, made up of epithelial cells, is known as the tunica mucosa (Bearden and Fuquay, 1984b).

The outer serosal layer and the middle muscularis layers are continuous through the oviduct and perform the same functions in both regions. The serosa is a protective layer of connective tissue that helps keep the outer surface of the oviduct moist. The function of the muscularis layer is to move the contents of the oviduct by contraction.

The mucosa consists of a single layer of columnar epithelial cells containing ciliated and secretory cells. The total number of epithelial cells is greatest in the ampulla, because the lining is highly folded creating greater surface area (Lewis, 1990).

The secretory cells of the oviductal mucosa are nonciliated cells that have numerous long, slender microvilli and contain secretory granules. The size and number of secretory granules seen in these cells vary widely among individuals and with the phase of the estrous cycle (Hafez, 1993a). Secretory cell height reaches a

maximum at estrus. After ovulation, many granules are released and the cell height is reduced to a minimum during mid-cycle. These changes suggest that changes in ovarian steroids associated with estrus induce specific histological changes in the secretory cells.

The ciliated cells of the oviductal mucosa have slender motile cilia that extend into the lumen. Control of ciliary activity and numbers by systemic hormone levels has been described in several species, with increased numbers and motility seen near ovulation (Hunter, 1988).

The percentage of ciliated cells decreases in the ampulla toward the isthmus and reaches a maximum in the fimbriae and infundibulum. In the ampulla, cilia currents are barely detectable. In the isthmus, cilia tend to maintain a rapid and strong current directed towards the utero-tubal junction, presumably to facilitate embryo movement into the uterus at the 8- to 16-cell stages. The activity of cilia keeps oviductal eggs in constant rotation. This activity is thought to be essential for bringing the egg and sperm together (fertilization) and preventing oviductal implantation.

#### Oviduct Vasculature and Lymphatic Vessels

The vasculature of the oviduct is derived from the uterine and ovarian arteries. During estrus, blood flow to the oviduct increases (Weeth and Herman, 1952), presumably because of a change in ovarian estrogen which enhances the secretory activity of the tubal mucosa. Lymphatic vessels, which are more prevalent in the isthmus, dilate in the follicular phase, adding to the edema seen at estrus (Ellington, 1991).

#### Oviduct Innervation

The degree of innervation of the oviduct varies in the different muscle layers and in different regions of the oviduct (Hafez, 1993a). The ampullary and infundibular regions of the oviduct have limited innervation; whereas, the well developed circular muscle layer of the isthmus and the ampullary-isthmus junction contain rich adrenergic innervation where adrenergic nerve terminals are in close contact with individual smooth muscle cells (El-Banna and Hafez, 1970; Isla et al., 1989). In most species the isthmus contains mostly alpha-adrenergic receptors, so norepinephrine causes intense periovulatory contraction of the isthmus as a physiologic sphincter, which may be

important for regulating egg transport (Brundin, 1969).

### Muscularis Activity

The oviductal musculature undergoes various types of complex contractions. In general the ampulla is less active than the isthmus. Oviductal muscularis activities are stimulated by contractions of two major membranes that are attached to the fimbriae, ampulla, and ovary: the mesosalpinx and the mesotubarium superius, which contain smooth musculature. Contractions usually proceed in an abovarian direction, adovarian contractions occur less frequently (Boling and Job, 1965). The frequency of contractions varies with the phase of the estrous cycle. Bennett et al. (1988) found that frequencies and amplitudes of contractions increased 3 to 5 days before estrus, resulting from segmental activity of the circular muscularis layer. These contractions decrease the internal isthmic diameter. Greatest muscle contractility is seen at estrus, especially at the utero-tubal junction in cows (Ruckebush and Bayard, 1975).

The varying patterns of oviductal contraction may be associated with cyclic changes in glycogen content of oviductal musculature. Glycogen in the oviduct is more

abundant in the inner circular musculature than in the outer longitudinal musculature.

Boling and Job (1965) found that the oviductal muscular activity in the rat increased when estrogen was withdrawn, but the pattern of contractility does not resemble the normal pattern as closely as that induced by progesterone. Whether this is the case in ewes is not known because there is no significant increase of progesterone during this phase of the estrous cycle in ewes.

#### Prostaglandin Activity

It has been suggested that in sheep, prostaglandin (PG) $F_{2\alpha}$  causes tubal contractions and PGE causes tubal relaxation (Harper, 1988). Both hormones increase tonus of the proximal part of the oviduct and cause relaxation in the remainder of the oviduct. However, PGE<sub>3</sub> relaxes the whole oviduct (Hafez, 1993a). On the other hand, PGF $_{2\alpha}$  stimulates contractions of the oviduct, aids sperm transport in female, and causes contraction of blood vessels.

## Oviduct Fluid and Its Secretion

The fluid found in the oviductal lumen provides a suitable environment for fertilization and cleavage of fertilized eggs (Hafez, 1993a). The volume of oviductal fluid varies during the estrous cycle. The volume is low during the luteal phase of the cycle and begins to rise at the onset of estrus. Maximum amounts of fluid are secreted on the day after the onset of estrus, and the quantity thereafter declines rapidly to luteal phase levels (Perkins et al., 1965). The rate of accumulation of oviductal fluid is regulated by ovarian steroid hormones.

The directional movement of oviductal secretions may contribute to ovum transport to the uterus. The direction of flow of oviductal fluid is toward the ovary, because the isthmus blocks the flow of fluids into the uterus. In sheep, most of the oviductal secretions pass out of the oviductal ostium early in the estrous cycle. On day 4, however, when ova usually enter the uterus, fluid flow through the uterotubal junction increases remarkably.

Several important reproductive events occur in oviductal fluid. These include: 1) final maturation of the oocyte, 2) sperm capacitation, 3) fertilization, and 4) early embryonic development. Oviductal fluid is a

combination of blood transudate and secretory products of the granules from the secretory cells of the oviductal epithelium (Oliphant et al., 1984a and b).

A chemical analysis of the fluid from the sheep oviduct indicates that estrogen increases the secretion of potassium, bicarbonate, and lactate; whereas, progesterone decreases lactate secretion. Lactate could serve as a substrate for sperm metabolism (Restall and Wales, 1966a). Oviductal fluids harvested at different stages of the estrous cycle uniformly depress the respiratory activity of ram spermatozoa (Restall and Wales, 1966b). Cyclical changes occur in the pH of the oviduct in sheep. The lowest values occur during diestrus (6 to 6.4), the highest during estrus and metestrus (6.8 to 7.0) (Hadek, 1953).

Secretion of oviductal fluid by the epithelial cells provides the media for all of the events of reproduction that take place in the oviduct. By actively transporting serum components into the lumen, the secretory cells create a fluid medium with most of the necessary elements for cell survival. Proteins that do not originate in the serum make up the remaining portion of the oviductal fluid.

### Oviductal Proteins

Fluid from the oviduct contains proteins from serum and proteins produced by the secretory cells of the oviductal lining (Leese, 1988; Brackett and Mastroianni, 1974). Features of regional specificity and temporal release due to estrogen stimulation have been identified for protein components that are common to oviductal fluid and serum. Two types of glycoproteins are secreted by the tubal epithelium. One type is secreted throughout the cycle and the other is only produced in the peri-ovulatory phase. These proteins have been the subject of studies in a wide range of species. Oviduct specific glycoproteins have been found in all mammals studied, from laboratory rats to humans (Abe and Abe, 1993 and Verhage et al., 1988).

In 1984, Sutton et al. performed an experiment to identify protein levels of sheep during the estrous cycle. Using a indwelling catheter technique they determined that protein concentration of the oviductal fluid increased 2- to 4-fold during the estrous cycle compared to non-cycling ewes.

Ellington et al. (1993) described experiments that involved culturing oviductal epithelial cells and analyzing the resulting protein secretions. Not only did they find oviduct-specific proteins, but they found that the types of proteins secreted depended upon whether the cells were in

contact, directly or indirectly, with sperm cells. The secretory cells changed the type of proteins they produced dependent on the environment. Cultured cells in contact with the sperm produced proteins that were not found in control cultures without sperm. This change in output could be an answer as to how sperm are capacitated in the oviduct.

An estrogen-dependent glycoprotein is produced by the epithelium of the ewe oviduct during the time the egg or fertilized ova are transported through the tube. Murray (1993) showed that proteins are secreted in response to hormonal control and are secreted in a region specific manner in the oviduct. She found that a 90,000 to 92,000 MW glycoprotein was secreted in response to estrogen in the ampulla, but not the isthmus, of cycling ewes on days 0 to 6 and Day 16 of the estrous cycle.

Many studies have shown a relationship between the improved survival of embryo transplants that are cultured with oviduct cells or fluids. Gandolfi et al. (1989b) discussed the role of the oviduct and its secretions on embryonic development. They concluded that oviducts improved the survivability of the embryos in vitro, and that some oviductal secretions may be essential for embryonic survival.

## Role of Oviductal Secretion in Embryo Development

The oviduct provides a unique environment for sustaining embryonic development. It is known that the oviductal environment exerts a functional role in early embryonic development (Bavister, 1988). In particular, oviductal proteins are associated with early embryonic development in sheep (Gandolfi et al., 1989b; Murray, 1994). Most of these proteins are derived directly from the serum and are primarily serum albumin and immunoglobulins (Leese, 1988). The passage of proteins from the serum into the oviductal lumen is thought to be a case of selective transudation (Oliphant et al., 1978). Endocytosis plays a major role in this selective transudation process (Parr et al., 1988). The other source of oviductal proteins, primarily, high molecular weight glycoproteins, is that secreted by the oviductal epithelial cells in a wide variety of species (rabbit, Barr and Oliphant, 1981; sheep, Sutton et al., 1984; mouse, Kapur and Johnson, 1985; baboon, Fazleabas and Verhage, 1986; human, Verhage et al., 1988). The combined findings from biochemical and culture studies demonstrate that epithelial cells of the oviduct secrete proteins which are glycosylated (Sutton et al., 1985; Robitaille et al., 1988) and that these proteins

selectively interact with the embryo (Kapur and Johnson, 1986; Robitalle et al., 1988).

Oviductal glycoproteins are found associated with the zona pellucida, perivitelline space, and plasma membranes of blastomeres (Gandolfi et al., 1989a and b; Murray, 1993; Buhi et al., 1993). Gandolfi et al. (1989b) reported that the period of time during which the early embryo bound oviductal glycoproteins was reduced in vitro compared to that for in vivo embryos. They suggested that this might be due to lower secretion rates of these proteins by oviductal cells in vitro. Gandolfi et al. (1989a) found that in the sheep a protein specifically secreted by the epithelium at the time of embryonic passage through the oviduct is bound to the zona and incorporated into the cytoplasm. These findings demonstrate the possibility that other oviduct proteins may be translocated to the embryo and that the oviduct and embryo have a close biochemical relationship.

#### Effect of Nutrition

Adequate nutrients are required for many functions by animals, and the quantity required increase during times of increased production. Thus, nutritional needs are greater and more critical for many aspects of production, such

maintaining reproduction, lactation, and growth. Influences of nutrition on reproductive functions have been recognized for many years. However, underlying mechanisms are complex and in many cases are not well understood. Effects of early nutrition that influence the outcome of reproductive events are becoming a major research area (Lucas, 1992). Thus, it is important to understand the mechanisms when alterations in the supply of nutrients evoke changes in reproductive performance.

Early embryonic growth patterns may be related to nutritional status of dams (Robinson, 1990; Ashworth, 1994). During early embryonic and fetal development in ewes, dietary nutrient changes can affect ovulation rate and the size, vigor, and viability of the newborn (Robinson, 1996). The majority of studies about nutritional effects on embryo survival do not distinguish between fertilization failure and embryonic mortality. Some studies have shown that when ewes were "flushed" (increase in nutrient intake) they were slower to conceive (Tassell, 1967a and b) and that ova from ewes on a high plane of nutrition have lower fertilization rates than those from ewes fed a low plane of nutrition (Lamond, 1970).

High levels of degradable intake protein in the diets of domestic ruminants have been associated with increased

fertilization failure (Blanchard et al., 1990). Protein level of diets can affect reproduction through toxic effects of ammonia and its metabolites on gametes and/or the early embryo (Ferguson and Chalupa, 1989). Toxic by-products of nitrogen metabolism from the rumen may impair sperm, ova, or early embryo survival. After feeding excess degradable intake protein, elevations of ammonia can interfere with intermediary metabolism and influence blood concentrations of glucose, lactate, free fatty acids, urea, and metabolic hormones (Visek, 1984) and corpus luteum functions (Garwacki et al., 1979).

Embryonic mortality is a major source of reproductive losses in most species of animals. In sheep, there is evidence that feeding either a low (Edey, 1966; Cumming et al., 1975; Hamra and Bryant, 1982) or a high (Hamra and Bryant, 1982; Parr et al., 1987) plane of nutrition during early pregnancy is detrimental to embryo survival. In a review of nutritional influences on embryo survival in cattle, sheep, and pigs, Robinson (1986) concluded that an extended period of under nutrition is required to cause significant reductions in embryonic growth and survival.

By using embryo transfer methods, Mckelvey and Robinson (1986) and Mckelvey et al. (1988) found that ewes fed a low plane of nutrition had higher embryonic survival

rates than ewes fed a high plane of nutrition. Specifically, the results of their reciprocal embryo-transfer experiments imply that it is more important for embryo survival than an increase in the plane of nutrition occurs during the pre- and peri-ovulatory periods than during early pregnancy; indeed, a high plane of nutrition during early pregnancy appears to be detrimental.

The mechanisms involved in the reduction of embryo survival arising from high-plane feeding in early pregnancy may be its affect on corpus luteum function or the secretion of progesterone. One of the physiological roles of progesterone is the maintenance of pregnancy. An inverse relationship has been observed between plane of nutrition and circulating progesterone concentrations in ewes (Parr et al., 1982; Williams and Cumming, 1982; Mckelvey et al., 1988; Rhind et al., 1985). Thus, increased progesterone metabolism occurs as a result of increased hepatic mixed-function oxidase activity in well-fed animals. Parr et al. (1987) demonstrated that ewes fed a high-plane of nutrition after mating had reduced progesterone concentrations and showed an increase in embryonic mortality. It would appear that feeding a high-plane diet, through its stimulatory effects on both hepatic blood flow and the metabolic clearance rate of progesterone (Symonds and Prime, 1989),

decrease progesterone concentrations which in turn are related to embryo development and survival. Changes in circulating progesterone concentrations modify the production of either the trophoblastic proteins and/or the endometrial secretory proteins (Knight et al., 1974; Roberts et al., 1988; Ashworth and Bazer, 1989). Some of these proteins can transport water-soluble nutrients across the placenta (Roberts et al., 1986). Progesterone-induced secretory proteins from the trophoblast play a critical role in the bi-directional signaling between mother and conceptus that is essential for maternal recognition of pregnancy and embryonic survival (Bazer, 1989; Ashworth, 1992). Pregnancy depends on a specific sequence of concentrations of progesterone and estrogen, and embryonic mortality may be caused by excesses or inadequate amounts of either of these ovarian steroids (Wilmot et al., 1986; Archibong et al., 1987).

The proposed mechanisms involved in the effects of high protein diets on the fertility of dairy cows have been reported. Elrod and Butler (1993) found that feeding high levels of degradable intake protein were associated with a reduction in pH of the uterine environment caused by excessive ammonia production by the rumen. Ammonia and urea differentially affected endometrial ion transport (Elrod,

1992) and contributed to reduced embryonic survival. Urea is one metabolite of dietary protein that is formed from detoxification of  $\text{NH}_4$  by the liver. The concentration of urea in the plasma or blood (PUN or BUN) is reflective of the quantity and degradability of the protein. Plasma urea nitrogen (PUN) concentrations have often been used as a correlate between dietary protein level and fertility. High concentrations of PUN are associated with increased rumen ammonia. High degradable intake protein diets cause excessive ammonia production in the rumen and conversion of ammonia to urea (Oltjen et al., 1972). At high ruminal ammonia concentrations, the capacity of the liver for urea synthesis is exceeded, ammonia would accumulate in the blood, and ammonia toxicity may result.

The hypothesis that excess rumen ammonia may affect early embryonic growth and development is illustrated by the following results of in vivo and in vitro embryo culture and embryo transfer experiments. Thompson et al. (1994) found that birth weights of lambs from embryos cultured in synthetic oviduct fluid (SOF) were greater than those from spontaneously ovulating ewes. For spontaneously ovulating animals, Ferguson and Chalupa (1989) have expressed concern at the low conception rates to first insemination in high-producing dairy cows. They suggested

that a major cause might be an excess of degradable intake protein leading to toxic effects of ammonia and its metabolites on gametes and/or embryos. Embryos cultured in SOF had higher concentrations of ammonia and induced high birth weights and dystocia in ewes (Thompson et al., 1994). The toxic effects of ammonia on cells (Visek, 1984), and specifically reproductive tissues (Stalheim and Gallagher, 1977), have been demonstrated. Elevated plasma ammonia (>100  $\mu\text{mol/l}$ ) induced higher occurrence of embryonic death in sheep (Bishonga et al., 1994).

## STATEMENT OF THE PROBLEM

Dietary nutrients can affect many reproductive processes in ruminants. It is well known that excess degradable intake protein and high feeding levels affect early embryonic development and survivability. The mechanism of this effect may be related to the liver's ability to accommodate the challenge of excess  $\text{NH}_4$  production, which in turn will affect reproductive process. The goal of our research is to investigate the relationship between high protein diets and reproductive changes, and influences of excess degradable intake protein on oviductal secretion and early embryonic development. The review of the literature indicates that the effects of excess degradable intake protein on embryonic survival may occur very early in embryonic development. Specifically, it may occur during the initial stages of development in the oviduct. The effect(s) may be carried over into later stages of development or cause a change in the ability of the embryo to synchronize its development with uterine changes necessary to sustain it. Presently, there are no data in the literature regarding the effect of protein nutrition on oviductal protein secretion or on the ovarian steroid environment during the first few days of embryonic

development in ruminants. Therefore, the objectives of this study were to determine if: 1) feeding mature ewes a diet high in degradable intake protein (HP) alters blood urea nitrogen and ovarian steroid concentrations during the estrous cycle or soon after fertilization, 2) oviductal protein concentrations or their patterns are altered in ewes fed a HP diet, and 3) early embryo development is altered by feeding ewes a HP diet.

It is our hypothesis that feeding HP diets reduces embryo survival through its influences on oviductal functions. The importance for this reproductive limitation is obvious if considered from the producer standpoint. Low rates of embryo survival of animals causes economic loss to the producer by reducing reproductive efficiency. By understanding the cause of poor reproductive performance, we may be able to change this effect and increase production efficiency. Furthermore, we may obtain information regarding a biologically important mechanism whereby the effect of a specific macronutrient influences reproductive processes.

## MATERIALS AND METHODS

### Animals

This study was performed between October and December of 1996. Thirty-one mature, western white-faced ewes, which were multiparous and 3- to 6- yr-old, were used. Ewes were housed at the Fort Ellis Sheep Research Station of Montana State University, Bozeman.

### Estrous Synchronization

Medroxy-aceto-progesterone (MAP) intravaginal sponges were inserted into each ewe to synchronize estrous cycles. Sponges were removed 14 days after insertion. Ewes were observed for estrus with the aid of sterile epididectomized (teaser) rams beginning 36 hours after the removal of the sponges. Ewes were checked twice daily for 4 days. Only ewes that would stand to be mounted by rams were assigned to treatments. Day of estrus was defined as Day 0 of an estrous cycle.

### Experimental Treatments

## Diets

At the synchronized estrus each ewe was assigned randomly to receive either a maintenance diet (C; control) or the maintenance diet plus supplemental protein (HP; high protein). Ewes assigned to the control diet were fed mixed-grass hay that had a crude protein (CP) content of 0.123 kg. Ewes assigned to the HP diet were fed mixed-grass hay (CP = 0.123 kg) and a soybean supplement. The crude protein content of the soybean meal was 44%. The supplement was fed at a rate of  $0.088 \text{ kg}\cdot\text{hd}^{-1}\cdot\text{d}^{-1}$ . The total CP for HP diet was  $0.211 \text{ kg}\cdot\text{hd}^{-1}\cdot\text{d}^{-1}$ . This level of crude protein provided approximately 200% of the NRC requirement for maintenance (NRC, 1985) for these ewes. Water and mineralized salt were provided ad libitum. Ewes were placed into separate pens and fed hay and/or HP supplement beginning on Day 2 of the synchronized estrous cycle. Ewes were fed the supplement and given the hay ration in the morning of each day throughout the synchronized cycle. The number of ewes on each diet is given in Table 1. Composition of diets is given in Table 2.

## Day of Estrous Cycle for Surgical Removal of Oviduct and Uterine Horns

On Day 16 of the synchronized estrous cycle, ewes on each diet were placed with teaser rams and observed for estrus twice daily. When a ewe was observed to display estrus she was mated to two different fertile rams. She was then assigned to undergo surgical removal of the oviducts and uterine horns on either Day 2 or 4 after estrus. The number of ewes on each diet that were assigned to each day of the estrous cycle is given in Table 1.

Table 1. Experimental design and numbers of ewe per treatment.

Day of Estrous Cycle	Diet		
	Control (C)	High Protein (HP)	Total
2	7	8	15
4	8	8	16
Total	15	16	31

Table 2. Chemical composition (% of DM) of diets.

Item	Mixed-grass hay <sup>a</sup>	Soybean meal <sup>b</sup>
Crude Protein	10.0	44.0
Crude Fat		0.5
Crude Fiber		7.0

<sup>a</sup>Chemical analysis determined by laboratory procedures.

<sup>b</sup>Chemical analysis provided by feed manufacturer (Westfeeds, Big Sky Division, Billings, MT)

### Surgical Procedures

Thiopental Sodium (10 ml; 4 %) was injected into a jugular vein to anesthetize each ewe. A mid-ventral incision was used to exteriorize the ovaries, oviducts, and uterine horns. Side and number of ovulations were recorded. Oviducts were ligated at the utero-tubal junction, the isthmic-ampullary junction, and the ampullary-infundibular junction to insure that no fluid was lost and that no transfer of fluids or contents occurred between regions. Uterine horns were ligated at the external bifurcation of the uterus. The blood supplies to the oviducts and uterine horns were then ligated, and the oviducts and uterine horns removed. Immediately after removal, the oviduct and uterine tissues were transported to the laboratory in a pre-warmed Styrofoam container. After surgery, Sodium Pentobarbital (3ml; 35%) was injected into a jugular vein for euthanasia.

### Blood Samples

Blood samples (10 ml) were collected daily from all ewes by jugular venipuncture each morning beginning on Day 2 of the synchronized estrous cycle until the day of surgery of the next cycle. Blood samples were allowed to clot at 25°C for 3 to 4 hours. They were then centrifuged at 1,285 x g for 20 minutes at 4°C. Serum from each sample was decanted into 12 x 75 mm plastic culture tubes and stored at -22°C for later assay of blood urea nitrogen (BUN), progesterone (P4), and estradiol-17 $\beta$  (E2).

#### Processing Oviduct and Uterine Tissue

Weight and length of each segment of oviduct and uterus were recorded in the laboratory. In vivo oviduct and uterine tissue secretions were collected from each ampulla (AMP), isthmus (IST), and uterine horn (UTH) by flushing each twice with 3, 1.5, and 10 mL of Delbecco's phosphate-buffered saline. Flushings from AMP, IST, and UTH ipsilateral to the ovulating ovary were searched microscopically for the presence of ova or embryos. Flushings of AMP, IST, and UTH from each side were then pooled. Pooled flushings were placed on ice and PMSF was added to a final concentration of 10 mM. Samples were flash

frozen in liquid N<sub>2</sub> and stored at -22°C for assay of protein content and concentration.

### Assays

#### Blood Urea Nitrogen Assay

The concentration of blood urea nitrogen (BUN) is regulated by the metabolism of proteins and by the renal excretion. Blood urea nitrogen concentrations were measured by coupled enzyme reactions involving urease and glutamate dehydrogenase. One mL of BUN Endpoint reagent and 0.005 mL (5 µL) of serum was pipetted into each tube and incubated at 37°C for 5 minutes. Sample absorbance was read and recorded at 340 nm. Range of the standard curve was 0 to 40 mg/dL. The sensitivity of the assay was 5 mg/dL. Inter- and intra-assay CV's were less than 5%.

#### Bicinchoninic Acid (BCA-Protein) Assay

A detergent compatible formulation based on BCA for the colorimetric detection and quantification of total protein was used to assay oviductal and uterine protein

content. A fresh set of protein standards was prepared by diluting the 2.0 mg/mL BSA stock standard (Stock). Fifty parts of BCA Reagent A with 1 part of BCA Reagent B were mixed (BCA Working Reagent) just before the assay. Two hundred  $\mu\text{L}$  of each standard or AMP, IST, or UTH flushing was pipetted into appropriately labeled test tubes. Two mL of the working reagent was added to each tube. All tubes were incubated at  $60^{\circ}\text{C}$  for 30 minutes. All tubes were cooled to room temperature and the absorbance measured at 562 nm using water as a reference. The range of the standard curve was 0 to 40  $\mu\text{g}/200\mu\text{L}$ . The sensitivity of the assay was 5  $\mu\text{g}/200\mu\text{L}$  and the inter- and intra-assay CV were less than 5%. Concentration was calculated by multiplying the assay content by the volume of the flushing then dividing by the weight of the tissue segment.

#### Progesterone Assay

Progesterone was assayed by a solid-phase radioimmunoassay (RIA) with kits purchased from Diagnostic Products Corp. (Los Angeles, CA). Briefly the procedure was as followed. Four plain (uncoated) 12 x 75 mm polypropylene tubes were labeled in duplicates for total

counts and non-specific binding tubes. Fourteen coated Progesterone Ab-Coated Tubes A (maximum binding) and B through G were labeled in duplicate. Additional antibody-coated tubes were also prepared for samples in duplicate. One hundred  $\mu\text{L}$  of the zero calibrator A was pipetted into the NSB and A tubes and 100  $\mu\text{L}$  of each of the calibrators B through G into correspondingly labeled tubes. One hundred  $\mu\text{L}$  of each sample was placed into the appropriately labeled tubes. One mL of  $^{125}\text{I}$ -progesterone was added to all the tubes and the tubes were incubated overnight at room temperature. Each tube, except the total count tubes, was decanted thoroughly and counted for 1 minute in a gamma counter. The sensitivity of the assay was 0.02 ng/mL. The inter- and intra-assay CV were less than 12%.

#### Estrogen Assay

Estradiol-17 $\beta$  was quantified by a double antibody RIA using kits purchased from Diagnostic Products Corp. (DPC; Los Angeles, CA). Samples were extracted before each assay. For the extraction procedure 200  $\mu\text{L}$  of serum or standards were pipetted into 16 x 100 mm borosilicate disposable culture tubes, and 75  $\mu\text{L}$  of  $^{125}\text{I}$ -E2 was pipetted into four

tubes to estimate recovery of the steroid. Two mL of ethyl acetate was added to each of the tubes. Samples were vortexed for 3 minutes then centrifuged at  $600 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes. The supernatants were removed with pasteur pipettes and placed into 12 x 75 mm culture tubes labeled with the same numbers as the 16 x 100 mm tubes from which we removed the supernatants. Then the 12 x 75 culture tubes were dried in a warm ( $37^{\circ}\text{C}$ ) water bath with  $\text{N}_2$ . The extraction was repeated. Then 200  $\mu\text{L}$  of 0.1% gelatin in 0.1 M Phosphate buffer saline was added to all the tubes. They were then incubated at  $4^{\circ}\text{C}$  overnight.

One hundred  $\mu\text{L}$  of the extracted sample was pipetted into another identically labeled tube. Thirty  $\mu\text{L}$  of E2 antibody from DPC was added to all tubes except the total count tubes (TCT), non-specific binding tubes (NSB), and buffer-blank tubes. Samples were incubated at room temperature for two hours. Seventy-five  $\mu\text{L}$  of  $^{125}\text{I}$ -E2 (25,000 cpm) was added to all tubes which were then incubated at room temperature for 1 hour. One mL of Precipitating Solution was added to all tubes except the TCT tubes and incubated for 10 minutes at  $4^{\circ}\text{C}$ . After this incubation all of the tubes except the TCT tubes were centrifuged at  $1,285 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ . The

supernatants were decanted, except the TCT tubes, and dried down on blotting paper. The samples were counted for 5 minutes in a gamma counter. The sensitivity of the assay was 0.16 pg/mL. The inter- and intra-assay CV were 15% and 10%, respectively.

### Statistical Analyses

#### Synchronized Estrous Cycle Length

Estrous cycle length for ewes was analyzed by a one-way ANOVA using the GLM procedure of SAS (SAS, 1996). The model included only diet.

#### Blood Urea Nitrogen Concentrations During the Synchronized Estrous Cycle

Blood urea nitrogen concentrations throughout the synchronized estrous cycle of two ewes from each diet were evaluated visually. After this assessment it was decided that the statistical analyses would include samples obtained from ewes on Day 15 of the synchronized estrous cycle through Day 4 of the next estrous cycle. These data were analyzed with a split-plot ANOVA for a completely random

design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

#### Progesterone Concentrations During the Synchronized Estrous Cycle

Progesterone concentrations during the synchronized estrous cycle of ewes fed the control or HP diets were analyzed with a split-plot ANOVA for completely random design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

#### Estradiol-17 $\beta$ Concentrations During the End of the Synchronized Estrous Cycle and Beginning of the Next Cycle

Estradiol-17 $\beta$  Concentrations data from Day 17 of the synchronized cycle through Day 4 of the next cycle were analyzed with a split-plot ANOVA for completely random

design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

#### BUN, P4, and E2 Concentrations on Days 2 and 4 of the Estrous Cycle of Breeding

Blood urea nitrogen, P4, and E2 concentrations in blood samples taken from ewes on either Day 2 or 4 after estrus and mating to fertile rams were analyzed by an ANOVA using the GLM procedure of SAS (SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet, day of surgery, and their interaction. Within class correlation coefficients were generated among BUN, P4, and E2 concentrations using the CORR procedure of SAS (SAS, 1996).

#### Oviductal Protein Content and Concentration

Protein content and concentrations of the AMP, IST, and UTH were analyzed separately using ANOVA for a completely random design using the GLM procedure of SAS

(SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet, day of surgery, and their interaction.

Ovulation Rates and Embryo Development and Location Within the Reproductive Tract

Ovulation and embryo recovery rates, proportion of embryos damaged, and the proportion of embryos located in either the AMP, IST, or UTH were analyzed by separate contingency Chi-square tests. Cell stage of embryos was analyzed by ANOVA for a completely random design using the GLM procedure of SAS (SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet (control and high protein), day of surgery (Days 2 and 4), and their interaction.

## RESULTS

Estrous Cycle Length

Estrous cycle lengths did not differ ( $P > .10$ ) between ewes fed a high protein (HP) or control (C) diet, and were  $17.4 \pm .3$  (mean  $\pm$  se) and  $17.3 \pm .3$  days, respectively.

Patterns of Blood Urea Nitrogen During the SynchronizedEstrous Cycle

There was no interaction ( $P > .10$ ) between diet and day of the estrous cycle for BUN concentrations from Day 15 of the synchronized cycle through Day 4 of the next estrous cycle (Figure 1). However, BUN concentrations were higher ( $P < .05$ ) in ewes fed the HP diet than in ewes fed the C diet during the synchronized estrous cycle and the beginning of the next estrous cycle (Table 3).

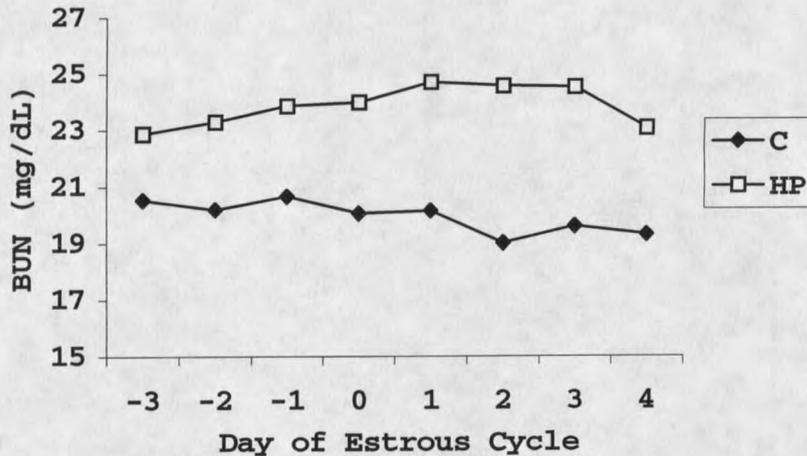


Figure 1. Blood urea nitrogen (BUN) concentrations from Day 15 of the synchronized estrous cycle through Day 4 of the next estrous cycle. Numbers of ewes through Day 2 for the control (C) and high protein (HP) diets were 15 and 16, respectively. Data for Days 3 and 4 represent 8 and 8 ewes from the C and HP diets, respectively.

Table 3. Least square means of BUN concentrations (mg/dL) for ewes fed either the control or high protein diet during the synchronized estrous cycle and the beginning of the next estrous cycle.

Item	Diet <sup>a</sup>		ANOVA
	Control (15)	High Protein (16)	
BUN (mg/dL)	19.0	24.0	P < .05

<sup>a</sup>SEM = 3.6.

Progesterone Concentrations During the Synchronized Estrous  
Cycle

Progesterone concentrations were not affected ( $P > .10$ ) by diet, and there was no interaction ( $P > .10$ ) between diet and day of the estrous cycle (Figure 2).

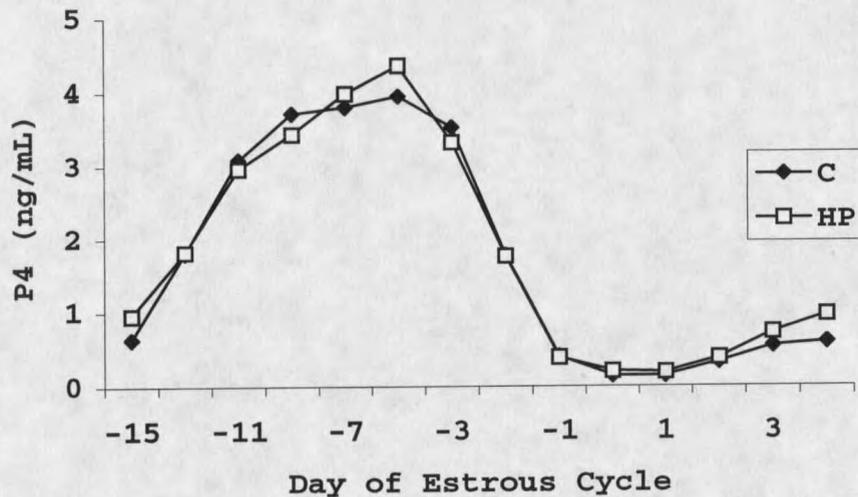


Figure 2. Progesterone (P4) concentrations from Day 15 of the synchronized estrous cycle through Day 4 of the next estrous cycle of ewes fed either a control (C) or high protein (HP) diet. Numbers of ewes through Day 2 for the C and HP diets were 15 and 16, respectively. Data for Days 3 and 4 represent 8 and 8 ewes from the C and HP diets, respectively.

Estradiol-17 $\beta$  Concentrations During the Peri-ovulatory  
Period

There was no interaction ( $P > .10$ ) between diet and day of the estrous cycle for E2 concentrations in samples collected 24 hours before estrus through Day 4 of the next estrous cycle (Figure 3). However, E2 concentrations were lower ( $P < .05$ ) in ewes fed the HP diet than in ewes fed the C diet (Figure 3).

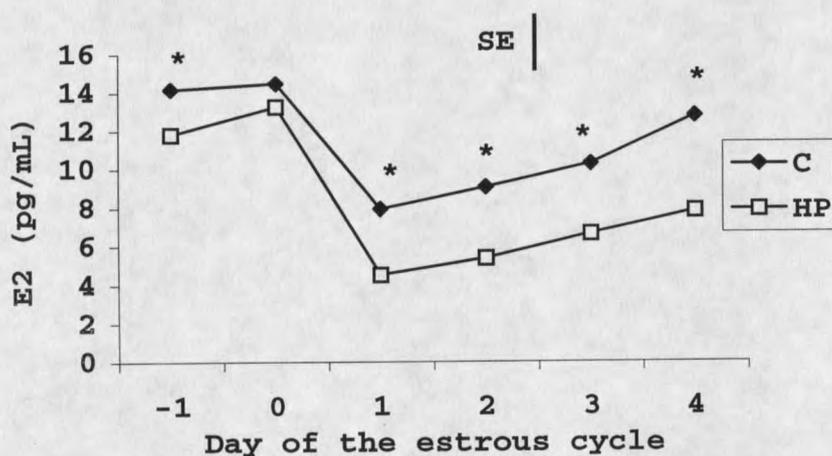


Figure 3. Estradiol-17 $\beta$  (E2) concentrations from 24 hours before estrus (Day 0) through Day 4 of the next estrous cycle of ewes fed either a control (C) or high protein (HP) diet during a synchronized estrous cycle. Numbers of ewes through Day 2 for the C and HP diets were 15 and 16, respectively. Data for Days 3 and 4 represent 8 and 8 ewes from the C and HP diets, respectively. An asterisk above a point indicates a difference between means at  $P < .05$ .

BUN, P4, and E2 Concentrations on Days 2 and 4 of the  
Estrous Cycle of Breeding

Blood urea nitrogen concentrations did not differ ( $P > .10$ ) between Days 2 and 4 after estrus associated with breeding in ewes fed either diet. However, BUN concentrations were higher ( $P < .05$ ) on Days 2 and 4 in ewes fed the HP diet than in ewes fed the C diet (Table 4).

Table 4. Least square means of BUN concentrations (mg/dL) for ewes fed either the control or high protein diet on Days 2 and 4 of the estrous cycle associated with breeding.

Day of the Estrous Cycle <sup>b</sup>	Diet <sup>a</sup>		Combined
	Control	High Protein	
2	17.7	25.2	21.5
4	18.5	21.9	20.7
Combined	18.1	23.5	

<sup>a</sup>Effect of diet,  $P < .05$ ; SEM = 3.6.

<sup>b</sup>Effect of day of estrous cycle,  $P > .10$ ; SEM = 3.6.

There was an interaction ( $P < .05$ ) between diet and day of the estrous cycle for progesterone concentrations. Progesterone increased to a greater ( $P < .05$ ) concentration by Day 4 of the estrous cycle in ewes fed the HP diet than in ewes fed C diet (Figure 4).

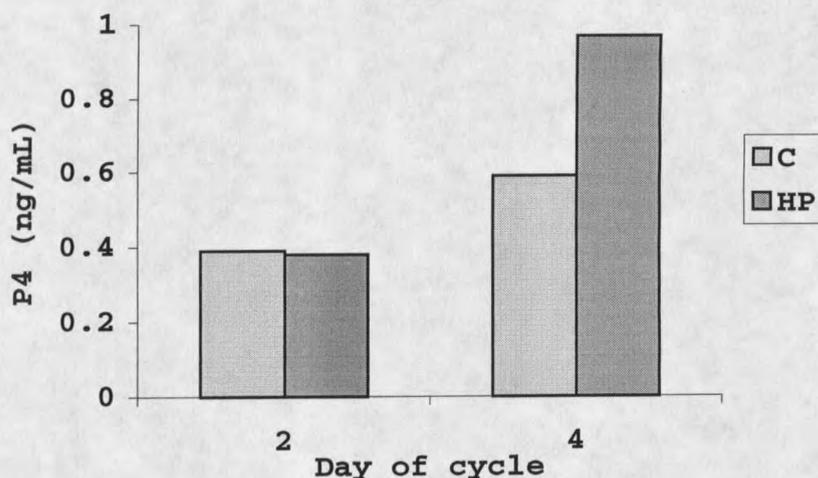


Figure 4. Least squares means of progesterone (P4) concentrations (ng/mL) on Days 2 and 4 of the estrous cycle associated with breeding in ewes fed either a control (C) or high protein (HP) diet. Diet x Day,  $P < .05$ ; SEM = .24.

There was no interaction ( $P > .10$ ) between diet and day of the estrous cycle associated with breeding for E2 concentrations. However, E2 concentrations were greater ( $P < .05$ ) in ewes on Day 4 than in ewes on Day 2 after estrus (Table 5). E2 concentrations were lower ( $P < .05$ ) in ewes fed the HP diet than in ewes fed the C diet after estrus associated with breeding (Table 5).

Table 5. Least square means of estradiol-17 $\beta$  concentrations (pg/mL) for ewes fed either the control or high protein diet on Days 2 and 4 of the estrous cycle associated with breeding.

Day of the Estrous Cycle <sup>b</sup>	Diet <sup>a</sup>		Combined
	Control	High Protein	
2	9.3	5.8	7.5
4	12.8	7.8	10.3
Combined	11.0	6.8	

<sup>a</sup>Effect of Diet,  $P < .05$ ; SEM = 3.2.

<sup>b</sup>Effect of Day,  $P < .05$ ; SEM = 3.2.

#### Correlation Coefficients among BUN, E2 and P4

##### Concentrations

There were no significant within class correlations among concentrations of BUN, E2, and P4 on Days 2 and 4 of the estrous cycle associated with breeding for ewes fed either the C or HP diets.

#### Oviductal Protein Contents and Concentrations

There was no interaction ( $P > .10$ ) between diet and day of the estrous cycle after breeding for AMP, IST, and UTH protein contents or concentrations. However, AMP protein content and concentration were higher ( $P > .05$ ) on

Day 2 than on Day 4 of the estrous cycle for ewes fed either diet after breeding (Table 6). Protein content and concentrations of the IST and UTH did not differ ( $P > .10$ ) on Days 2 and 4 after breeding (Table 6).

Table 6. Oviductal and uterine protein content and concentrations on Days 2 and 4 after estrus in ewes fed a control or high protein diet. Number of ewes is shown in parentheses.

Item	Diet			
	Control		High Protein	
	Day 2	Day 4	Day 2	Day 4
	(7)	(8)	(8)	(8)
Ampulla protein <sup>a</sup>				
Content ( $\mu\text{g}/\text{mL}$ ) <sup>b</sup>	62	55	67	51
Concentration ( $\mu\text{g}/\text{mg}$ ) <sup>c</sup>	464	312	457	328
Isthmus protein				
Content ( $\mu\text{g}/\text{mL}$ )	43	54	44	47
Concentration ( $\mu\text{g}/\text{mg}$ )	361	449	370	384
Uterine horn protein				
Content ( $\mu\text{g}/\text{mL}$ )	57	59	38	44
Concentration ( $\mu\text{g}/\text{mg}$ )	44	46	45	30

<sup>a</sup>Effect of day,  $P < .05$ .

<sup>b</sup>SEM = 28.

<sup>c</sup>SEM = 105.

#### Early Embryonic Development

There was no interaction ( $P > .10$ ) between diet and day of the estrous cycle after breeding for ovulation and recovery rates or embryo damage. However, embryos recovered on Day 4 had more cells than those collected on Day 2 ( $P < .05$ ; Table 7). There was a higher ( $P < .05$ )

percentage of embryos collected on Day 4 in the AMP of ewes fed the HP diet than in the AMP of ewes fed the C diet (Table 7). Whereas, there was a tendency ( $P < .10$ ) for more embryos to be collected from the UTH of ewes fed the C diet than in ewes fed the HP diet (Table 7).

Table 7. Early embryo development on Days 2 and 4 after estrus in ewes fed a control or a high protein diet. Number of ewes is shown in parentheses.

Item	Diet			
	Control (C)		High Protein (HP)	
	Day 2	Day 4	Day 2	Day 4
	(7)	(8)	(8)	(8)
No. of ovulation	11 (157%)	9 (150%)	10 (163%)	11 (163%)
Embryo recovery	11	9	10	11
Damaged	1	1	0	2
Cell Stage <sup>a</sup>	1.60	6.38	2.1	5.56
Location				
AMP (%)	100	12.5 <sup>c</sup>	100	43 <sup>d</sup>
IST (%)	0	50.0	0	43
UTH (%)	0	37.5 <sup>e</sup>	0	14 <sup>f</sup>

<sup>a</sup>Effect of day,  $P < .05$ ; SEM = .5.

<sup>c,d</sup>Chi-square = 5.2, d.f. = 1;  $P < .05$ .

<sup>e,f</sup>Chi-square = 2.6, d.f. = 1;  $P < .10$ .

## DISCUSSION

Feeding excess degradable intake protein did not change the estrous cycle length of ewes in this study. Estrous cycle length in ruminants is primarily regulated by the life-span of the corpus luteum (CL). The CL is responsible for the synthesis and secretion of progesterone. Regression of the CL and loss of progesterone secretion is caused by the release of prostaglandin  $F_{2\alpha}$  from the uterus in the ewe during the latter half of the luteal phase of the estrous cycle (Niswender and Nett, 1994). Prostaglandin  $F_{2\alpha}$  release by the uterus is controlled by oxytocin, acting via endometrial oxytocin receptors (Sheldrick et al., 1980; Sheldrick & Flint, 1985; Hooper et al., 1986). Oxytocin receptor concentrations during the estrous cycle are primarily regulated by progesterone (Lamming et al., 1991). In the present study, we found that progesterone concentrations during the synchronized estrous cycle were not affected by diet. Therefore, feeding ewes excess degradable intake protein during a synchronized estrous cycle does not affect the system that regulates CL function and/or regression.

One major finding of this experiment is that E2 concentrations at the end of the synchronized cycle and

during the first 4 days of the next cycle were reduced by feeding ewes the HP diet. To our knowledge, this is the first report in the literature that feeding excess degradable intake protein to ewes alters the systemic blood concentrations of the ovarian steroid estrogen. Adams et al. (1994) reported that fecal excretion of E2 increased in well-fed ewes. They postulated that a reduction in circulating E2 by fecal elimination might play an important role in mediating so-called "nutritional effects" on reproductive events. Reducing circulating E2 concentrations may be associated with a reduction in estradiol feedback that would be expected to enhance ovulation rate (Payne et al., 1991). However, in our study, neither the occurrence of estrus nor ovulation rates were affected by feeding ewes the HP diet that decreased periovulatory concentrations of E2. This result differs from results of Robinson (1990, 1996) and Smith (1985, 1988) who found that high protein supplements enhanced ovulation rates in sheep.

Blood urea nitrogen concentrations in serum of ewes increased within 48 hours after the start of feeding the excess degradable intake protein. Concentrations of BUN were approximately 28% higher throughout the estrous cycle and during the first 4 days of the next cycle in ewes fed the HP diet than in ewes fed the C diet. These results

support those of Oltjen et al. (1972) who found that feeding ewes excess degradable intake protein diets increased BUN concentrations. It is known that high BUN concentrations are associated with increased ruminal ammonia production, which in turn is associated with high ammonia concentrations in blood. Ferguson and Chalupa (1989), and Bishonga et al. (1994) reported that elevated plasma ammonia (>100  $\mu\text{mol/L}$ ) impaired fertility in sheep, supposedly as a result of toxic effects of ammonia, urea, and/or other unidentified nitrogenous compounds on functions of ova, sperm, and early embryos. Jordan and Swanson (1979) suggested that high plasma urea nitrogen and ammonia concentrations might increase the pH within the reproductive tract and reduce motility and survival of sperm. However, Elrod and Bulter (1993) demonstrated that excess degradable intake protein acts through some undefined mechanism to decrease uterine pH during the luteal phase, which may play a role in the observed reduction of fertility.

The focus of the present study was on the effects of excess degradable intake protein upon events associated with oviductal function and early embryonic development. Oviductal function is primarily regulated by ovarian steroid hormones (see Harper, 1994; Brenner and Slayden,

1994). Gamete transport, fertilization, and early embryonic development take place in an E2-dominated oviduct. Usually, transport of ova through the oviduct to the uterus takes 3 to 4 days in the ewes (see Harper, 1994). In sheep, the embryo leaves the oviduct and enters the uterus at approximately Day 3 after ovulation. During this phase of the cycle systemic P4 concentrations are rising while E2 concentrations are falling (Hafez, 1993b). In the present study, we found that that feeding ewes a HP diet altered ovarian steroid concentrations during the first 4 days of the estrous cycle associated with breeding. Progesterone concentrations increased to greater concentrations by Day 4 after estrus in ewes fed the HP diet than in ewes fed the C diet. Whereas, E2 concentrations were lower on both Days 2 and 4 after estrus in ewes fed the HP diet than in ewes fed the C diet. Increasing concentrations of E2 act upon the oviductal epithelium to increase cell number and stimulate differentiation into ciliated and secretory cells (Brenner and Slayden, 1994). Under the influence of E2, the oviduct exhibits an increase in contractility supposedly as a result of an increase in  $\alpha$ -adrenergic and prostaglandin F<sub>2 $\alpha$</sub>  activity (Hafez, 1993a; Harper, 1994). The increase in contractility reduces transport through the oviduct. Progesterone inhibits this estrogenic effect causing

relaxation and allowing the embryo to be released into the uterus. Feeding ewes a diet high in degradable intake protein altered the secretion pattern of E2 at the end of the synchronized cycle and altered both E2 and P4 concentration patterns during the first 4 days of the next cycle. These changes might be expected to alter oviductal epithelial differentiation and secretory ability, and contractility. The mechanism involved in altering ovarian steroid secretion in ewes fed the HP diet is not clear. However, it does not appear to be related to increased concentrations of BUN because there were no significant within class correlations among BUN, E2, and P4.

In sheep, E2 induces the synthesis and release of a  $M_r$  90,000 to 92,000 glycoprotein from the ampulla but not the isthmus of the oviduct (Murray, 1993). Oviductal secretory proteins induced by E2 and repressed by P4 are localized to the nonciliated epithelial cells of the oviduct. Estrogen-dependent oviductal glycoproteins are released into the oviduct lumen until Day 4 of pregnancy (Murray, 1993). They are localized to the zona pellucida and perivitelline space in early cleavage-stage sheep embryos (Gandolfi, 1989b, 1991). The functional significance of the glycoproteins and smaller polypeptides is unclear. However, if one takes into account that they are: secreted

at a time when sperm capacitation, fertilization, and early embryonic growth occurs; bind to zona pellucidae and sperm (Gandolfi et al., 1989a and b; Murray, 1993; Buhi et al., 1993; Malette and Bleau, 1993; Lippes and Wagh, 1989); found in the perivitelline space (Gandolfi et al., 1989; Murray, 1993; Buhi et al., 1993); associated with plasma membranes of blastomeres (Buhi et al., 1993); enhance in vitro embryonic development (Gandolfi et al., 1989; Rexroad, 1989); and may provide protection from proteolytic enzymes during oviductal transport (Malette and Bleau, 1993; Broerman et al., 1988), then the conclusion must be that oviduct-specific proteins play essential roles in processes regulating early embryonic development and survival. These biosynthetic and reproductive events occur when E2 is the predominant ovarian steroid before a rise in P4 concentrations. Although there are no data for oviductal secretory proteins in our experiment, specific E2-induced oviductal-derived secretory proteins should be reduced by decreased E2 concentrations in the oviduct of ewes fed the HP diet. Furthermore, the HP diet might affect the cell stage of cleavage of the embryos by its effect on estrogen concentrations that are related to E2-dependent oviductal proteins.

The results of our study indicate that the feeding ewes a diet high in degradable intake protein did not affect ovulation rate. Nutrition is one of the most significant influences on the ovulation rate of sheep. Over the past 20 years considerable effort has been devoted to distinguishing between the relative importance of protein and energy intake as the stimulus leading to increase in ovulation rate arising from flushing. Many of these studies are confounded by the fact that ruminal degradation of dietary protein together with synthesis of microbial protein, consequent to the ruminal digestion of carbohydrate, poses major problems in the interpretation of the effects of dietary protein and energy on reproductive function. Dufour and Matlon (1977) suggested that feeding high energy or low energy levels from Day 10 of the estrous cycle did not influence ovulation rate. On the other hand, Fletcher (1981) found that increased protein only stimulated ovulation rate in ewes previously fed low levels of protein. Ovulation rate was increased when available crude protein rose from 35 to 70 g.day<sup>-1</sup>, but unlike our results when protein intake increased from 70 to 150 g.day<sup>-1</sup> there was no increase in ovulation rate.

The most significant finding of our study was the fact that transport of embryos through the oviduct to the uterus

was delayed in ewes fed the HP diet. However, the timing of embryonic cleavage events was not affected by this delay in transport: indicating that the timing of cleavage events is independent of the location in the oviduct. This is the first report of the effect of feeding a diet high in degradable protein on early embryonic events in sheep. The mechanism for this is not clear. Transport of the embryo through the oviduct is related to the frequency and amplitude and direction of smooth muscle contractions, frequency and direction of ciliary activity, and intraluminal hydrostatic pressure gradients (see Brenner and Slayden, 1994). Clearly, these activities are regulated by the ovarian steroid environment during the periovulatory phase of the estrous cycle. Furthermore, these activities are modulated by intrinsic (intracellular regulatory system) and extrinsic (adrenergic system and prostaglandins) factors, and possibly by the interaction of the embryo with the oviductal mucosa.

Feeding excess degradable intake protein has been associated with lower fertility in domestic ruminants (Visek, 1984; Garwacki, 1979; Parr et al., 1988; Blanchard et al., 1990). Generally, the mechanism for this effect is that excess ammonia escapes ruminal recycling superceding the ability to convert ammonia to urea. This drives urea

concentrations to increase in the blood. If the ability of the liver to convert ammonia to urea is surpassed then blood ammonia increases. This in turn interferes with intermediary metabolism and disrupts cellular activities, which in the reproductive tract, leads to embryonic loss. In the present experiment, BUN concentrations were increased indicative of increased ammonia in the blood, however, BUN concentrations were not correlated with embryonic loss or with concentrations of ovarian steroids. Therefore, one can conclude that high concentrations of BUN are associated with but not directly related to delayed embryo transport in this study.

The results of this experiments indicate that feeding excess degradable intake protein alter ovarian steroid secretion. Our results show that embryos appear to be retained within the oviduct longer in ewes fed the HP diet than in ewes fed the C diet. Estrogen concentrations were lower in ewes fed the HP diet than in ewes fed the C diet. Whereas, P4 concentrations increased sooner in HP ewes than in ewes fed the C diet. In some species, low doses of exogenous estrogen cause a phenomenon known as "tube locking" where ova are retained at the isthmus-ampulla junction; larger doses of estrogen hastened transport through the isthmus and into the uterus (Dukelow and

Riegle, 1974). Harper (1964) found that in rabbits progesterone alone has little effect on transport of ovum surrogates; whereas, estrogen alone cause either "locking" of ova in the oviduct or premature expulsion to the uterus, depending on dose and timing of administration relative to entry of ova into the oviducts (Greenwald, 1967; Noyes et al., 1959; Harper, 1964). Therefore, low E2 concentrations coupled with high P4 concentrations in HP ewes may have altered both extrinsic and intrinsic factors that regulate contractility of the oviduct reducing its ability to transport embryos. The consequence of this activity may be that embryos from ewes fed HP diets are more mature before they enter the uterus resulting in a asynchrony between uterine changes and embryonic changes necessary for maternal recognition of pregnancy. Such asynchrony may result in embryonic loss.

Associated with this hypothesis is the idea that changing the steroid milieu of the oviduct will change the secretory capacity of the epithelium. Many studies have suggested that oviductal secretory macromolecules are involved in early embryonic development or cleavage (Whittingham and Biggers, 1967; Krisher et al., 1989; Minami et al., 1993; Boatman et al., 1994). Many others have described estrogen-dependent oviductal secretory

glycoproteins (sheep: Murray, 1992,1993; pigs: Buhi et al., 1992; cows: Boice et al., 1990a). In mammals, for the first 4 days of pregnancy the estrogen-dominated oviductal milieu provides the immediate surrounding for fertilization and cleavage of the fertilized ovum until it enters the progesteronally receptive uterus (Murray, 1994). An estrogen dependent glycoprotein is produced by the endothelium of the ewe oviduct during the time the fertilized ova are transported the tube. Murray (1993) reported that in the sheep a 90,000-92,000 MW E2-dependent glycoprotein was synthesized and released by the ampulla during early embryonic development at a time that corresponds to fertilization and when the first mitotic divisions of the fertilized ovum were take place. Steroid-regulated oviductal secretory proteins have been contributed to the microenvironment of the embryos by associating with the zona pellucida and entering the perivitelline space (Kan et al., 1988, 1993; Gandolfi, 1991; Boice et al., 1990b; Abe, 1992; Buhi et al., 1993). It is known that filamentous-actin, a plasma membrane protein, is involved in cell cleavage of embryos. In the zona pellucida of 1- and 2-cell embryos, the oviduct protein displayed an intertwining reticular organization that was replaced by a diffuse and more intense

accumulation in 4-, 8-, and > 16-cell embryos. Murray and Messinger (1994) found that in early cleavage-stage hamster embryos, the intensity and pattern of staining for an E2-dependent oviductal protein ( $M_r$  200,000) which is released into the oviductal lumen during embryo transport can be distinguished on the basis of membrane f-actin display and blastomere number and shape.

We did not observe a change in protein content or concentration in any segment of the oviduct or the uterine horns in ewes fed either the HP or C diet. Therefore, feeding excess degradable intake protein does not appear to impact protein secretion of the oviduct. However, one cannot exclude the possibility that feeding excess degradable intake protein alters the quantity or the quality of specific oviductal protein types. This possibility remains to be tested.

In conclusion, the results of this experiment support the hypothesis that feeding excess degradable intake protein alters ovarian steroid concentration patterns during the periovulatory period in sheep and delays transport of embryos from the oviduct to the uterus. We propose that periovulatory changes in E2 and P4 concentrations alter  $PGF_{2\alpha}$  and norepinephrine secretion or activity to inhibit contractions or hydrostatic pressure of

the oviduct, which in turn delays embryo transport. These results do not support the hypothesis that feeding excess degradable intake protein alters the protein secretory capacity of the oviduct.

## IMPLICATIONS

Feeding excess degradable intake protein decreases reproductive rates in domestic ruminants. The mechanism for this effect is not well understood. We have found that feeding excess degradable intake protein to ewes during a synchronized estrous cycle and during the first 4 days of the next cycle alters periovulatory ovarian steroid concentrations. Further, we found that embryo transport was delayed in ewes fed excess degradable intake protein. Delay in embryo transport through the oviduct to the uterus may result in an asynchrony between the embryo and uterine such that maternal recognition may not occur. This would lead to embryonic loss. This possibility remains to be tested. We propose that the change in ovarian hormone secretion during the first 4 days after breeding in ewes fed excess degradable intake protein alters oviductal contractility by interfering with the action of the ovarian steroids on intrinsic cellular factors and extrinsic factors such as norepinephrine and prostaglandin. Lastly, we propose that the alteration in ovarian steroid patterns induce different types of protein to be either synthesized or released by the oviductal epithelium. Such a change may

be detrimental to embryonic growth and development and increase reproductive loss.

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